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Test battery for the effect determination of chemicals in soils: Suitability of test systems with mycorrhizal fungi for the risk assessment

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Abstract: ERAMYC - Test battery for the effect determination of chemicals in soils: Suitability of test systems with mycorrhizal fungi for the risk assessment

Arbuscular Mycorrhizal Fungi (AMF) are recognized as important providers of ecosystem services, supporting soil health and key soil functions. Their ecological relevance and sensitivity to chemicals led the European Food Safety Authority (EFSA) to indicate AMF as a potential group of non-target test organisms for future Ecological Risk Assessment schemes (ERA) for Plant Protection Products (PPPs). However, AMF sensitivity to chemicals is poorly understood and the impact of contaminants on the AMF life cycle, including on spore viability (AMF pre-symbiotic phase), root colonization and subsequent production of spores and soil mycelium (AMF symbiotic phase), are of growing concern due to the crucial importance of these microorganisms on soil ecosystems.

The ERAMYC project included an extensive literature review on the effects of organic chemicals on the pre-symbiotic and symbiotic phases of the AMF life cycle which guided the development and refinement of test protocols for AMF testing for risk assessment. A series of laboratory experiments, including the use of different test methodologies, AMF species, host plants, test soils and test substances, culminated on the production of test protocols which were submitted to ring tests, with the objective of drafting a new OECD guideline for AMF pre-symbiotic and symbiotic phase testing.

Despite the steps given towards standardization of AMF testing, the methods tested here could not be considered reproductible and/or precise due to high variability of the estimated endpoints and the low number of valid data sets resulting from the ring test experiments. AMF intra-specific genetic variation and the technical knowledge needed for the measurement of AMF parameters (e.g. root colonization and hyphae measurements) could be the main factors contributing to the high variability of the results.

Data produced in the ERAMYC project constitutes an undeniable contribution for the standardization of methods based on AMF colonization and highlights the current insufficiency of ERA of PPPs in European Union to protect this group of microorganisms in terrestrial systems.

Kurzbeschreibung: ERAMYC - Testbatterie zur Wirkungsbestimmung von Chemikalien in Böden: Eignung von Testsystemen mit Mykorrhizapilzen zur Risikobewertung

Arbuskuläre Mykorrhizapilze (AMF) gelten als wichtige Anbieter von Ökosystemdienstleistungen, da sie die Bodengesundheit und wichtige Bodenfunktionen unterstützen. Aufgrund ihrer ökologischen Relevanz und Empfindlichkeit gegenüber Chemikalien hat die Europäische Behörde für Lebensmittelsicherheit (EFSA) AMF als potenzielle Gruppe von Nichtziel-Testorganismen für zukünftige ökologische Risikobewertungssysteme (ERA) für Pflanzenschutzmittel (PPPs) bezeichnet. Die Empfindlichkeit von AMF gegenüber Chemikalien ist jedoch noch wenig erforscht und die Auswirkungen von Schadstoffen auf den Lebenszyklus von AMF, einschließlich der Sporenlebensfähigkeit (präsymbiotische Phase von AMF), der Wurzelbesiedlung und der anschließenden Produktion von Sporen und Bodenmyzel (symbiotische Phase von AMF), geben aufgrund der entscheidenden Bedeutung dieser Mikroorganismen für Bodenökosysteme zunehmend Anlass zur Sorge.

Das ERAMYC-Projekt umfasste eine umfassende Literaturrecherche zu den Auswirkungen organischer Chemikalien auf die präsymbiotischen und symbiotischen Phasen des Lebenszyklus von AMF, die als Leitfaden für die Entwicklung und Verfeinerung von Testprotokollen für AMF-Tests zur Risikobewertung diente. Eine Reihe von Laborexperimenten, bei denen unterschiedliche Testmethoden, AMF-Arten, Wirtspflanzen, Testböden und Testsubstanzen verwendet wurden, mündete in der Erstellung von Testprotokollen, die Ringversuchen unterzogen wurden, mit dem Ziel, eine neue OECD-Richtlinie für Tests der präsymbiotischen und symbiotischen Phase von AMF zu erstellen.

Trotz der unternommenen Schritte zur Standardisierung von AMF-Tests können die hier getesteten Methoden aufgrund der hohen Variabilität der geschätzten Endpunkte und der geringen Anzahl gültiger Datensätze aus den Ringversuchsexperimenten nicht als reproduzierbar und/oder präzise angesehen werden. Die intraspezifische genetische Variation von AMF und das technische Wissen, das zur Messung von AMF-Parametern erforderlich ist (z. B. Wurzelbesiedlung und Hyphenmessungen), könnten die Hauptfaktoren sein, die zur hohen Variabilität der Ergebnisse beitragen.

Die im Rahmen des ERAMYC-Projekts gewonnenen Daten stellen einen unbestreitbaren Beitrag zur Standardisierung von Methoden dar, die auf der AMF-Kolonisierung basieren, und unterstreichen die derzeitige Unzulänglichkeit der ERA von PSM in der Europäischen Union zum Schutz dieser Gruppe von Mikroorganismen in terrestrischen Systemen.

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List of abbreviations

AF	Assessment factor
a.i.	Active ingredient
BA	Boric acid
AMF	Arbuscular mycorrhizal fungi
AZX	Azoxystrobin
$CuSO_4$	Copper sulphate
EC	Effect Concentration
EFSA	European Food Safety Authority
ERA	Ecological Risk Assessment
FAO	Food and Agriculture Organization of the United Nations
FLZ	Fluazinam
IC	Inhibitory Concentration
ITPS	Intergovernmental Technical Panel on Soils
LOEC	Lowest Observed Effect Concentration
NOEC	No-Observed Effect Concentration
PCP	Personal Care Products
PDM	Pendimethalin
PPP	Plant Protection Products
TBC	Tebuconazole
TCC	Triclocarban
TCS	Triclosan
TRZ	Terbuthylazine
UC	University of Coimbra
UDESC	University of Santa Catarina State
UT	University of Tartu

Summary

Introduction

Arbuscular Mycorrhizal Fungi (AMF) are organisms that live in obligate symbiosis with most vascular plant species. They have an important role on soil ecosystems by increasing plant nutrient acquisition (namely of phosphorous) and tolerance to various environmental stressors (e.g., metals, pathogens, drought, salinity, and others) and by increasing soil carbon sequestration, and soil aggregation and stabilization. Due to their recognized ecological relevance in supporting key soil functions as well as their sensitivity to chemicals, AMF have been identified by EFSA as a potential group of non-target test organisms to be included in the standard test battery to assess the effects of PPP on in-soil organisms (EFSA, 2017).

As in-soil organisms, AMF are exposed to a wide range of chemicals found, namely, in agricultural soils (e.g., PPP, veterinary pharmaceuticals, metals) and the potential risk derived from this exposure is largely unknown and usually disregarded in ecological risk assessment (ERA) schemes. Moreover, in addition to the limited information on the effects of chemicals on AMF, the methodologies adopted to evaluate those effects (e.g. selection of AMF and host plant species, endpoints considered) are often inconsistent among published studies.

The ERAMYC project was founded with the main objective of filling some of the gaps identified in the EFSA's scientific opinion (EFSA, 2017). This included the development and standardization of new test protocols and the refinement of existing guiding documents concerning AMF testing, with the final objective of including these organisms in ERA schemes.

To accomplish these objectives, the work developed during the ERAMYC project was divided into four main sections. **Section 1** included a literature review conducted in WP1 about the effects of organic contaminants on the AMF pre-symbiotic and symbiotic phases; **Section 2** encompassed the work conducted for the development and testing of a protocol for AMF symbiotic phase testing based on the results of laboratory experiments conducted without chemicals and with chemicals over the two phases of WP2. Experiments aiming to define a reference substance, the chemicals and the strategy to be used in the symbiotic and pre-symbiotic ring tests were conducted in a second phase of WP2; **Section 3** comprised the work developed in WP3, that included the performance of the symbiotic and pre-symbiotic ring tests; **Section 4** comprised the refinement and the adaptation of protocols tested during the symbiotic and pre-symbiotic testing for future publication/proposal (WP4) for a new OECD guideline, based on the results obtained in experiments of WP2 and WP3.

WP1 - Systematic literature review and a comparative assessment of the studies with AMF exposed to chemicals

The main objective of WP1 within the ERAMYC project was to conduct a literature review, based on studies where AMF have been exposed to chemicals, to collect information at different levels (e.g., chemicals assessed, AMF and host plant species used, test conditions adopted, and parameters measured including their sensitivity to the tested chemicals) and synthesize that information to help defining different aspects of the protocol to be developed and ring tested during the next phases of the project. The information collected was decisive to select the best combination of AMF, plant species, test conditions and test endpoints to be used to assess effects of organic chemicals on both pre-symbiotic and symbiotic phases of AMF life cycle. The selection of AMF species was also supported by an additional search on the worldwide distribution of AMF taxa to evaluate their geographical representativeness.

Methods adopted in the literature review

Two independent searches were performed:

1. A search to evaluate AMF biogeographical distribution was performed and ranked the 20 most reported groups of AMF worldwide based on their occurrence. This search was conducted on specialized AMF databases, namely PlutoF (Abarenkov et al., 2010), MaarjAM (Öpik et al., 2010, Stürmer et al., 2018), Fungal Traits (Põlme et al., 2021) and NCBI website[1].
2. A systematic literature search using different databases (see below) was performed to retrieve information on (i) effects of chemicals on AMF and (ii) influence of abiotic parameters on AMF performance both on the pre-symbiotic and symbiotic phases. This search was performed on platforms Scopus[2], ScienceDirect[3], PubMed[4], Web of Science[5], SciELO[6] and Google Scholar[7] in April and May of 2021. Specific search terms are indicated in the respective sections where the results are presented.

Results obtained in the literature review

A total of 322 research papers were found, but only 53 of them met the goals of this review and were analysed in more detail. The information about chemicals and active ingredients (a.i.) tested, AMF species and host plants used, and test conditions adopted in ecotoxicological studies can be synthesized as follows:

1. The most studied chemicals were fungicides (F; 41 a.i.), herbicides (H; 16 a.i.), veterinary and human-use pharmaceuticals (15), biological pesticides (6), disinfectant agents (4), personal care products (PCP; 2), insecticides (I; 2 a.i.), and mixtures of pesticides (12 formulations, 2 with fungicides+insecticides; 8 with fungicides; 2 with herbicides).
2. The most studied a.i. were Glyphosate (H) (11 studies), Benomyl (F) (9), Carbendazim (F) (8), Fenhexamid (F) (8), Captan (F) (6), Azoxystrobin (F) (5), Chlorothalonil (F) (5), Mancozeb (F) (5), Pentachloronitrobenzene (F) (5), Flutholanil (F) (4), Triclosan (PCP) (4) Alachlor (H) (3), Dicamba (H) (3), Dimethoate (I) (3), Fenpropimorph (F) (3), Fosethyl-aluminium (F) (3), and Pencycuron (F) (3).
3. The AMF species mostly used were *Funneliformis mosseae* (27 studies), *Claroideoglomus etunicatum* (14), *Rhizophagus intraradices* (13), *Rhizophagus clarus* (8), *Gigaspora margarita* (6), *Glomus aggregatum* (4), *Rhizophagus irregularis* (4), *Scutellospora heterogama* (4), *Acaulospora laevis* (3), *Funneliformis caledonium* (3), *Gigaspora rosea* (3), and *Glomus monosporum* (3). This is in close agreement with the findings presented in section 2.3 about AMF worldwide distribution.
4. 28 species of host plants were used in experiments reported in existing literature. The most used plant was *Zea mays* (6 studies), followed by *Glycine max* (5), *Daucus carota* (*in vitro* transformed roots) (5), *Triticum aestivum* (4), *Pisum sativum* (3), *Capsicum annum* (2),

[1] https://www.ncbi.nlm.nih.gov/

[2] https://www.scopus.com/search/form.uri?display=basic

[3] https://www.sciencedirect.com/

[4] https://pubmed.ncbi.nlm.nih.gov/

[5] https://www.webofknowledge.com

[6] https://scielo.org

[7] https://scholar.google.com.br

Citrus aurantium (2), *D. carota* (2), *Leucaena leucocephala* (2), *Phaseolus vulgaris* (2), and *Trifolium subterraneum* (2).

5. Most papers focused on the pre-symbiotic phase (27), the symbiotic phase (15) or both (11). Experiments have been performed using natural soils (20 studies), natural soil mixed with sand (11), artificial soils (3), sand (5) and culture medium (*in vitro*) (17) as test medium. Studies using natural soils have reported pH values between 6.3-8.2; pH values of artificial soil were adjusted to 6.0 (± 0.5), and of sand varied between 6.0 and 7.0.
6. Most studies focusing on the pre-symbiotic phase reported experiments carried out in the dark and only two studies have used a photoperiod of 16:8h light/dark. Most experiments have used temperatures of 25 °C, 27 °C, and 28 °C ± 2 °C with two exceptions where temperatures of 22 °C and 26 °C were tested.

Conclusions from the literature review

On this first approach, we found that the most studied group of chemicals were fungicides, and the most studied a.i. was Glyphosate. The AMF species more often used were *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Rhizophagus intraradices*. It is important to highlight that there is a predominance of studies reporting the effect of chemicals only at pre-symbiotic phase level and the scarcity of data regarding effects on the symbiotic phase. In general, there is a lack of data regarding the effect of biocides, veterinary pharmaceuticals, and insecticides on AMF species.

WP2 - Performance of ecotoxicological tests with AMF – development phase

The strategy defined for WP2 was based on the review presented in Section 1 of this Final report and on the experience of the consortium members.

WP2 was divided into two phases (two clusters of experiments) with the objective of defining and refining methodologies for the performance of symbiotic phase tests. In the first phase, no chemicals were used, and the aim was to evaluate the performance/suitability of the selected AMF species in the selected soils under specific test conditions (environmental conditions and selected host plants). The most relevant parameters were assessed to evaluate the operational aspects of their measurement, the intra-specific variability and to define adequate validity criteria for combinations of AMF species/measured parameters. The second phase included the use of test chemicals aiming to assess how the different AMF species and parameters measured react to the selected chemicals. The results obtained in this second phase were used to select test substances to be applied in the ring tests (interlaboratory tests) of WP3.

Results obtained in WP2

The data produced in WP2 were essential to define the methodologies to be applied in the next steps of the project, especially in the ring tests of WP3 and, therefore, for the preparation of the proposal for the new OECD standard method.

Before starting the experiments, the selection of soils, AMF species and host plant had to be supported:

- Soils for the tests were selected according to their characteristics through a Principal Component Analysis (PCA), availability, and suitability for AMF development. Five soils were selected: a modified artificial soil (with 2.5% of organic matter) and four natural soils

(one natural soil per each country of the consortium partners, i.e. Portugal, Estonia and Brazil and a natural reference soil from Germany);

- Four species of AMF, *Rhizophagus irregularis*, *Rhizophagus clarus*, *Gigaspora margarita* and *Funneliformis mosseae*, were selected based on data obtained in the literature review from WP1 and on the availability of inoculant suppliers;
- Two species of host plants, *Solanum lycopersicum* (tomato) and *Allium ampeloprasum* (leek), were selected due to their use in previous ecological studies with AMF.

After the selection of these components, the first part of the experimental phase of WP2 began, with the main objective of refining the methodology for symbiotic phase experiments and to evaluate the suitability of the selected AMF. The following aspects were evaluated:

- Mode of AMF inoculation (number of spores *vs* mixed inoculum weigh): inoculation with mixed inoculum was the most suitable to achieve adequate percentages of AMF colonization;
- Potential of inoculum: to estimate the amount (i.e. weigh) of mixed inoculum necessary for an acceptable AMF colonization;
- Host plant: the host plant selected for the following phases was leek, as AMF colonization was generally higher in this species than in tomato;
- Development phase of the host plant for test beginning: turgid seeds, with no signs of germination, are more suitable for these experiments as the initial phase of plant development is essential to the establishment of the plant-AMF symbiosis;
- Parameter endpoints and acceptable outcomes (for test validation): intra-radicular colonization (total and arbuscular) and extra-radicular hyphae length were the selected parameters to be used in symbiotic phase tests, as they reflect the strength of the AMF-plant symbiosis. A percentage of 30% total colonization was considered the minimum required to validate the tests.

Other technical parts of the methodology such as the staining method, the necessity of using a nutrient solution for plant irrigation and of adding sand/glass beads in the top of the soil to prevent soil water losses over the experiments, were also approached in this phase.

Based on the work developed in this first phase, initial drafts of the SOPs were produced. Methods described in these drafts were followed by the consortium during testing with contaminants, in the second phase of WP2.

The main objectives of the second phase of WP2 were 1) to define a reference substance to be used in positive controls and 2) to select suitable chemicals to be used in the ring tests of laboratory AMF symbiotic and pre-symbiotic phase tests.

Boric acid was the first chemical selected to be suggested as reference substance for AMF symbiotic phase testing as this chemical has been used as reference substance for other organisms in ecotoxicological testing. However, there was an apparent higher toxicity of Boric acid to the host plant when compared to the AMF root colonization that appeared unaffected in replicates with plant biomass production even in the highest tested concentrations. Therefore, it was concluded that Boric acid is not suitable to be used as reference substance in AMF symbiotic phase tests.

A second chemical was proposed for reference substance: Copper sulphate. The results obtained with Copper sulphate were inconclusive regarding its suitability to be used as reference substance, due to the unclear relationship between the concentrations of Copper sulphate and the effects on plant biomass production and root AMF colonization of *F. mosseae*. For *R. irregularis*, toxic effects on the host plant appeared to be higher than the effects on AMF colonization, but this could be due to the concentration gradient used, possibly too large between test concentrations.

Since neither Boric acid nor Copper sulphate showed to be adequate to be used as reference substance in AMF symbiotic phase tests, it was decided to select 2 chemicals from a list of a.i. commonly used in agriculture for the ring tests and, according to the results obtained, select one of them to be used as reference substance for the method.

Five a.i. (3 fungicides: Azoxystrobin, Fluazinam and Tebuconazole + 2 herbicides: Pendimethalin and Terbuthylazine) were tested using both artificial and natural soils and using three AMF species with leek as host plant. The main objective of these experiments was to select chemicals to be used in the ring tests of WP3. Azoxystrobin and Fluazinam were the selected chemicals to be used in the ring tests. Both substances were tested in the artificial soil (i.e. OECD artificial soil with 2.5% of OM) and in the natural soils from Portugal, Brazil, Estonia and a reference soil from Germany.

Data obtained in experiments of WP2 were used in predictive Generalized Linear Models to assess the influence of soil properties on AMF colonization and on the sensitivity of AMF to chemicals in soil.

The influence of soil properties on AMF colonization was evaluated considering the average measurements of AMF total (TC) and arbuscular colonization (AC) and extra-radicular hyphae length (EHL) in negative controls of experiments with *R. irregularis* and *F. mosseae* and leek from both phases of WP2 as response variables and soil properties - soil pH, organic matter content, cation exchange capacity, sand, silt and clay contents, aluminium exchangeable concentration and phosphorus, iron and manganese extractable concentrations - as continuous explanatory variables and AMF species and laboratory as nominal explanatory variables. Average TC and AC measurements did not show a statistically significant influence on the measured endpoints raised by changed soil properties. On the other hand, average EHL was significantly influenced by iron and phosphorous extractable concentrations, soil pH and cation exchange capacity. The laboratory also had a significant influence on EHL measurements.

The effect of soil properties on the AMF sensitivity to chemicals in soil was assessed considering the endpoints estimated for the three AMF parameters (i.e. TC, AC and EHL) in the experiments conducted during the second phase of WP2 with *R. irregularis* and Azoxystrobin or Fluazinam as response variables. The same soil properties considered in the previous evaluation were used as continuous explanatory variables and the laboratory and test substance were used as nominal explanatory variables. No significant explanatory variables were found in GLMs using the estimated endpoins for the three AMF parameters considered as response variable, except for a GLM with “Laboratory” as explanatory variable for EC10 values based on TC measurements.

Although GLMs generally did not evidence significant explanatory variables, they explained a higher percentage of the observed variance when considering the “Laboratory” and “Chemical” variables for all AMF parameters (TC explained the highest percentages, followed by AC and EHL). The absence of statistical significance for the “Chemical” variable in the GLMs suggests that a dataset with endpoints estimated for a larger number (and a greater variety) of substances would be needed to enhance the robustness of the analysis.

Data obtained in the second phase of WP2 were also analyzed from the perspective of risk assessment to understand at which point AMF are being protected by the current ERA of PPPs in the European Union (EU). Endpoints reported in the literature for the seven test substances using standard species were compared with the endpoints estimated from TC measurements obtained in ERAMYC. This comparison showed that AMF are more than 5 times more sensitive to three of the seven substances tested in WP2 (i.e. Tebuconazole, Azoxystrobin and Pendimethalin) than the standard organisms currently considered in ERA. Therefore, AMF are probably not being sufficiently protected considering the assessment fator of 5 currently used in ERA schemes, which highlights the urgency of including AMF for the refinement of future PPP risk assessment schemes in EU.

Data regarding nominal and initial measured concentrations of the test substances were compared and used to infere on the relevance of performing chemical determination on soil samples to complement and support risk assessment. Chemical analysis showed, that already at test start 80 % of nominal concentrations were not reached for the majority of test substances. During the project, the estimated endpoints based on nominal concentrations were, generally, within a factor of 5 of the estimated endpoints based on initial measured concentrations. However, in some cases (namely for Tebuconazole, Terbuthylazine and Azoxystrobin), the endpoints derived from nominal concentrations were more than 5 times – up to 50000 fold - higher than those derived from measured concentrations, which suggests that the use of nominal concentrations may lead to insufficiently protective measures for AMF. Chemical analyses can produce relevant data to complement and confirm the accuracy of the methods applied during the performance of the tests and to produce more precise endpoints, enabling the refinement of risk evaluation.

WP3 – Performance of a ring test

The test protocols developed and agreed in WP2 were used to a ring test (interlaboratory tests), involving the members of the consortium and other laboratories among Europe that expressed interest in participating.

The ring test was carried out under the coordination of CloverStrategy and implied the elaboration of clear and detailed test protocols that each laboratory had to follow. In WP3, AMF pre-symbiotic and symbiotic phase tests were carried out with 2 chemicals (the fungicides Fluazinam and Azoxystrobin, both as pure a.i.), 1 AMF species (*Rhizophagus irregularis*) and 1 soil (the OECD 2.5% artificial soil). The *in vivo* symbiotic phase tests were carried out using one host plant (leek, *Allium ampeloprasum*).

Results obtained in WP3

In the symbiotic phase ring test, from the eighteen experiments conducted, eleven were valid (six for Azoxystrobin and five for Fluazinam), being seven not valid due to the low root colonization and/or high coefficients of variation in controls. Valid tests showed a tendency (in general more evident for Azoxystrobin, but not statistically significant) to reduce percentages of total and arbuscular colonization and extra-radicular hyphae length along the increase of the test concentrations, despite the high variability observed, especially in AC and EHL measurements. The reproducibility of the method could not be verified as there were not sufficient confidence intervals to allow the estimation of the repeatability of the method. Precision of the method could not be confirmed either, due to the high variability of the

estimated endpoints between laboratories and the reduced number of data sets considered that made the warning limits too wide.

In the pre-symbiotic phase tests, from the twelve experiments conducted, three were valid (two for Azoxystrobin and one for Fluazinam). Most tests were not valid due to low germination rate and low number of recovered spores at the end of the tests (the percentage of germinated spores was lower than 75% in control and/or the number of recovered spores was not higher than 25 in all test treatments). The number of data sets obtained in this ring test was insufficient to evaluate the reproducibility and/or precision of the method.

The inclusion of more participanting laboratories, the adaptation of some of the methodologies applied during the ring test experiments (e.g. use of more replicates in the negative control and/or use of propagules from in-vitro cultivations), and a more customized or in person training of the less experienced laboratories could contribute to reduce the variability observed in the results of the ring test and to increase the number of valid data sets in both AMF pre-symbiotic and symbiotic testing.

WP4 – Drafting of an OECD Test Guideline

Two drafts for OECD guidance documents were prepared, contemplating both pre-symbiotic and symbiotic phases of the AMF life cycle. These documents were based on the methodologies discussed and agreed during the ERAMYC project, being supported by the systematic literature review of WP1, on the data obtained in the experiments of WP2, on data generated in the ring test of WP3 and on the information available in existing international standards for laboratory testing with AMF.

Zusammenfassung

Einleitung

Arbuskuläre Mykorrhizapilze (AMF) sind Organismen, die in obligatorischer Symbiose mit den meisten Gefäßpflanzenarten leben. Sie spielen eine wichtige Rolle in Bodenökosystemen, indem sie die Nährstoffaufnahme der Pflanzen (insbesondere von Phosphor) und ihre Toleranz gegenüber verschiedenen Umweltstressoren (z. B. Metalle, Krankheitserreger, Dürre, Salzgehalt und andere) erhöhen und die Kohlenstoffbindung im Boden sowie die Bodenaggregation und -stabilisierung verbessern. Aufgrund ihrer anerkannten ökologischen Relevanz bei der Unterstützung wichtiger Bodenfunktionen sowie ihrer Empfindlichkeit gegenüber Chemikalien wurden AMF von der EFSA als potenzielle Gruppe von Nichtziel-Testorganismen identifiziert, die in die Standardtestbatterie aufgenommen werden sollen, um die Auswirkungen von PSM auf Bodenorganismen zu bewerten (EFSA, 2017). Als Bodenorganismen sind AMF einer Vielzahl von Chemikalien ausgesetzt, die insbesondere in landwirtschaftlichen Böden vorkommen (z. B. Pflanzenschutzmittel, Tierarzneimittel, Metalle). Das potenzielle Risiko, das sich aus dieser Exposition ergibt, ist weitgehend unbekannt und wird in der Regel bei der Bewertung ökologischer Risiken (ERA) außer Acht gelassen. Zusätzlich zu den begrenzten Informationen über die Auswirkungen von Chemikalien auf AMF sind die zur Bewertung dieser Auswirkungen angewandten Methoden (z. B. Auswahl von AMF und Wirtspflanzenarten, berücksichtigte Endpunkte) in veröffentlichten Studien häufig inkonsistent.

Das ERAMYC-Projekt wurde mit dem Hauptziel entworfen, einige der in der wissenschaftlichen Stellungnahme der EFSA festgestellten Lücken zu schließen (EFSA, 2017). Dies umfasste die Entwicklung und Standardisierung neuer Testprotokolle und die Verfeinerung bestehender Leitliniendokumente zur AMF-Prüfung mit dem Endziel, diese Organismen in ERA-Programme aufzunehmen.

Um diese Ziele zu erreichen, wurde die im Rahmen des ERAMYC-Projekts entwickelte Arbeit in vier Hauptabschnitte unterteilt. Abschnitt 1 umfasste eine in WP1 durchgeführte Literaturübersicht über die Auswirkungen organischer Schadstoffe auf die präsymbiotischen und symbiotischen Phasen von AMF; Abschnitt 2 umfasste die Arbeiten zur Entwicklung und Prüfung eines Protokolls für die Prüfung der symbiotischen Phase von AMF basierend auf den Ergebnissen von Laborexperimenten, die ohne Chemikalien und mit Chemikalien in WP2 durchgeführt wurden. In einer zweiten Phase von WP2 wurden Experimente durchgeführt, die darauf abzielten, eine Referenzsubstanz, die Chemikalien und die in den symbiotischen und präsymbiotischen Ringtests zu verwendende Strategie zu bestimmen; Abschnitt 3 umfasste die in WP3 entwickelte Arbeit, die die Durchführung der symbiotischen und präsymbiotischen Ringtests beinhaltete; Abschnitt 4 umfasste die Verfeinerung und Anpassung der während der symbiotischen und präsymbiotischen Tests getesteten Protokolle für eine künftige Veröffentlichung/einen künftigen Vorschlag (WP4) für eine neue OECD-Richtlinie, basierend auf den in den Experimenten von WP2 und WP3 erzielten Ergebnissen.

WP1 – Systematische Literaturrecherche und vergleichende Bewertung der Studien mit AM-Pilzen, die Chemikalien ausgesetzt wurden

Das Hauptziel von WP1 im Rahmen des ERAMYC-Projekts war die Durchführung einer Literaturrecherche auf der Grundlage von Studien, in denen AMF Chemikalien ausgesetzt wurden, um Informationen auf verschiedenen Ebenen zu sammeln (z. B. bewertete Chemikalien, verwendete AMF- und Wirtspflanzenarten, gewählte Testbedingungen und gemessene Parameter, einschließlich ihrer Empfindlichkeit gegenüber den getesteten Chemikalien) und diese Informationen zu synthetisieren, um verschiedene Aspekte des Protokolls zu definieren, das in den nächsten Phasen des Projekts weiterentwickelt und ringgetestet werden soll. Die gesammelten Informationen werden entscheidend dazu beitragen, die beste Kombination aus AMF, Pflanzenarten, Testbedingungen und Testendpunkten auszuwählen, die zur Bewertung der Auswirkungen organischer Chemikalien auf die präsymbiotischen und symbiotischen Phasen des AMF-Lebenszyklus verwendet werden soll. Die Auswahl der AMF-Arten wird auch durch eine zusätzliche Suche nach der weltweiten Verbreitung von AMF-Taxa unterstützt, um ihre geografische Repräsentativität zu bewerten.

Methoden der Literaturrecherche

Es wurden zwei unabhängige Suchvorgänge durchgeführt:

1. Es wurde eine Suche zur Bewertung der biogeografischen Verbreitung von AMF durchgeführt und die 20 am häufigsten gemeldeten AMF-Gruppen weltweit wurden anhand ihres Vorkommens bewertet. Diese Suche wurde in spezialisierten AMF-Datenbanken durchgeführt, nämlich PlutoF (Abarenkov et al., 2010), MaarjAM (Öpik et al., 2010, Stürmer et al., 2018), Fungal Traits (Põlme et al., 2021) und der NCBI-Website.
2. Es wurde eine systematische Literatursuche in verschiedenen Datenbanken (siehe unten) durchgeführt, um Informationen zu (i) den Auswirkungen von Chemikalien auf AMF und

(ii) dem Einfluss abiotischer Parameter auf die AMF-Leistung sowohl in der präsymbiotischen als auch in der symbiotischen Phase abzurufen. Diese Suche wurde im April und Mai 2021 auf den Plattformen Scopus, ScienceDirect, PubMed, Web of Science, SciELO und Google Scholar durchgeführt. Spezifische Suchbegriffe sind in den jeweiligen Abschnitten angegeben, in denen die Ergebnisse präsentiert werden.

Ergebnisse der Literaturrecherche

Insgesamt wurden 322 Forschungsarbeiten gefunden, aber nur 53 davon erfüllten die Ziele dieser Recherche und wurden genauer analysiert. Die Informationen über die getesteten Chemikalien und Wirkstoffe (a.i.), die verwendeten AMF-Arten und Wirtspflanzen sowie die in ökotoxikologischen Studien angewandten Testbedingungen können wie folgt zusammengefasst werden:

1. Die am häufigsten untersuchten Chemikalien waren Fungizide (F; 41 a.i.), Herbizide (H; 16 a.i.), Arzneimittel für Tier- und Humanmedizin (15), biologische Pestizide (6), Desinfektionsmittel (4), Körperpflegeprodukte (PCP; 2), Insektizide (I; 2 a.i.) und Pestizidmischungen (12 Formulierungen, 2 mit Fungiziden+Insektiziden; 8 mit Fungiziden; 2 mit Herbiziden).
2. Die am häufigsten untersuchten Wirkstoffe waren Glyphosat (H) (11 Studien), Benomyl (F) (9), Carbendazim (F) (8), Fenhexamid (F) (8), Captan (F) (6), Azoxystrobin (F) (5), Chlorothalonil (F) (5), Mancozeb (F) (5), Pentachlornitrobenzol (F) (5), Flutholanil (F) (4), Triclosan (PCP) (4), Alachlor (H) (3), Dicamba (H) (3), Dimethoat (I) (3), Fenpropimorph (F) (3), Fosethylaluminium (F) (3) und Pencycuron (F) (3). 3. Die am häufigsten verwendeten AMF-Arten waren *Funneliformis mosseae* (27 Studien), *Claroideoglomus etunicatum* (14), *Rhizophagus intraradices* (13), *Rhizophagus clarus* (8), *Gigaspora margarita* (6), *Glomus aggregatum* (4), *Rhizophagus irregularis* (4), *Scutellospora heterogama* (4), *Acaulospora laevis* (3), *Funneliformis caledonium* (3), *Gigaspora rosea* (3) und *Glomus monosporum* (3). Dies steht in enger Übereinstimmung mit den in Abschnitt 2.3 vorgestellten Erkenntnissen zur weltweiten Verbreitung von AMF.
3. In den in der vorhandenen Literatur beschriebenen Experimenten wurden 28 Arten von Wirtspflanzen verwendet. Die am häufigsten verwendete Pflanze war *Zea mays* (6 Studien), gefolgt von *Glycine max* (5), *Daucus carota* (in vitro transformierte Wurzeln) (5), *Triticum aestivum* (4), *Pisum sativum* (3), *Capsicum annum* (2), *Citrus aurantium* (2), *D. carota* (2), *Leucaena leucocephala* (2), *Phaseolus vulgaris* (2) und *Trifolium subterraneum* (2).
4. Die meisten Arbeiten konzentrierten sich auf die präsymbiotische Phase (27), die symbiotische Phase (15) oder beide (11). Es wurden Experimente mit natürlichen Böden (20 Studien), natürlichem Boden gemischt mit Sand (11), künstlichen Böden (3), Sand (5) und Kulturmedium (in vitro) (17) als Testmedium durchgeführt. Studien mit natürlichen Böden ergaben pH-Werte zwischen 6,3 und 8,2; die pH-Werte des künstlichen Bodens wurden auf 6,0 (± 0,5) eingestellt und die des Sandes variierten zwischen 6,0 und 7,0.
5. Die meisten Studien, die sich auf die präsymbiotische Phase konzentrierten, berichteten von Experimenten, die im Dunkeln durchgeführt wurden, und nur zwei Studien verwendeten eine Photoperiode von 16:8 Stunden Licht/Dunkelheit. Bei den meisten Experimenten wurden Temperaturen von 25 °C, 27 °C und 28 °C ± 2 °C verwendet, mit zwei Ausnahmen, bei denen Temperaturen von 22 °C und 26 °C getestet wurden.

Schlussfolgerungen aus der Literaturrecherche

Bei diesem ersten Ansatz stellten wir fest, dass die am Besten untersuchte Gruppe von Chemikalien die Gruppe der Fungizide war. Der am Besten untersuchte Wirkstoff war Glyphosat. Die am häufigsten verwendeten AMF-Arten waren Funneliformis mosseae, Claroideoglomus etunicatum und Rhizophagus intraradices. Es ist wichtig hervorzuheben, dass es überwiegend Studien gibt, die die Wirkung von Chemikalien nur auf der Ebene der präsymbiotischen Phase berichten, und dass es nur wenige Daten zu den Auswirkungen auf die symbiotische Phase gibt. Im Allgemeinen fehlen Daten zur Wirkung von Bioziden, Tierarzneimitteln und Insektiziden auf AMF-Arten.

WP2 – Durchführung ökotoxikologischer Tests mit AMF – Entwicklungsphase

Die für AP2 festgelegte Strategie basierte auf der in Abschnitt 1 dieses Abschlussberichts vorgestellten Übersicht und den Erfahrungen der Konsortiumsmitglieder.

AP2 war in zwei Phasen (zwei Versuchscluster) unterteilt, um Methoden für die Durchführung von Tests in der symbiotischen Phase zu definieren und zu verfeinern. In der ersten Phase wurden keine Chemikalien verwendet. Ziel war es, die Leistungsfähigkeit/Eignung der ausgewählten AMF-Arten in den ausgewählten Böden unter spezifischen Testbedingungen (Umweltbedingungen und ausgewählte Wirtspflanzen) zu bewerten. Die wichtigsten Parameter wurden bewertet, um die operationellen Aspekte ihrer Messung und die intraspezifische Variabilität zu beurteilen und geeignete Validitätskriterien für Kombinationen von AMF-Arten/gemessenen Parametern zu definieren. In der zweiten Phase wurden Testchemikalien eingesetzt, um zu beurteilen, wie die verschiedenen gemessenen AMF-Arten und Parameter auf die ausgewählten Chemikalien reagieren. Die Ergebnisse dieser zweiten Phase dienten der Auswahl von Testsubstanzen für die Ringversuche (Ringversuche) von AP3.

Ergebnisse aus WP2

Die in Arbeitspaket 2 gewonnenen Daten waren entscheidend für die Definition der Methoden für die nächsten Projektschritte, insbesondere für die Ringversuche in Arbeitspaket 3, und damit für die Ausarbeitung des Vorschlags für die neue OECD-Standardmethode.

Vor Beginn der Experimente musste die Auswahl der Böden, der AMF-Arten und der Wirtspflanze unterstützt werden:

- Die Böden für die Tests wurden anhand ihrer Eigenschaften mittels einer Hauptkomponentenanalyse (PCA), ihrer Verfügbarkeit und ihrer Eignung für die AMF-Entwicklung ausgewählt. Fünf Böden wurden ausgewählt: ein modifizierter Kunstboden (mit 2,5 % organischer Substanz) und vier natürliche Böden (ein natürlicher Boden pro Land der Konsortialpartner, d. h. Portugal, Estland und Brasilien, sowie ein natürlicher Referenzboden aus Deutschland);
- Vier AMF-Arten – *Rhizophagus irregularis*, *Rhizophagus clarus*, *Gigaspora margarita* und *Funneliformis mosseae* – wurden basierend auf den Daten aus der Literaturrecherche in Arbeitspaket 1 und der Verfügbarkeit von Impfmittellieferanten ausgewählt;
- Zwei Wirtspflanzenarten, *Solanum lycopersicum* (Tomate) und *Allium ampeloprasum* (Lauch), wurden aufgrund ihrer Verwendung in früheren ökologischen Studien mit AMF ausgewählt.

Nach der Auswahl dieser Komponenten begann der erste Teil der experimentellen Phase von WP2 mit dem Hauptziel, die Methodik für Experimente in der symbiotischen Phase zu verfeinern und die Eignung der ausgewählten AMF zu bewerten. Folgende Aspekte wurden bewertet:

- Art der AMF-Inokulation (Anzahl der Sporen vs. Gewicht des gemischten Inokulums): Die Inokulation mit gemischtem Inokulum war am besten geeignet, um einen angemessenen Prozentsatz an AMF-Besiedlung zu erreichen.
- Potenzial des Inokulums: Abschätzung der Menge (d. h. des Gewichts) des gemischten Inokulums, die für eine akzeptable AMF-Besiedlung erforderlich ist.
- Wirtspflanze: Als Wirtspflanze für die folgenden Phasen wurde Lauch ausgewählt, da die AMF-Besiedlung bei dieser Art generell höher war als bei Tomaten.
- Entwicklungsphase der Wirtspflanze zu Beginn des Versuchs: Pralle Samen ohne Anzeichen einer Keimung eignen sich für diese Versuche besser, da die anfängliche Phase der Pflanzenentwicklung für die Etablierung der Pflanze-AMF-Symbiose entscheidend ist.
- Parameterendpunkte und akzeptable Ergebnisse (zur Testvalidierung): Die intraradikuläre Besiedlung (gesamt und arbuskulär) und die extraradikuläre Hyphenlänge wurden als Parameter für die Tests in der symbiotischen Phase ausgewählt, da sie die Stärke der AMF-Pflanzen-Symbiose widerspiegeln. Ein Prozentsatz von 30 % Gesamtbesiedlung wurde als Mindestanforderung zur Validierung der Tests angesehen.

Weitere technische Aspekte der Methodik, wie die Färbemethode, die Notwendigkeit der Verwendung einer Nährlösung zur Pflanzenbewässerung und die Zugabe von Sand-/Glasperlen in die Bodenoberfläche zur Vermeidung von Bodenwasserverlusten während der Experimente, wurden ebenfalls in dieser Phase behandelt.

Basierend auf den in dieser ersten Phase erzielten Ergebnissen wurden erste Entwürfe der Standardarbeitsanweisungen (SOPs) erstellt. Die in diesen Entwürfen beschriebenen Methoden wurden vom Konsortium in der zweiten Phase von WP2 bei Tests mit Schadstoffen angewendet.

Die Hauptziele der zweiten Phase von WP2 waren 1) die Definition einer Referenzsubstanz für Positivkontrollen und 2) die Auswahl geeigneter Chemikalien für die Ringversuche von Labortests in der symbiotischen und präsymbiotischen Phase von AMF.

Borsäure wurde als erste Chemikalie als Referenzsubstanz für Tests in der symbiotischen Phase von AMF vorgeschlagen, da diese Chemikalie bereits in ökotoxikologischen Tests als Referenzsubstanz für andere Organismen verwendet wurde. Allerdings zeigte sich eine höhere Toxizität von Borsäure für die Wirtspflanze im Vergleich zur AMF-Wurzelbesiedlung, die in Replikaten mit Pflanzenbiomasseproduktion selbst bei den höchsten getesteten Konzentrationen unbeeinflusst blieb. Daher wurde der Schluss gezogen, dass Borsäure nicht als Referenzsubstanz in Tests der symbiotischen Phase von AMF geeignet ist.

Als Referenzsubstanz wurde Kupfersulfat vorgeschlagen. Die mit Kupfersulfat erzielten Ergebnisse ließen hinsichtlich seiner Eignung als Referenzsubstanz keine eindeutigen Schlüsse zu, da der Zusammenhang zwischen den Kupfersulfatkonzentrationen und den Auswirkungen auf die Pflanzenbiomasseproduktion und die AMF-Wurzelbesiedlung von *F. mosseae* unklar war. Bei *R. irregularis* schienen die toxischen Effekte auf die Wirtspflanze stärker zu sein als die Effekte auf die AMF-Besiedlung. Dies könnte jedoch auf den verwendeten

Konzentrationsgradienten zurückzuführen sein, der möglicherweise zu groß zwischen den Testkonzentrationen war.

Da sich weder Borsäure noch Kupfersulfat als Referenzsubstanz in Tests zur symbiotischen AMF-Phase als geeignet erwiesen, wurde beschlossen, zwei Chemikalien aus einer Liste landwirtschaftlicher Wirkstoffe für die Ringversuche auszuwählen und je nach den erzielten Ergebnissen eine davon als Referenzsubstanz für die Methode auszuwählen.

Fünf Wirkstoffe (3 Fungizide: Azoxystrobin, Fluazinam und Tebuconazol + 2 Herbizide: Pendimethalin und Terbuthyazin) wurden in künstlichen und natürlichen Böden sowie an drei AMF-Arten mit Lauch als Wirtspflanze getestet. Das Hauptziel dieser Experimente war die Auswahl von Chemikalien für die Ringversuche von AP3. Azoxystrobin und Fluazinam waren die ausgewählten Chemikalien für die Ringversuche. Beide Substanzen wurden im künstlichen Boden (d. h. künstlichem OECD-Boden mit 2,5 % OM) und in natürlichen Böden aus Portugal, Brasilien, Estland und einem Referenzboden aus Deutschland getestet.

Die in den Experimenten von AP2 gewonnenen Daten wurden in prädiktiven verallgemeinerten linearen Modellen verwendet, um den Einfluss der Bodeneigenschaften auf die AMF-Kolonisierung und auf die Empfindlichkeit der AMF gegenüber Chemikalien im Boden zu bewerten.

Der Einfluss der Bodeneigenschaften auf die AMF-Besiedlung wurde unter Berücksichtigung der Durchschnittswerte der AMF-Gesamt- (TC) und Arbuskulärbesiedlung (AC) sowie der extraradikulären Hyphenlänge (EHL) in Negativkontrollen von Experimenten mit *R. irregularis* und *F. mosseae* sowie Lauch aus beiden Phasen von WP2 als Antwortvariablen und der Bodeneigenschaften – Boden-pH-Wert, Gehalt an organischer Substanz, Kationenaustauschkapazität, Sand-, Schluff- und Tongehalt, austauschbare Aluminiumkonzentration sowie extrahierbare Phosphor-, Eisen- und Mangankonzentrationen – als kontinuierliche erklärende Variablen sowie der AMF-Arten und des Labors als nominale erklärende Variablen bewertet. Durchschnittliche TC- und AC-Messungen zeigten keinen signifikanten Einfluss der Bodeneigenschaften. Andererseits wurde die durchschnittliche EHL signifikant durch die extrahierbaren Eisen- und Phosphorkonzentrationen, den Boden-pH-Wert und die Kationenaustauschkapazität beeinflusst. Auch das Labor hatte einen signifikanten Einfluss auf die EHL-Messungen.

Der Einfluss der Bodeneigenschaften auf die AMF-Empfindlichkeit gegenüber Chemikalien im Boden wurde unter Berücksichtigung der für die drei AMF-Parameter (d. h. TC, AC und EHL) geschätzten Endpunkte in den Experimenten der zweiten Phase von WP2 mit *R. irregularis* und Azoxystrobin oder Fluazinam als Antwortvariablen bewertet. Dieselben Bodeneigenschaften wie in der vorherigen Bewertung wurden als kontinuierliche erklärende Variablen verwendet, Labor und Testsubstanz als nominale erklärende Variablen. In den GLMs, die die geschätzten Endpunkte für die drei AMF-Parameter als Antwortvariable verwendeten, wurden keine signifikanten erklärenden Variablen gefunden, mit Ausnahme eines GLMs mit „Labor“ als erklärender Variable für EC10-Werte basierend auf TC-Messungen.

Obwohl GLMs im Allgemeinen keine signifikanten erklärenden Variablen aufwiesen, erklärten sie einen höheren Prozentsatz der beobachteten Varianz, wenn die Variablen „Labor“ und „Chemikalien“ für alle AMF-Parameter berücksichtigt wurden (TC erklärte die höchsten Prozentsätze, gefolgt von AC und EHL). Das Fehlen statistischer Signifikanz für die Variable „Chemikalie“ in den GLMs lässt darauf schließen, dass zur Verbesserung der Robustheit der Analyse ein Datensatz mit geschätzten Endpunkten für eine größere Anzahl (und eine größere Vielfalt) von Substanzen erforderlich wäre.

Die in der zweiten Phase von WP2 erhobenen Daten wurden auch aus der Perspektive der Risikobewertung analysiert, um zu verstehen, ab wann AMF durch die aktuelle Risikobewertung für Pflanzenschutzmittel in der Europäischen Union geschützt sind. Die in der Literatur für die sieben Testsubstanzen unter Verwendung von Standardspezies berichteten Endpunkte wurden mit den aus den in ERAMYC ermittelten TC-Messungen geschätzten Endpunkten verglichen. Dieser Vergleich zeigte, dass AMF gegenüber drei der sieben in WP2 getesteten Substanzen (Tebuconazol, Azoxystrobin und Pendimethalin) mehr als fünfmal empfindlicher reagieren als die derzeit in der Risikobewertung berücksichtigten Standardorganismen. Daher sind AMF unter Berücksichtigung des derzeit in Risikobewertungssystemen verwendeten Bewertungsfaktors von 5 wahrscheinlich nicht ausreichend geschützt. Dies unterstreicht die Dringlichkeit, AMF in die Weiterentwicklung zukünftiger Risikobewertungssysteme für Pflanzenschutzmittel in der EU einzubeziehen.

Die chemische Analyse zeigte, dass bereits zu Beginn der Prüfung 80 % der Nennkonzentrationen für die meisten Prüfsubstanzen nicht erreicht wurden. Daten zu nominalen und anfänglich gemessenen Konzentrationen der Testsubstanzen wurden verglichen und verwendet, um Rückschlüsse auf die Relevanz chemischer Bestimmungen an Bodenproben zur Ergänzung und Unterstützung der Risikobewertung zu ziehen.

Während des Projekts lagen die auf Nominalkonzentrationen basierenden geschätzten Endpunkte auf Grundlage der nominalen Konzetrationen größtenteils um den Faktors 5, verglichen mit Endpunkte auf Basis initial gemessener Konzentrationen In einigen Fällen (insbesondere bei Tebuconazol, Terbuthylazin und Azoxystrobin) waren die aus Nominalkonzentrationen abgeleiteten Endpunkte jedoch mehr als fünfmal – bis zum 50000 fachen - höher als die aus gemessenen Konzentrationen abgeleiteten. Dies deutet darauf hin, dass die Verwendung von Nominalkonzentrationen zu unzureichenden Schutzmaßnahmen für AMF führen kann. Chemische Analysen können relevante Daten liefern, um die Genauigkeit der bei der Durchführung der Tests angewandten Methoden zu ergänzen und zu bestätigen sowie präzisere Endpunkte zu ermitteln und so die Risikobewertung zu verfeinern.

WP3 – Durchführung eines Ringtests

Die in WP2 entwickelten und vereinbarten Testprotokolle wurden für einen Ringtest (Ringversuche) verwendet, an dem die Mitglieder des Konsortiums und andere Labore in Europa teilnahmen, die Interesse an einer Teilnahme bekundeten.

Der Ringtest wurde unter der Koordination von CloverStrategy und CloverStrategy durchgeführt und beinhaltete die Ausarbeitung klarer und detaillierter Testprotokolle, die jedes Labor befolgen musste. In WP3 wurden AMF-Tests in der präsymbiotischen und symbiotischen Phase mit 2 Chemikalien (den Fungiziden Fluazinam und Azoxystrobin, beide als reine Wirkstoffe), 1 AMF-Art (*Rhizophagus irregularis*) und 1 Boden (dem 2,5 %igen künstlichen Boden der OECD) durchgeführt. Die Tests in der symbiotischen Phase in vivo wurden mit einer Wirtspflanze (Lauch, Allium ampeloprasum) durchgeführt.

Ergebnisse aus WP3

Im Ringtest der symbiotischen Phase waren von den 18 durchgeführten Experimenten elf valide (sechs für Azoxystrobin und fünf für Fluazinam). Sieben Experimente waren aufgrund der geringen Wurzelbesiedlung und/oder hoher Variationskoeffizienten in den Kontrollen ungültig. Valide Tests zeigten eine Tendenz (im Allgemeinen deutlicher für Azoxystrobin, jedoch nicht statistisch signifikant) zu einer Abnahme der Prozentsätze der Gesamt- und

Arbuskelbesiedlung sowie der extraradikulären Hyphenlänge mit zunehmender Testkonzentration, trotz der beobachteten hohen Variabilität, insbesondere bei AC- und EHL-Messungen. Die Reproduzierbarkeit der Methode konnte nicht überprüft werden, da die Konfidenzintervalle für die Abschätzung der Wiederholbarkeit der Methode nicht ausreichten. Auch die Präzision der Methode konnte aufgrund der hohen Variabilität der geschätzten Endpunkte zwischen den Laboren und der geringen Anzahl berücksichtigter Datensätze, die die Warngrenzen zu weit gefasst machten, nicht bestätigt werden.

In den Tests der präsymbiotischen Phase waren von den zwölf durchgeführten Experimenten nur drei valide (zwei für Azoxystrobin und eines für Fluazinam). Die meisten Tests waren ungültig, da am Ende der Tests der Anteil gekeimter Sporen in der Kontrolle unter 75 % lag und/oder die Anzahl der wiedergefundenen Sporen in allen Testbehandlungen nicht höher als 25 war. Die Anzahl der in diesem Ringtest erhaltenen Datensätze reichte nicht aus, um die Reproduzierbarkeit und/oder Präzision der Methode zu bewerten.

Die Einbeziehung weiterer teilnehmender Labore, die Anpassung einiger der während der Ringtestexperimente angewandten Methoden (z. B. Verwendung von mehr Replikaten in der Negativkontrolle und/oder Verwendung von Propagule aus In-vitro-Kultivierungen) und eine individuellere oder persönliche Schulung der weniger erfahrenen Labore könnten dazu beitragen, die beobachtete Variabilität der Ringtestergebnisse zu verringern und die Anzahl gültiger Datensätze sowohl in den präsymbiotischen als auch in den symbiotischen AMF-Tests zu erhöhen.

WP4 – Erstellung einer OECD-Testrichtlinie

Es wurden zwei Entwürfe für OECD-Leitlinien erstellt, die sowohl die präsymbiotische als auch die symbiotische Phase des AMF-Lebenszyklus berücksichtigen. Diese Dokumente basieren auf den im ERAMYC-Projekt diskutierten und vereinbarten Methoden und werden durch die systematische Literaturrecherche von AP1, die in den Experimenten von AP2 gewonnenen Daten, die im Ringversuch von AP3 generierten Daten und die in bestehenden internationalen Normen für Labortests mit AMF verfügbaren Informationen unterstützt.

1 Section 1 - WP1: Systematic literature review and a comparative assessment of the studies with AM fungi exposed to chemicals

1.1 Introduction

Soil is a non-renewable resource hosting an enormous diversity of organisms in a dynamic and interactive system, providing a variety of essential ecological, economic, and social functions (EC, 2003). Interaction between soil organisms results in important ecological functions such as organic matter decomposition, nutrient cycling, biotic regulation, and maintenance of soil structure (Delgado-Baquerizo et al., 2016; Pretty, 2018).

Expansion and intensification of human activities contribute to the deposition of large amounts of chemical compounds in the soil. Not occurring naturally in the environment, these contaminants are generated from key anthropogenic activities like agriculture (e.g. plant protection products (PPP) applications), zoonoses control, disposal of urban waste as soil amendments and accidental disposals of industrial wastes (Rajput et al., 2020). Soil contamination can lead to multiple negative consequences to soil organisms and soil food webs, reducing biodiversity of terrestrial ecosystems (Tibbett et al., 2020). A loss in diversity can, consequently, impair the function of biological systems and contribute to the loss of several key soil functions (Cardinale et al., 2012; Wagg et al., 2014).

One of the groups of organisms that can be affected by soil pollution are arbuscular mycorrhizal fungi (AMF) (Mallmann et al., 2018; Malfatti et al., 2021; Rabab & Reda, 2019). AMF form specialized associations (symbiosis) with roots of about 72% of vascular plant species, including native and most cultivated species (Brundrett & Tedersoo, 2018). AMF are components of soil microbiota and are widely distributed across terrestrial ecosystems (McGuire et al., 2008; Beauregard et al., 2013, Davison et al., 2015). In the symbiosis, the host plant supplies the fungi with sources of energy that allow their maintenance and growth via photosynthates (Smith & Read, 2008). On the other hand, the fungi provide several direct (e.g., enhance nutrient absorption, promote water transference to the plant, and improve the plant pathogen defense system) and indirect benefits to the plant (e.g., promote formation and stabilization of soil structure) (Louarn et al., 2012; Demenois et al., 2018; Zhang et al., 2019).

Although the AMF life cycle depends on the establishment of the symbiosis (obligatory symbiotrophy), spore germination can occur without a host – the so-called pre-symbiotic phase. Germination, germ tube formation, and asymbiotic hyphal growth are important phases for the establishment of the symbiosis and could be affected by the presence of pollutants in the soil (Hage-Ahmed et al., 2019; Mallmann et al., 2018; Malfatti et al., 2021). Effects of chemicals on these phases can impact AMF life cycle directly, resulting in no-formation, reduction, or even inhibition of mycorrhizal colonization of plant roots (Maia et al., 2010; Mallmann et al., 2018; Malfatti et al., 2021).

Nowadays, ecotoxicological evaluations of soil pollutants are carried out using various tests with living organisms (especially soil invertebrates and plants). These assays are standardized by entities such as the Organization for Economic Cooperation and Development (OECD) and the International Organization for Standardization (ISO). The standardization of methods used in this type of tests has been widely recommended, since it allows comparison of results obtained under different circumstances and laboratories, thus improving robustness of the

generated data. These protocols are based on the use of standard species representatives of key groups of organisms. Most of the soil ecotoxicological studies are based on non-target invertebrates, particularly earthworms (ISO, 2023b; OECD, 2016a), collembolans (ISO, 2023c; OECD, 2016c), mites (OECD, 2016b) and enchytraeids (ISO, 2023) that are used as bioindicators of toxicity. Standard species usually have a broad geographic distribution, relevant ecological roles, live in permanent contact with soil, have fast reproduction, and are easily cultured in laboratory conditions (Römbke et al., 1996; OEHHA, 2009).

In addition to the organisms belonging to soil mesofauna and macrofauna, microorganisms could provide significant information that can support the interpretation of the effects attributed to soil disturbances provoked by the presence of pollutants (Bossio et al., 2005). In general, tests using microorganisms allow to assess community functions like carbon (OECD, 2000b) and nitrogen transformation in soil (OECD, 2000a).

Although it is thought that most of the soil microorganism's species are still unknown (Torsvik & Øvreås, 2007), among those already described, many have well-known ecological functions. In this context, AMF prove to have advantages as non-target test organisms, considering that AMF contribute for the majority of soil fungal biomass in arable systems (van-Groenigen et al., 2010), are relevant to terrestrial ecosystems, have functional specificity, and can be used in ecotoxicological tests (Basile, 2008). AMF comply with most of the criteria defined to consider an organism as a bioindicator. They are sensitive to soil pollutants (Mallmann et al., 2018; Malfatti et al., 2021) and variations in edaphoclimatic factors (Carvalho et al., 2012), are widely distributed around the globe (McGuire et al., 2008; Beauregard et al., 2013, Davison et al., 2015), and ecologically relevant to plant and ecosystem health (Begum et al., 2019). Thus, it is important to include AMF in ecological risk assessments of chemicals to be applied in soils, as suggested by the European Food Safety Authority (EFSA) (EFSA, 2017).

Concerning effects on the AMF symbiotic phase, the technical guidance document published by the Association Française de Normalisation (AFNOR) (AFNOR, 2014), describes a methodology to evaluate the effects of contaminants on this phase by evaluating the frequency of mycorrhization on the host plant roots. A protocol describing *in vivo* and *in vitro* experiments was proposed by Mallmann (2020) to evaluate the effects of PPPs in the symbiotic phase of AMF, using the fungicide chlorothalonil as a reference substance.

So, although there have been studies on these subjects, the knowledge gap related to the effect of soil contaminants on AMF species remains, especially when considering the symbiotic phase. Therefore, in order to comply with the main objective of the ERAMYC project, a compilation of the existing information regarding effects of chemicals towards AMF is important to take decisions at several levels (species and parameter selection, substrate to use, exposure conditions, etc.) and to steer the work on the next phases of the project culminating on the development of a standard guideline to test effects of chemicals on AMF pre-symbiotic and symbiotic phases.

The aim of this section is to provide a literature review, and a comparative assessment of the studies conducted to investigate the effect of chemicals in AMF species, focusing on both the pre-symbiotic and symbiotic phase of AMF life cycle. This review is structured in four topics. First, a brief introduction placing this review into the context of the ERAMYC project is presented (present topic 1.1) followed by an overview of AMF ecology with a biogeographical distribution survey about the most relevant AMF species and the influence of abiotic (temperature, soil pH and nutrients) and biotic (intraspecific variability) factors of AMF performance on topic 1.2. Topic 1.3 provides information about the effects of organic chemicals that reach the focus of the project (plant protection products, biocides and pharmaceuticals)

on AMF, both on the pre-symbiotic and symbiotic phases, with information on AMF species selected, host plants and soil types used, and exposure conditions (photoperiod and temperatures) adopted in the experiments. Topic 1.4 contains the main conclusions of the review.

1.2 Overview on the ecology and geographical distribution of AMF

1.2.1 Ecological relevance of investigating the AMF life cycle

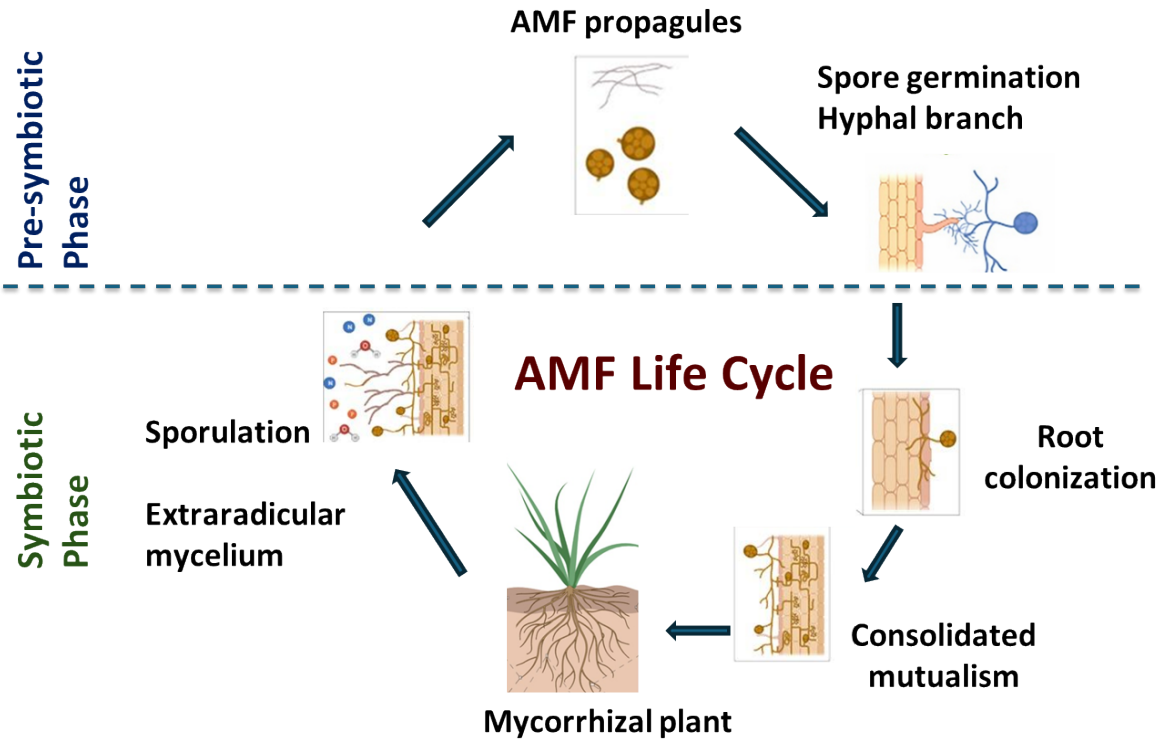
AMF are important components of soil microbiota, capable of forming mutualist symbiotic associations with most plant species (Öpik et al., 2006). Symbiosis between AMF and plant roots – named arbuscular mycorrhiza (AM) – is obligatory for fungi to complete their life cycle (Bentivenga et al., 2013; Powell & Rillig, 2018), which is divided in two typical phases: the pre-symbiotic and the symbiotic phase (Figure 1).

The pre-symbiotic phase consists of spore germination, germ tube formation and production/elongation of pre-symbiotic mycelium (Giovannetti et al., 2010; Maia et al., 2010). Spore germination can occur even without a host, utilizing the spores' own energetic reserves. Initially, spores absorb water/nutrients and swell, starting the synthesis of diverse compounds necessary to keep the process ongoing (Giovannetti et al., 2010; Maia et al., 2010). Sequentially, cellular division occurs, and cells are deposited in the place where the germ tube will be formed (some species can form various germ tubes). Afterwards, an initial aseptate mycelium is formed and develops into a mycelium network with variable extension, which will promote the contact between the host plant and the fungus (Maia et al., 2010). Thus, the symbiotic phase of the AMF life cycle relies on the success of spore germination (although other colonization routes by other propagules such as infected root fragments or extra-radical hyphae also occur in soil), pre-symbiotic mycelium growth and chemical signalling between fungi and the host plant, which allows recognition and promotes changes in both partners metabolism, allowing for the onset of the symbiosis (Berbara et al., 2006; Smith & Read, 2008; Stürmer & Siqueira, 2008). Once the pre-symbiotic hyphae contacts with the host plant roots, a structure called appressorium is differentiated. Rapidly, appressoria penetrates the root epidermis, setting the root cortex free to be colonized by the fungus. Colonization can be either intercellular or intracellular, depending on the AMF type. Inside the plant cell, between the cellular wall and the plasma membrane, AMF hyphae can turn into arbuscles. During the symbiosis, the arbuscules are responsible for most of the nutrient exchanges that occur between the host plant and the fungi (Stürmer & Siqueira, 2008). The plant provides photosynthates to the fungi, allowing them to grow and reproduce and, on the other hand, the fungi provide a range of services to the plant, notably increased mineral nutrition (especially in P and N), facilitated growth and productivity, pathogen protection, C sequestration, and resistance to stress caused by drought and soil pollutants (ISO, 2009; Louarn et al., 2012; Öpik et al., 2006; Smith & Read, 1997; Song et al., 2010; Souza et al., 2008).

Once intraradical colonization is established, hyphae begin to grow outside the root, forming an extra-radical mycelium network, responsible for "exploring" the soil (reaching places that roots cannot reach). This soil mycelium represents an important sink of carbon from the roots to the soil while allowing connections between plants (Smith & Read, 2008). The extra-radical mycelium also provides nutrient uptake, carrying them to the intraradical mycelium and finally transferring to the host (Berbara et al., 2006; Smith & Read, 2008; Stürmer & Siqueira, 2008). For some AMF species, intraradical hyphae can turn into reserve structures denominated vesicles. Extra-radical mycelium is also responsible for new propagule production,

differentiating into asexual spores (but spores not the only source of AMF propagules). Spore differentiation can occur in apical or intercalary portions of hyphae (in the latter case generally forming sporocarps) (Berbara et al., 2006). Some species can even produce spores inside host roots (Berbara et al., 2006; Stürmer & Siqueira, 2008).

Figure 1: AMF life cycle and development of a functional symbiosis.



Based on Akiyama (2007). Source: own illustration using BioRender; UDESC.

AMF live in close relationship with plants and distinct taxon composition in AMF communities can induce different growth responses in plants and influence structure and composition of vascular plant communities (Öpik et al., 2006). Information about the diversity and factors that affect the structure and functions of natural AMF communities contributes to understanding and identifying the factors shaping plant communities (Öpik et al., 2006; Souza et al., 2010). AMF relevance to the ecosystem, however, goes beyond nutritional improvements and enhanced plant growth (Vos et al., 2013; van der Heijden et al., 2015; Rahman et al., 2018). These fungi are directly and indirectly involved in the provision of ecosystem services such as soil formation, aggregate stability (Purin & Rillig, 2007; Rillig et al., 2015; Demenois et al., 2018; Wilkes et al., 2021), carbon stocks increase (Jansa & Treseder, 2017; McGowan et al., 2019), nutrient cycling (Cavagnaro et al., 2015; Frew, 2019), food web support (Antunes & Koyama, 2017), positive effects on primary (Nara, 2006) and secondary (Lerat et al., 2002; Schoonmaker et al., 2007; Teste et al., 2009, Vahter et al., 2020) successions establishment in terrestrial ecosystems (for example, nutrient acquisition, exchange of carbon and nutrients), also establishing a complex linked network between roots of plant communities (Rillig, 2004; Selosse & Duplessis, 2006). These effects shape the composition, interactions, and diversity within plant and AMF communities (Simard et al., 2012; Wubs et al., 2016; Vahter et al., 2020).

Because they are relevant to ecosystems and because they are sensitive to soil pollutants (Twanabasu et al., 2013a), AMF comprise a key-group to assess ecological risk of substances deposited in soil. This relevance has been highlighted by regulatory agencies like EFSA that pointed out the need to establish viable methodologies to assess effect of PPP on different phases of the AMF life cycle (EFSA, 2017).

1.2.2 AMF spore formation and germination

AMF are included on the group of Glomeromycetes (one of the divisions of the fungi kingdom) and reproduce mainly asexually by cloning their spores, denominated glomerospores (Goto & Maia, 2006). They constitute the main form of AMF propagation and constitute some of the biggest spores in the kingdom of fungi (22 to 1050 µm) (Souza et al., 2010). Spores are considered indicators of ecological processes and are able to reflect anthropogenic influence on AMF communities, since their presence in the soil is evidence of adequate root colonization, although they are not present during the entire life cycle of the fungi (Souza et al., 2008).

According to Stürmer & Siqueira (2008) and Maia et al. (2010), glomerospores present different formations, as described so far:

- Glomoid – presents glomerospore formation at terminal or intercalary portion of hyphae, it is common in *Glomus* genera, although present in *Ambispora*, *Archeospora*, *Diversispora*, *Pacispora*, and *Paraglomus* as well.

- Radial-glomoid – glomerospore formation from the terminal portion of hyphae, but radially, from a complex of central hyphae, occurs in *Glomus* species.

- Gigasporoid – spores are formed terminally in a bulbous hypha of *Gigaspora*, *Racocetra*, *Cetraspora*, *Fuscutata*, *Dentiscutata*, *Quatunica*, and *Scutellospora*.

- Acaulosporoid – present in *Acaulospora*, *Ambispora*, *Archeospora*, and *Otosporora*, they are formed laterally in the hyphae (hyphae neck), that connects to the terminal sporiferous saccule, which leaves a scar on the surface of the spore.

- Entrofosporoid – glomerospores are formed inside the hyphae that connects sporiferous saccule, which leaves two scars on the surface of the spore, typical for *Entrophospora*, *Kuklospora*, and *Intraspora*.

Although this characterization of groups based on spores exists, Azevedo & Souza (2007) pointed out the existence of dimorphism in *Glomus* species, since a same strain of AMF can have glomerospores with different sizes. In *Ambispora* as well, dimorphism has already been identified in species that present glomoid and acaulosporoid formation (Maia et al., 2010). Some authors consider this trait as a conservative strategy that possibly confers better adaptation capacity of some species (Azevedo & Souza, 2007).

If glomerospores are fundamental structures to initiate AMF life cycle - organisms known by their obligatory symbiosis - germination and hyphal growth are the precursory phases of symbiosis establishment and fungal survival (Maia et al., 2010).

Initial studies about glomerospores germination are dated to 1950 and refer to the germination process of *Endogone* species (Maia et al., 2010). Siqueira et al. (1985) published a review focused on data description and discussion concerning the germinative process of AMF spores. They indicated that germination proceeds following three main steps: peripheral shares, through the glomerospore wall and by regrowth of the sustentation hyphae, this last one occurring in *Glomus*. Further studies provided more information about these aspects of

glomeromycotans but are still far from being conclusive and are outdated for some genera (Maia et al., 2010).

Glomerospores are biological unities in dormancy that can be activated to start metabolic processes that induce germination and subsequent growth. The duration of the state of dormancy depends on AMF species (Maia et al., 2010), as well as the mechanisms that induce morphogenesis and growth of germ tube. Juge et al. (2002) observed that spore storage at low temperatures is a factor that could contribute to breaking dormancy, though the mechanisms that guide this process are not completely elucidated and are not present in all species. After water and nutrient absorption during the wet extraction process, spores could grow their volume by up to 40%, which triggers membrane modifications and activation of proteolytic enzymes previously inactive in the spore, leading to biochemical chains that finally induce germination (Moreira & Siqueira, 2006).

Germination can occur without a host. It starts after spore imbibition and continues until complete depletion of spore reserves. In the initial steps, after dormancy break, nucleic acids and essential proteins are synthetized to support morphogenesis and growth of the germ tube that can reach a few centimetres, and the following grouping and branching of cells forming an initial mycelium (Maia et al., 2010). Pre-symbiotic mycelium can grow only for a brief period by itself. If the host is absent, cytoplasm recoil occurs and mycelium growth stops, and the fungi enters in a new dormancy state, which characterize its obligatory biotrofism (Maia et al., 2010).

The main biochemical and phenotypical events of AMF spores and mycelium growth *in vitro* are chronologically present in Table 1. Outstanding factors that influence the speed and final rate of germination include pH, temperature, moisture, luminosity and radiation, oxygen and CO_2 concentration, nutrient content, microorganisms' presence, substrate composition, and pollutant effects.

Table 1: Biochemical and phenotypical events of *in vitro* spores' germination from 0 to 30 days.

Time (min/hours/days)	Events
0	Soaking and swelling
15 minutes	Start of ARN and protein synthesis
35 minutes	Proteins and neutral carbohydrates synthesis
45 minutes	Amino acid, ATP, and fatty acids synthesis
5 hours	Lipid synthesis
13 hours	Polysaccharide synthesis
29 hours	Non-detectable DNA synthesis
3-4 days	Germ tube primordium
5-7 days	Germ tube emergence
7-20 days	Growth and branching
20-30 days	Growth arrest and new dormancy

Adapted from Moreira & Siqueira, 2006.

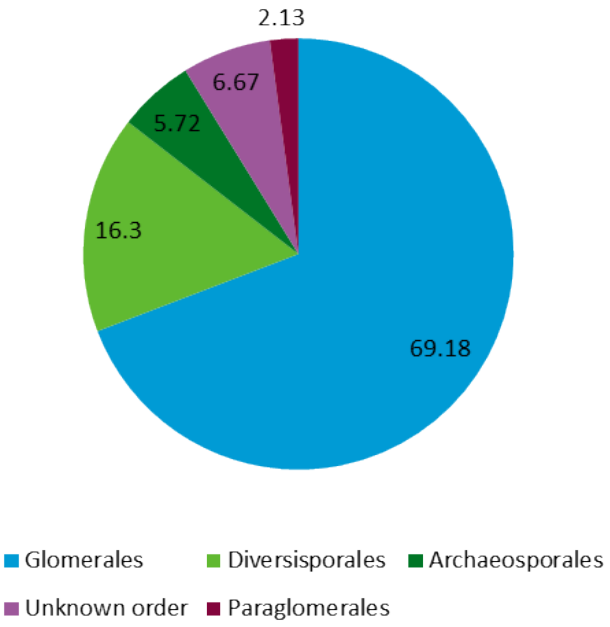
1.2.3 AMF Classification and Biogeography

Currently, four established orders are recognized: Glomerales, Archaeosporales, Paraglomerales, and Diversisporales[8]. Although AMF have global distribution, some species are known to be cosmopolitan, while others are not (Öpik et al., 2006, 2010; Davison et al., 2015; Sturmer et al., 2018). In this section, our goal was to report global occurrence of most frequently recorded species in Databases and Web Sites that compile studies about AMF occurrence. This is important to help selecting potential test species for the project.

1.2.3.1 Global occurrence

Occurrence of AMF species is reported based both on morphology and molecular data. First, a database regarding morphological records of mycorrhizal associations (PlutoF) was used as data source (Abarenkov et al., 2010). This database compiles information published worldwide regarding several types of mycorrhizae and allows evaluation of biogeographical distribution based on several parameters such as location, year of occurrence and basis of record. A total of 113 601 AMF records were found, distributed among 4 taxa and 1 unknown order (Figure 2).

Figure 2: Percentage of AMF species distributed in Glomerales, Archaeosporales, Paraglomerales, Diversisporales or unknown order.



Data obtained from PlutoF database, April 2021 (Abarenkov et al., 2010). Source: own illustration, UFSC.

Secondly, a search was performed to rank the most reported groups of AMF worldwide based on their occurrence, and selected the 20 most frequent species. In addition, we included data obtained from 3 other databases: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), Fungal Traits (Põlme et al., 2021) and NCBI website. MaarjAM´s database includes 44 545 Glomeromycetes sequences: 39 255 from Glomerales, 5 273 from Diversisporales, 1 492 from Archaeosporales, and 1 110 from Paraglomerales. Although NCBI contains mostly sequencing

[8] http://www.amf-phylogeny.com/

data, information was used with the goal to provide an overview of which are the most studied species worldwide.

The number of records regarding each species is presented in Table 2 and used as ranking criteria. If classification of the 5 most reported species were to be considered, a similar pattern to Figure 2 is observed, i.e., most species belong to Glomerales, followed by Diversisporales.

Note that, currently, the most correct nomenclature for the genus *Rhizophagus* is in fact *Rhizoglomus* (Robert et al., 2005). However, and due to its wide use in literature, the nomenclature *Rhizophagus* will be used in this report.

Table 2: Rank of the 20 AMF species with the highest frequency of occurrence worldwide. Morphology data were obtained from PlutoF database, whereas molecular data were obtained from both MaarjAM and NCBI databases.

Rank	Species	Maarj AM	Stürmer	PlutoF	Fungal Traits	NCBI	Total
1	***Gigaspora margarita***	**109**	**145**	**228**	**193**	**90 507**	**91 182**
2	***Rhizophagus irregularis***	**29**	**0**	**349**	**359**	**77 314**	**78 051**
3	***Rhizophagus intraradices***	**115**	**280**	**340**	**299**	**49 059**	**50 093**
4	***Rhizophagus clarus***	**23**	**146**	**210**	**299**	**4 305**	**4 983**
5	***Funneliformis mosseae***	**427**	**432**	**1 558**	**310**	**1 378**	**4 105**
6	*Claroideoglomus etunicatum*	92	311	480	147	2088	3 118
7	*Glomus versiforme*	1	48	549	71	1519	2 188
8	*Claroideoglomus claroideum*	68	167	460	144	393	1 232
9	*Funneliformis caledonium*	42	102	727	55	143	1 069
10	*Paraglomus occultum*	24	177	619	16	91	927
11	*Funneliformis geosporum*	31	217	247	60	155	710
12	*Acaulospora laevis*	0	132	385	31	89	637
13	*Glomus macrocarpum*	8	194	293	39	75	609
14	*Rhizophagus fasciculatus*	5	270	178	2	96	551
15	*Archaeospora trappei*	13	67	275	9	61	425
16	*Acaulospora scrobiculata*	9	208	141	11	47	416
17	*Funneliformis constrictum*	8	161	151	0	67	387
18	*Scutellospora calospora*	6	174	146	2	55	383
19	*Acaulospora brasiliensis*	6	2	171	42	51	272
20	*Diversispora eburnea*	10	21	155	12	32	230

1.2.3.2 Worldwide distribution

We searched for evidence of cosmopolitan distribution of the top five species highlighted in bold on Table 2. In a first approach, records from Stürmer et al. (2018) were used, which compiled a database with 7 105 records from 1960 to 2012. Among 4 orders, Diversisporales and Glomerales were the most frequent in all continents, countries, climatic zones, and biomes considered. This aspect corroborates global occurrence data from section 1.2.3.1. It is important to emphasize that some regions (e.g. in Africa and Asia) are less studied compared to the well-studied areas across Europe and America.

Among the 5 top species presented in Table 2, only *Rhizophagus irregularis* is not considered cosmopolitan (Stürmer et al. 2018). This species has been recorded only in non-contiguous countries from Europe, Asia and Africa, and therefore considered to have disjunct distribution. All other 4 species have been reported through the 5 continents (Stürmer et al., 2018) and, therefore, are classified as cosmopolitan.

In a second approach, all data from databases of Stürmer et al. (2018), PlutoF, MaarjAM, and FungalTraits were collected. All entries that contained information about country of origin or geographic coordinates were used, in order to build distribution maps. Entries with no information about the country or coordinates pointing to ocean locations (a mistake from the database itself), were excluded from the dataset.

Distribution maps were built for the 5 top-ranked AMF species, with data from single databases or all of them combined.

Gigaspora margarita

Data on *Gigaspora margarita* global distribution are presented in Figure 3 and Appendix A.1.

Figure 3: Global frequency of occurrence of *Gigaspora margarita* by continent, considering data from 4 databases combined: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010), and FungalTraits (Põlme et al., 2021). Numbers within each continent represent the total number of entries recorded.

Source: own illustration, UFSC.

Rhizophagus intraradices

Data on *Rhizophagus intraradices* global distribution are presented in Figure 4 and Appendix A.2.

Figure 4: **Global frequency of occurrence of *Rhizophagus intraradices* by continent, considering data from 4 databases combined: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010), and FungalTraits (Põlme et al., 2021). Numbers within each continent represent the total number of entries recorded.**

Source: own illustration, UFSC.

Rhizophagus irregularis

Data on *Rhizophagus irregularis* global distribution are presented in Figure 5 and Appendix A.3.

Figure 5: **Global frequency of occurrence of *Rhizophagus irregularis* by continent, considering data from 4 databases combined: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010), and FungalTraits (Põlme et**

al., 2021). Numbers within each continent represent the total number of entries recorded.

Source: own illustration, UFSC.

Rhizophagus clarus

Data on *Rhizophagus clarus* global distribution are presented in Figure 6 and Appendix A.4.

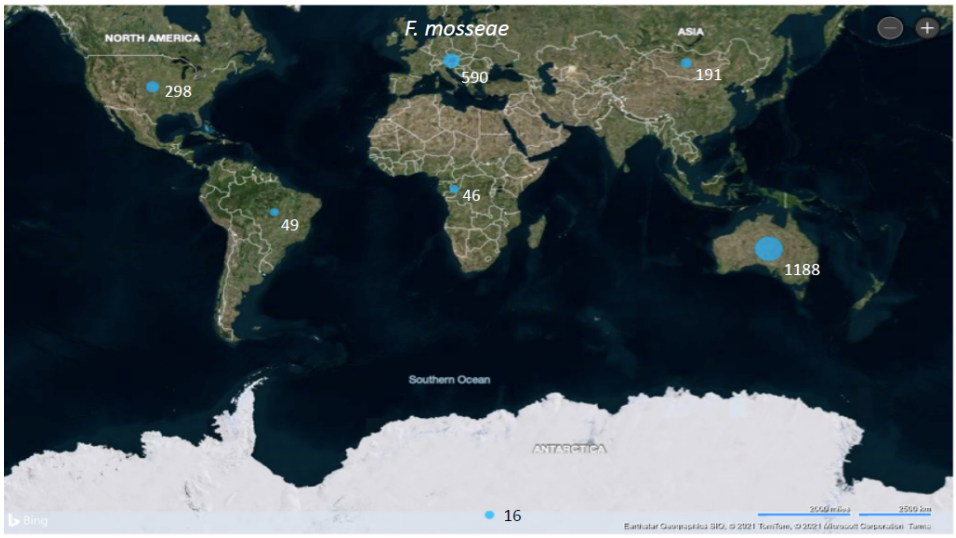
Figure 6: Global frequency of occurrence of *Rhizophagus clarus* by continent, considering data from 4 databases combined: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010) and FungalTraits (Põlme et al., 2021). Numbers within each continent represent the total number of entries recorded.

Source: own illustration, UFSC.

Funneliformis mosseae

Data on *Funneliformis mosseae* global distribution are presented in Figure 7 and Appendix A.5.

Figure 7: **Global frequency of occurrence of *Funneliformis mosseae* by continent, considering data from 4 databases combined: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010), and FungalTraits (Põlme et al., 2021). Numbers within each continent represent the total number of entries recorded.**



Source: own illustration, UFSC.

Overall, the 5 AMF species reported in section 1.2.3 seem to be the most promising candidates to be test species in laboratory tests, given their high frequency of occurrence and global distribution.

1.2.4 Availability of AMF species in culture collections and inoculum storage

A fundamental criterion to select a particular test species for ecotoxicological tests is the availability of AMF species at culture collections worldwide. This is mandatory for laboratories to purchase inoculum and then multiply these fungi at their own facilities.

We used information from the following culture collections (Table 3):

1. BEG: The International Bank of Glomeromycota, located in Dijon - France. Information can be checked at: https://www.i-beg.eu/
2. INVAM: International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi, located in West Virginia – United States. Information can be checked at: https://invam.wvu.edu/
3. CICG: Coleção Internacional de Cultura de Glomeromycota, located in Santa Catarina – Brasil. Information can be checked at: https://sites.google.com/site/cicgfma/home
4. GINCO: Glomeromycota *In vitro* Collection, located in Belgium. Information can be checked at: https://www.mycorrhiza.be/ginco-bel/index.php
5. CMCC: Centre for Mycorrhizal Culture Collection, located in India. Information can be checked at: http://mycorrhizae.org.in/cmcc/prod_res.php
6. SAF: Swiss culture collection of Arbuscular Mycorrhizal Fungi, located in Switzlerland. Information can be checked at: https://www.agroscope.admin.ch/agroscope/en/home/topics/environment-resources/soil-bodies-water-nutrients/swiss-collection-of-arbuscular-mycorrhizal-fungi.html.

Table 3: **Species that are currently available for purchase from different culture collections. BEG: The International Bank of Glomeromycota, INVAM: International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi, CICG: Coleção Internacional de Cultura de Glomeromycota, GINCO: Glomeromycota *In vitro* Collection, CMCC: Centre for Mycorrhizal Culture Collection, SAF: Swiss culture collection of Arbuscular Mycorrhizal Fungi.**

Species	BEG	INVAM	CICG	GINCO	CMCC	SAF
Gigaspora margarita	X	X	X			X
Rhizophagus irregularis	X			X		X
Rhizophagus intraradices		X		X		X
Rhizophagus clarus		X	X	X		
Funneliformis mosseae	X	X				X
Claroideoglomus etunicatum		X	X		X	
Glomus versiforme						
Claroideoglomus claroideum	X	X			X	X
Funneliformis caledonium	X	X				X
Paraglomus occultum		X	X			
Funneliformis geosporum	X	X				X
Acaulospora laevis		X				
Glomus macrocarpum						
Rhizophagus fasciculatus						
Archaeospora trappei		X	X			X
Acaulospora scrobiculata		X	X			
Funneliformis constrictum		X				
Scutellospora calospora	X	X				X
Acaulospora brasiliensis						
Diversispora eburnea		X				

Information regarding spore storage is scarce and often contradictory (Silva et al., 2007; Püschel et al., 2019). Different conditions and periods of storage of each species are important as they can influence further performance of the inoculum (Püschel et al., 2019). Significant reduction in viability over short storage periods may represent the need for frequent multiplication, which tends to cause instability in AMF responses obtained in laboratory tests. Moreover, recurrent AMF multiplication demands constant human resources and is a time-consuming process, thus hindering the development of experimental trials (Mallmann, 2020).

Inoculum storage temperature commonly used for research purposes is 4 °C, even in international AMF culture collections (INVAM, 2021; Stürmer, 2021). Louis & Lim (1988)

reported that a brief storage time in low temperature (4 °C at least 2 weeks) increases spore germination of *Rhizophagus clarus* under temperatures of 25-35 °C. Indeed, storage at low temperatures may be important to promote a dormancy break of some species (Juge et al., 2002). However, other species like *Gigaspora albida* may not present differences in infectivity if previously stored at 4 or 28 °C (Silva et al., 2007).

Storage time is another factor influencing future performance of the spores. Wagner et al. (2001) found that although storage temperature of 4 °C was better than 24 °C for long term survival of *Glomus claroideum*, spore viability decreases with time of storage, even after 272 weeks stored there was considerable number of viable spores. Practical observations have shown that while some species need to be multiplied frequently to maintain a viable inoculum (e.g., *Gigaspora* spp.), others are more resistant to degradation (e.g., *Acaulospora* spp.) (INVAM, 2021). Furthermore, the response to storage conditions can be variable even between isolates of the same species (Püschel et al., 2019).

The effects of storage time on spores may also pose practical implications, as evidenced by Mallmann (2020). The germination rate of *Gigaspora albida* spores remained the same over 5 years of storage, meeting the validation criteria of the ISO 10832 (ISO, 2009) ecotoxicological protocol during the entire period. However, for *Rhizophagus clarus* there was a significative reduction in spores' germination over the years, and from the third year of storage onwards, the inoculum no longer met the validity criteria. This leads to assume that inoculum of *Rhizophagus clarus* needs more frequent multiplication than inoculum of *Gigaspora albida* in testing routines (Mallmann, 2020). Besides the aspects inherent to the spores of the multiplication process itself, another practical implication is the existence of variation in germination response. Spores that do not remain viable for long periods, or do not tolerate prolonged storage time, will necessarily need more frequent reproduction cycles, implying an increase in the possibilities of genetic variation and, consequently, in the variation of the responses of these test organisms to the tested substances (Mallmann, 2020).

1.2.5 Effects of abiotic conditions on the performance of AMF species

In this section, we focused on papers describing the effects of abiotic factors on target AMF species. Databases used for this search were Web of Science[9], SciELO[10] and Google Scholar[11]. Other search tools mentioned in the Methods section were also used but are not reported here because no additional papers were obtained from those sources.

Only studies targeting the five most frequently used species presented in Table 2 were considered for this part of the literature review. Several papers regarding temperature and pH effects on plants that are colonized by AMF communities were found but not used in this part of the review since this is not the focus of the current project. Studies reporting the effects of temperature or pH on AMF species richness or diversity, were also not considered.

1.2.5.1 Temperature

Search terms used were: “Temperature AND arbuscular AND mycorrhiz* AND spore AND germination”; “Temperature AND Gigaspora AND spore AND germination”; “Temperature AND

[9] https://www.webofknowledge.com

[10] https://scielo.org

[11] https://scholar.google.com.br

Rhizophagus AND spore AND germination"; "Temperature AND Glomus AND spore AND germination"; Temperature AND arbuscular AND mycorrhiz* AND colonization"; "Temperature AND Gigaspora AND colonization"; "Temperature AND Rhizophagus AND colonization"; "Temperature AND Glomus AND colonization"; "Temperature AND arbuscular AND mycorrhiz* AND symbiosis"; "Temperature AND Gigaspora AND symbiosis"; "Temperature AND Rhizophagus AND symbiosis"; "Temperature AND Glomus AND symbiosis".

Most studies performed under laboratory conditions have reported the formation of AMF symbiosis at temperatures between 18 and 40°C, and the optimum temperature for most associations has been found between 28 and 30°C. This optimal condition is related to temperatures that promote spore germination, as well as photosynthesis and C flow from plants to roots (Entry et al., 2002). Effects of temperature on both spore germination (pre-symbiotic phase) and symbiotic parameters (symbiotic phase) for different species are shown in Tables 4 and 5, respectively. It must be acknowledged that temperature may also have an indirect effect on AMF, since the formation of the symbiosis can be influenced by the optimal growth temperature of the host plants.

Table 4: Percentage of spore germination (%) of distinct AMF species at different temperatures between 15 and 37 °C.

Reference	Species	15 °C	20 °C	22 °C	24 °C	25 °C	28 °C	30 °C	34 °C	35 °C	37 °C
Schenck et al. (1975)	*F. mosseae*	8	80	-*	-	73	-	-	5	-	-
Haugen & Smith (1992)	*R. intraradices*	-	-	57	-	-	75	-	-	-	25
Matsubara & Harada (1996)	*G. margarita*	15	50	-	-	85	-	90	-	82	-
Mallmann et al. (2018)	*R. clarus*	-	-	-	21	-	91	-	-	-	-
Mallmann et al. (2018)	*G. albida*	-	-	-	56	-	96	-	-	-	-
Mallmann et al. (2018)	*G. margarita*	-	-	-	40	-	73	-	-	-	-

* - not evaluated by the referred authors.

Table 5: Optimal temperature for different symbiotic characteristics of AMF species.

Reference	Species	Host	Intraradical root colonization	Extra-radical mycelium	Spores
Schenck & Smith (1982)	*R. clarus*	*Glycine max*	-*	-	24 °C

Reference	Species	Host	Intraradical root colonization	Extra-radical mycelium	Spores
Schenck & Smith (1982)	*F. mosseae*	*Glycine max*	-	-	30 °C
Haugen & Smith (1992)	*R. intraradices*	*Vigna radiata*	30 °C	-	-
Matsubara & Harada (1996)	*G. margarita*	*Asparagus officinalis*	25 °C	-	-
Liu et al. (2004)	*R. intraradices*	*Sorghum bicolor*	23 °C	-	-
Liu et al. (2004)	*R. intraradices*	Transformed carrot roots	23 °C	23 °C	23 °C
Gavito et al. (2005)	*R. intraradices*	Transformed carrot roots	-	30 °C	-
Martin & Stutz (2004)	*R. intraradices*	*Capsicum annuum*	20.7-25.4 °C	-	-
Heinemeyer & Fitter (2004)	*F. mosseae*	*Holcus lanatus* and *Plantago lanceolata*	20 °C	-	-
Costa et al. (2013)	*R. clarum*	Transformed carrot roots	-	-	28 °C

* - not evaluated in the study

Classic studies by Tommerup (1983) and Siqueira et al. (1985) have demonstrated differences among the preferential germination temperature to various species. Tommerup (1983) evaluated various temperatures and germination times and found maximum germination after 5 days, at temperatures between 25-30 °C to *Scutellospora heterogama* (*Gigaspora calospora*) spores, 7 days at 20-25 °C to *Funneliformis caledonoim* (*Glomus*) *caledonium*, and 16 days at 20 °C to *Acaulospora laevis*. Siqueira et al. (1985) observed that temperatures between 18 to 25 °C are optimum for *Glomus versiforme* germination and that temperatures below 15 °C or above 35 °C are prejudicial for *Gigaspora gigantea* in the pre-symbiotic development. Different temperatures could, however, reflect the disparity of various environments where fungi were collected. Under Brazilian conditions, the use of a temperature close to 28 ± 2 °C has resulted in satisfactory spore germination (Maia & Yano-Melo, 2001; Cardoso et al., 2002; Soares et al., 2009; Malfatti et al., 2021; Mallmann et al., 2018).

Some AMF isolates have shown an effect on spore germination only at a temperature of 28 °C of incubation. At lower temperatures, germination does not occur or is low (lower than the value suggested in ISO 10832:2009 for test validation). In addition, results have shown that shorter incubation times at higher temperatures lead to spore germination, even in isolates from colder regions. This indicates that the adoption of 28 °C for the same evaluation period proposed by the ISO 10832:2009 protocol is feasible. The temperature of 28 °C is also used for the evaluation of other microbial parameters such as the determination of soil basal respiration ($C\text{-}CO_2$) (Jenkinson & Powlson, 1976).

1.2.5.2 Soil pH

Search terms used were: “pH AND arbuscular AND mycorrhiz* AND spore AND germination”; “pH AND *Gigaspora* AND spore AND germination; “pH AND *Rhizophagus* AND spore AND germination”; “pH AND *Glomus* AND spore AND germination”; “pH AND arbuscular AND mycorrhiz* AND colonization”; pH AND *Gigaspora* AND colonization”; “pH AND *Rhizophagus* AND colonization”; “pH AND *Glomus* AND colonization”; “pH AND arbuscular AND mycorrhiz* AND symbiosis”; “pH AND *Gigaspora* AND symbiosis”; “pH AND *Rhizophagus* AND symbiosis”; “pH AND *Glomus* AND symbiosis”.

Effects of soil pH on spore germination (pre-symbiotic phase) and symbiotic parameters (symbiotic phase) for different species are shown in Tables 6 and 7. pH values between 6.0 and 7.0 are the most frequent values for optimal pre-symbiotic and symbiotic stages. It must be acknowledged that pH values may also have an indirect effect on AMF, once distinct host plants will likely have different growth optimum pH.

Table 6: Optimal pH identified for spore germination (%) of distinct AMF species.

Reference	Species	4.0	4.5	5.0	5.5	6.0	6.5	7.0
Siqueira et al. (1984)	*F. mosseae*							x
Siqueira et al. (1984)	*G. margarita*					x		

Table 7: Optimum pH identified for different symbiotic variables of AMF species.

Reference	Species	Host	Intra-radical root colonization	Extra-radical mycelium	Spores formation
Hayman & Tavares (1985)	*F. mosseae* *G. margarita* *R. clarus*	*Fragraria vesca*	7.0 7.0 5.0	-*	-
Medeiros et al. (1994)	*R. intraradices*	*Sorghum bicolor*	7.0	-	-
Rohyadi et al. (2004)	*G. margarita*	*Vigna unguiculata*	4.7	-	-
Rohyadi (2008)	*G. margarita*	*Vigna unguiculata*	5.2	4.9	-
Costa et al. (2013)	*R. clarus*	Transformed carrot roots	-*	-	4.5
Radha et al. (2015)	*R. intraradices*	Transformed carrot roots	-	-	5.5

Reference	Species	Host	Intra-radical root colonization	Extra-radical mycelium	Spores formation
Oehl et al. (2017)	*R. irregularis*	Grassland and croplands plant communities	-	-	7.0
	R. intraradices		-	-	7.0
	F. mosseae		-	-	7.0
	G. margarita		-	-	6.0

* - not evaluated in the study

The mutualism between plants and AM fungi is an essential component for ecosystems. The beneficial effects of AM fungal communities on their plant hosts and the occurrence and abundance of AM fungi are relatively well-known, and we highlight effects of pH, Al, N, and P soil content.

The pH value cannot be considered as an isolated factor since pH values between 6.0 and 7.0 are considered ideal for most AMF species germination and growth. Particularly, some species, like *Acaulospora caledonicum,* germinate in acid conditions (pH<5.5) and others, like *Funeliformis mosseae,* are capable to form the germ tube only in pH>5.0 (Moreira & Siqueira, 2006). On the other hand, *Claroideoglomus etunicatum* and *Rhizophagus clarus* are species more adapted to higher pH (pH>6.0) (Maia et al., 2010). Thus, differences in spore germination between species and genera are often related to the environment from which the fungi were isolated (Clark, 1997; Souza et al., 2010).

In acid soils, aluminium in its trivalent form (Al^{3+}) can reduce germination rates, i.e. germ tube growth rates, and can also affect the initial steps of symbiosis establishment. The magnitude of these effects, however, depends on species or strain tolerance (Maia et al., 2010). Clark (1997) found that *Gigaspora albida*, *Gigaspora gigantea*, and *Gigaspora margarita* spores have high tolerance towards an Al saturated soil, as their germinations rates were higher than 60%. Meanwhile, *Rhizophagus clarus* (*Glomus clarum*) and three strains of *Claroideoglomus* (*Glomus*) *etunicatum* were heavily affected, presenting germination rates lower than 3%. The presence of Al did not inhibit hyphal growth of *Gigaspora gigantea* (304 mm of hyphae) and *Gigaspora margarita* (197 mm) strains but reduced hyphal growth of *Gigaspora albida* (46 mm) and *Gigaspora clarum* (3 mm) and inhibited mycelial growth of *Claroideoglomus* (*Glomus*) *etunicatum*.

Differences between spore germination and hyphal growth of *Acaulospora morrowiae*, *Claroideoglomus claroideum*, *Rhizophagus clarus* (*Glomus clarum*), *Claroideoglomus* (*Glomus*) *etunicatum*, *Paraglomus brasilianum*, and *Scutellospora heterogama* in an Al saturated soil were found by Klugh-Stewart & Cumming (2009). Both germination and hyphal growth decreased with the Al presence, except for *Scutellospora heterogama* that exhibited increased germination. However, these responses did not interfere on short-term colonization, suggesting that Al might not be an impediment to symbiosis establishment.

On the other hand, in acid soils, P is fixed and thus in low availability, and, in turn, Al in low pH appears in its phytotoxic form, which is a problem for host plants development and consequently to symbiosis. AMF suppression increased at low pH across several soils (Cruz-Paredes et al., 2021) and different Al contents show a strong negative correlation with root colonization by AMF (Göransson et al., 2008). Thus, it may be necessary to adjust soil pH, so that the host plant and the AMF species may establish their symbiosis.

1.2.5.3 Soil nutrient content

Soil nitrogen (N) content has been shown to have little or no influence on spore germination (Daniels & Trappe, 1980), while phosphorus (P) in small quantities favours AMF germination and asymbiotic growth (Moreira & Siqueira, 2006). The combined effect of soil N and P on AMF was investigated by Bressan (2001). In this study, conducted in agar-water medium (0,8%), spore germination of *Claroideoglomus* (*Glomus*) *etunicatum* in low P medium (2 mg L^{-1}) was reduced by the addition of N doses (5, 10, and 50 mg L^{-1}) compared to the control medium. In low N mediums (5 mg L^{-1}), the increase of P content (2, 10, and 20 mg L^{-1}) stimulated spore gemination, suggesting that P and N interaction directly affects spore germination.

Soil nutrients is also considered an important factor in the control of mycorrhizal associations. Soils with low nutrient levels limit plant development and increase the dependence of plants on the mycorrhizal association (Martínez-García et al., 2012). On the other hand, high soil nutrient levels decrease colonization, which is probably related to the reduction in the dependence of plants on their association with mycorrhiza, restricting the development of these fungi to the root cortex of the host plants (Verlinden et al., 2018). Thus, the use of fertilizers in soil in the experiments should be undertaken with care, to avoid negatively influencing colonization of AMF in roots and mycorrhizal structures (e.g., hyphae, vesicles, and arbuscules).

1.2.6 Intraspecific variability

It has been assumed that AMF are asexual organisms that grow clonally (see Sanders & Croll, 2010 for further discussion). However, some species present high intraspecific diversity, resulting in a wide range of effects on terrestrial ecosystems (van der Heijden et al., 2015; Mathieu et al., 2018). These genetic variations may affect symbiotic performance and may affect the stress response of fungi, such as exposure to pesticides (Novais et al., 2019a; Mallmann, 2020).

Genetic variability is usually associated with heterokaryosis, i.e., the fact some fungi have spores and hyphae with multiple nuclei genetically different inside a common cytoplasm (Kuhn et al., 2001; Croll et al., 2009; Hijri & Sanders, 2005), although the subject raises controversy (Pawlowska & Taylor, 2004).

A study conducted by Koch et al. (2004) reported high genetic and phenotypic variation in a *Rhizophagus intraradices* population, which was supported by further studies with other species, such as the model species *Rhizophagus irregularis* (Chen et al., 2018). Note that *Rhizophagus irregularis* is the only AMF species to date with its genome is completely codified. Different lineages of *Funneliformis mosseae* originating from the same ancestor isolate, cultivated for 20 years in different locations in Europe, showed high levels of vegetative incompatibility and reduced ability to form anastomosis, suggesting the occurrence of potential genetic divergence (Sbrana et al., 2018). In addition to the intraspecific diversity, variations can also be found within the same isolate, as evidenced by Ehinger et al. (2012). These authors found remarkable genetic and even phenotypic variation between three clonal generations of *Rhizophagus* (*Glomus*) *irregularis*, originating from a common single spore. These findings agree with heterokaryotic hypothesis and evidence the dynamic nature of AMF genetics (Ehinger et al., 2012).

Phenotypic plasticity derived from genetic variations has great practical importance for agricultural and environmental purposes as different isolates may affect plant fitness in different ways (Koch et al., 2006; Mathieu et al., 2018; Peña et al., 2020). Beyond that, the existence of such plasticity may explain part of the variation in AMF responses against stressors. In fact, different isolates of the same species have demonstrated distinct responses towards pesticide exposure (Novais et al., 2019a; Mallmann, 2020). Moreover, each of three clonal generations of a same isolate (of *Gigaspora albida* or *Rhizophagus clarus*) presented distinct germination responses to pesticide exposure, which was hypothesized to be (at least partially) a consequence of a potential genetic variability within spores. The variability in fungal response, however, seems dependent on the test substance and the magnitude of the measured effect, being IC_{10} data less variable among generations than IC_{20} and IC_{50} (Mallmann, 2020).

This type of variation, originated by genetic and/or phenotypic plasticity, does exist also in all other test organisms currently used in ecotoxicology. In the case of AMF, the most important aspect, when thinking in terms of test standardization, is that the level of variation when using inocula from the same species, but from different origins, should be within the acceptable level of variability in the test endpoints (assessed, e.g., via a warning chart).

1.2.7 Interspecific variability

Almost all terrestrial plants form symbiotic associations with AMF, but they have no host specificity. Thus, the effectiveness of the symbiosis depends, among other factors, on the combination of plant and AM fungi and on the origin of the AMF isolates (Novais et al., 2014). For example, plant growth response to inoculation varied from – 19 to 232% and depended on AMF isolate, species, and family (Säle et al., 2021).

AMF responses to chemicals in the pre-symbiotic and symbiotic phases have been shown to be quite different, however it is dependent on the AMF species and chemical. Mallmann et al. (2018) and Malfatti et al. (2021), evaluated spore germination, in tropical artificial soil (TAS), for *Rhizophagus clarus* and *Gigaspora albida*, and concluded on different responses of the test species towards the application of fungicides and herbicides. The species *Rhizophagus clarus* was more sensitive to the use of herbicides (glyphosate and Diuron+Paraquat), while *Gigaspora albida* showed higher sensitivity to fungicides (Chlorothalonil, Mancozeb, and Metalaxyl - M). Other studies have shown differences in sensitivity of spore germination for the species *Gigaspora margarita*, *Glomus intraradices*, *Funneliformis mosseae*, and *Rhizophagus irregularis* when exposed to the same chemical (Giovannetti et al., 2006; Pasaribu et al., 2011; Dodd & Jeffries, 1989a,b; Malty et al., 2006; Zocco et al., 2008; Schreiner & Bethlenfalvay, 1997; Buysens et al., 2015). Despite the variability in sensitivity of spore germination across different AMF species, spore germination have been reported as adversely affected by most of the substances tested.

For symbiotic phase the variation in sensitivity is dependent on the same factors as during the pre-symbiotic phase. Jakobsen et al. (2021) evaluated the effect of mancozeb and carbendazim on the performance of the AMF species *Claroideoglomus claroideum*, *Funneliformis mosseae*, and *Funneliformis caledonium* and found a hormetic response for root colonization. The authors report effects for AMF species for both fungicides in the highest test concentrations, only (0.602 µg Carbendazim g^{-1} soil; 83.325 µg Mancozeb g^{-1} soil). Pasaribu et al. (2013) found different effects in AMF endpoints for the herbicides Alachlor and Glyphosate. None of the herbicide affected the external hyphal length. However, spore production and internal hyphal infectivity were significantly affected by Alachlor while glyphosate had no significant effects on those parameters. Therefore, the symbiotic functions with the host plant can be affected

differently according to the endpoint evaluated. Therefore, different endpoints should be evaluated to know which function is affected.

However, Dodd & Jeffries (1989a), Pasaribu et al. (2011), and Malty et al. (2006) found no effect of the application of the herbicides Difenzoquat methyl sulphate, Bifenox, Chlorotoluron, a mixture of Mecoprop+Ioxynil+Clopyralid, Alachlor, and Glyphosate on total intra-radicular colonization as well as in spore production for *Funneliformis mosseae* and *Gigaspora margarita*. The lack of effect may be raised by the increased production of isoflavonoids in the roots, which can promote mycorrhizal colonization (Siqueira et al. 1991).

Little is known about the influence of pharmaceuticals or personal care products on AMF. The effects of 12 pharmaceuticals on *Rhizophagus* (*Glomus*) *intraradices* hyphal length and spore production were evaluated by Hills et al. (2008). The authors found six pharmaceuticals toxic to hyphal length and five to spore production. Both parameters are valuable in assessing the potential impacts of chemicals on the mycorrhizal life cycle, considering the different modes of action of chemicals towards AMF.

These results demonstrate that there is a wide variation in AMF responses of different parameters to chemicals. This fact gives strength to the use of different parameters and AMF species in ecotoxicological experiments.

1.3 Effects of organic chemicals on AMF

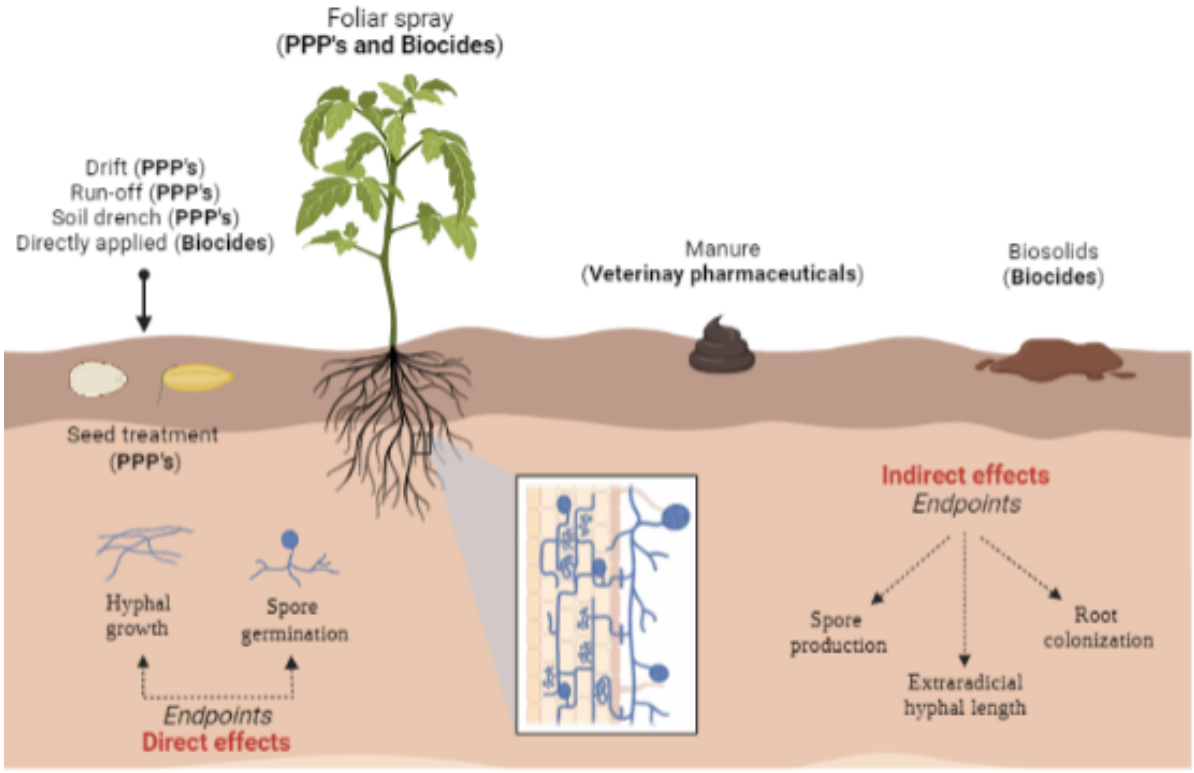
Soil organisms in agricultural systems are exposed to a wide range of chemicals (e.g., the so-called regulated chemicals as PPP, biocides, veterinary pharmaceuticals, among others) and the potential risks derived from this exposure must be accurately assessed with the ultimate goal of protecting soil biodiversity and safeguarding the soil functions they support.

According to FAO and ITPS (2017), PPP are pesticides used in plant or crop production, contributing to prevent, destroy or control any pest causing harm or interfering in the production, processing, storage, transport or marketing of food and agricultural commodities. Biocides are products that aim to control organisms/pests not involved in plant or crop production, and include disinfectants, preservatives, pest control, and other biocidal products (more details can be seen in Appendix A.6) (EU, 2012). Veterinary pharmaceuticals are products either for therapeutic or non-therapeutic use in animals and, depending on the different groups of animals, the same compound can be used for different purposes (Kim & Carlsson, 2005). According to Bártíková et al. (2016), veterinary pharmaceuticals belong to several pharmacological categories: antiparasitics (ectoparasiticides, endectocides, endoparasiticides including antiprotozoals and anthelmintics), antimicrobials (antibiotics including growth promoters and antiseptics), hormones, antifungals, anti-inflammatory (steroidal and non-steroidal) drugs, anaesthetics, euthanasia products, tranquilizers, sedatives, bronchodilators, antacids, diuretics, emetics and emulsifiers.

In the field, these chemicals can affect the pre-symbiotic and symbiotic phases of AMF (Figure 8). PPP are applied on the crop or directly on the soil surface, being sprayed, applied as granules or as seed treatments. Also, they can reach soil as wash-off from leaf application in treated areas, reach off-crop areas by drift or as run-off and soil drainage (Hage-Ahmed et al., 2019). Veterinary pharmaceuticals enter terrestrial systems through the common practice of fertilizing soil with manure or directly through livestock grazing (Kaczala & Blum, 2015). Other chemicals that have been of growing concern in terrestrial areas are human pharmaceuticals and personal care products (biocides). These chemicals may reach soils through the application of biosolids

resulting mainly from waste sludges of municipal wastewater treatment systems (Solomon et al., 2010). In this way, these chemicals have become a concern also to AMF. In the pre-symbiotic phase, structures such as spores and hyphae can be exposed to chemicals when these substances reach the ground (direct effects). In the symbiotic phase, indirect effects on AM symbiosis mediated by changes in the host plant physiology can occur (Hage-Ahmed et al., 2019), like in root colonization, extra-radicular hyphal length, and spore production.

Figure 8: Main ways of exposure of AMF to soil pollutants (chemicals as PPP, biocides, and veterinary pharmaceuticals).



Adapted from Hage-Ahmed et al. (2019).

1.3.1 Research terms used

A literature search was performed to collect information about AMF used in ecotoxicological tests, and the material was collected from peer-reviewed publications and reports. Articles were collected from the search platforms Scopus[12], ScienceDirect[13], PubMed[14], and Web of Science[15] in April and June 2021, and our search was restricted to title+abstract+key words.

The search was performed using the following terms: “arbuscular mycorrhizal fungi AND biocides”; "arbuscular mycorrhizal fungi AND herbicide”; "arbuscular mycorrhizal fungi AND insecticide”; "arbuscular mycorrhizal fungi AND fungicide”; "arbuscular mycorrhizal fungi AND pharmaceuticals”.

[12] https://www.scopus.com/search/form.uri?display=basic

[13] https://www.sciencedirect.com/

[14] https://pubmed.ncbi.nlm.nih.gov/

[15] https://www.webofknowledge.com

The exclusion criteria were articles published without an ecotoxicological point of view, effects of soil parameters, and metals to AMF, pure molecular analysis, and exposure experiments with other chemicals like hydrocarbonates.

1.3.2 Overview of the literature review

The number of publications recovered in the search with the terms arbuscular mycorrhizal fungi, fungicides, herbicides, insecticides, pharmaceuticals, and biocides, is presented in Table 8. In total, 618 publications were recovered, but there is an overlap between the searched databases.

Discounting the overlapping results, 323 publications were found. The chemicals investigated until date predominantly concern to fungicides (172 publications), followed by herbicides (82), pharmaceuticals (35), insecticides (26), and biocides (8). However, only a small percentage (less than 20%) of the publications met our objectives and, therefore, were further analysed. In total, 53 papers were analysed, of which 27 are involved mainly in symbiotic phase, 15 in pre-symbiotic phase, and 11 in both pre and symbiotic phases.

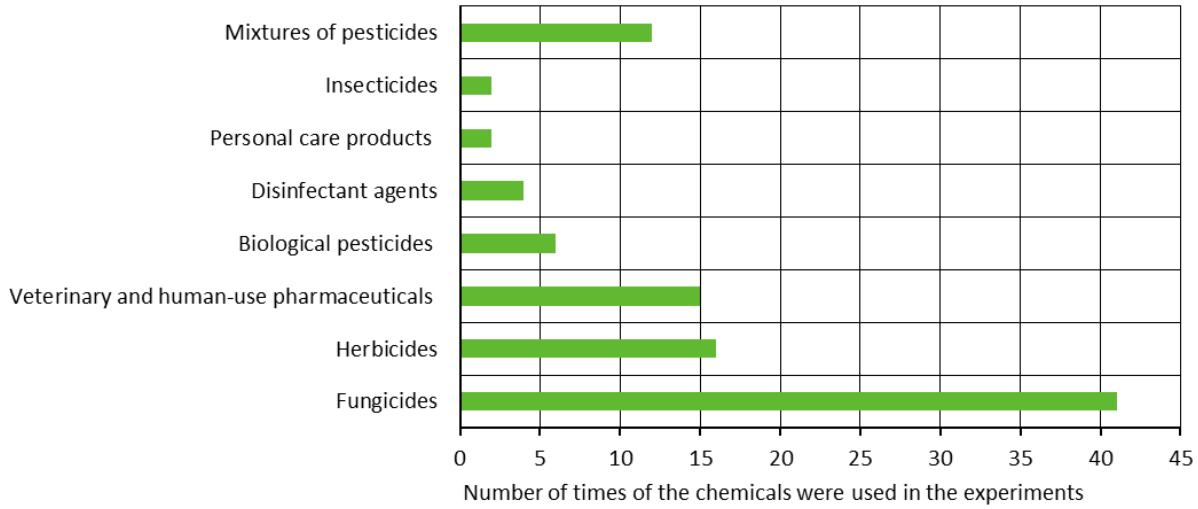
Table 8: Number of publications for AMF and each combination of key words.

Combinations	Scopus	Science Direct	PubMed	Web of Science
"arbuscular mycorrhizal fungi" AND fungicide	164	31	33	101
"arbuscular mycorrhizal fungi" AND herbicide	79	21	8	47
"arbuscular mycorrhizal fungi" AND insecticide	24	4	3	8
"arbuscular mycorrhizal fungi" AND pharmaceuticals	32	14	8	24
"arbuscular mycorrhizal fungi" AND biocides	7	4	0	6

Considering the papers analysed, among field and laboratory experiments, we found that:

1. The most studied chemicals (Figure 9) were fungicides (F; 41 active ingredients, a.i.), herbicides (H; 16 a.i), veterinary and human-use pharmaceuticals (15 a.i), biological pesticides (6 a.i), disinfectant agents (4 a.i), personal care products (PCP; 2 a.i), insecticides (I; 2 a.i), and mixtures of pesticides (12 formulations, 2 between fungicides+insecticides; 8 fungicides; 2 herbicides) (Appendix A.7).

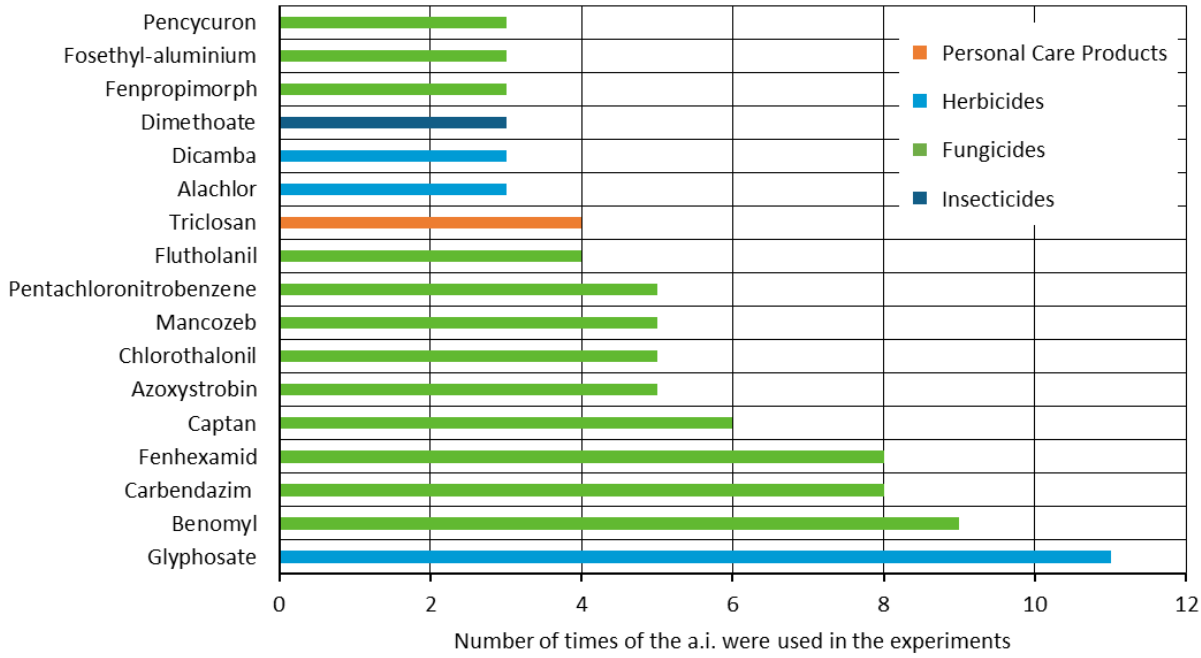
Figure 9: Main chemicals used in the experiments with AMF.



Source: own illustration, UDESC.

2. The most active ingredients studied (Figure 10) were Glyphosate (H) (11 studies), Benomyl (F) (9), Carbendazim (F) (8), Fenhexamid (F) (8), Captan (F) (6), Azoxystrobin (F) (5), Chlorothalonil (F) (5), Mancozeb (F) (5), Pentachloronitrobenzene (F) (5), Flutholanil (F) (4), Triclosan (PCP) (4) Alachlor (H) (3), Dicamba (H) (3), Dimethoate (I) (3), Fenpropimorph (F) (3), Fosethyl-aluminium (F) (3), and Pencycuron (F) (3) (Appendix A.7).

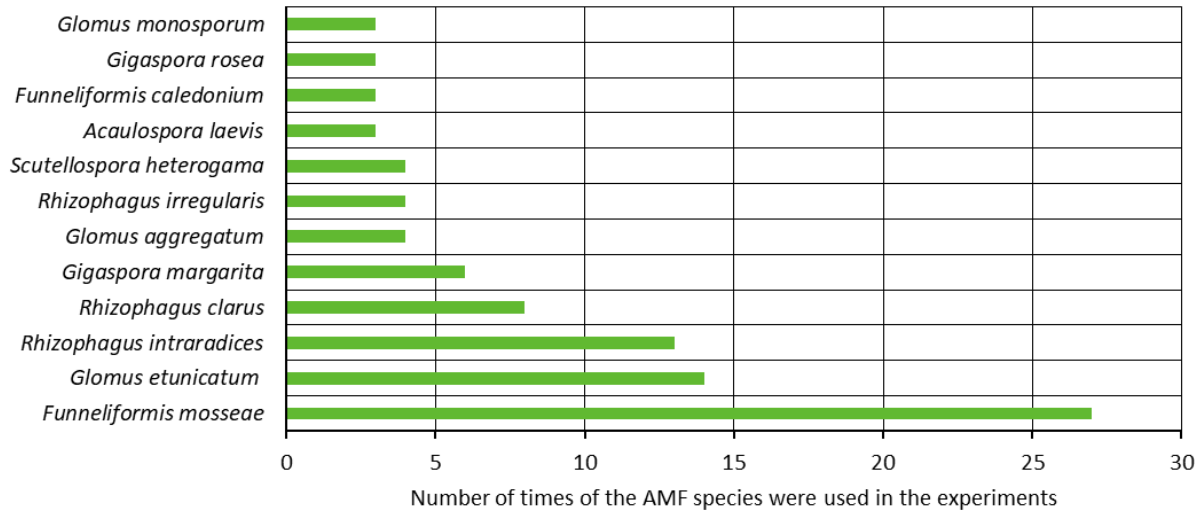
Figure 10: Main active ingredients of PPP used in the experiments.



Source: own illustration, UDESC.

3. The most studied AMF species (Figure 11) were *Funneliformis mosseae* (27 studies), *Claroideoglomus (= Glomus) etunicatum* (14), *Rhizophagus intraradices* (13), *Rhizophagus clarus* (8), *Gigaspora margarita* (6), *Glomus aggregatum* (4), *Rhizophagus irregularis* (4), *Scutellospora heterogama* (4), *Acaulospora laevis* (3), *Funneliformis caledonium* (3), *Gigaspora rosea* (3), and *Glomus monosporum* (3) (Appendix A.8). This is in accordance with our findings in section 1.2.3, about the worldwide distribution of AMF.

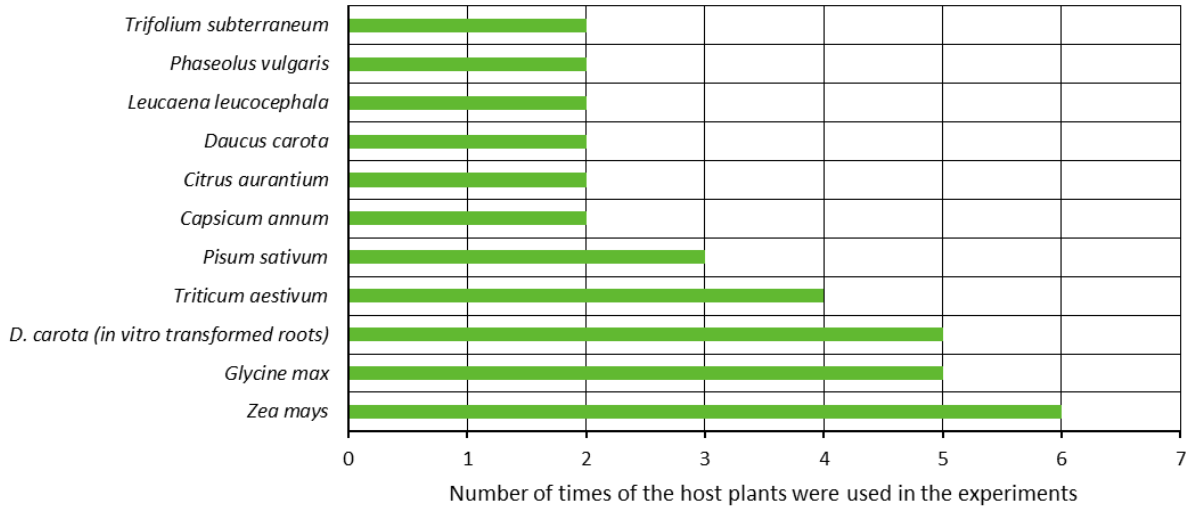
Figure 11: Main AMF species used in the experiments.



Source: own illustration, UDESC.

4. A total of 28 species of host plants were used in the experiments published until June 2021 (Figure 12). The most often used plant was *Zea mays* (6 studies), followed by *Glycine max* (5), *Daucus carota* (transformed roots) (5), *Triticum aestivum* (4), *Pisum sativum* (3), *Capsicum annum* (2), *Citrus aurantium* (2), *Daucus carota* (2), *Leucaena leucocephala* (2), *Phaseolus vulgaris* (2), and *Trifolium subterraneum* (2) (Appendix A.9).

Figure 12: Main host plants used in the experiments.



Source: own illustration, UDESC.

5. Studies focusing on the pre-symbiotic phase have used natural soils, artificial soil, sand and culture medium. The natural soils used had pH values of 6.3-6.5, 7.83, 8.24; the pH of the artificial soil has been adjusted to 6.0 (± 0.5); and sand, when used, had a pH varying between 6.0 and 7.0. Most studies that use culture medium, do not bring information about its pH value. Few exceptions reported pH values of 5.5, 5.8, and 7.5.
6. In general, most studies in pre-symbiotic phase were performed in the dark. Only two studies were conducted using a photoperiod of 16:8 h light/dark. In most cases, the temperatures used in the experiments were 25, 27, and 28 with a variation of ± 2 °C. Exceptionally, 22 and 26 °C have been also used.

The results of effects in pre and symbiotic phases are summarized on Table 9. A more descriptive explanation of these results is given sections 1.3.3 and 1.3.4.

Table 9: Overview of sensitivity of the AMF species and chemicals for different parameters. (+) Effect/Showed sensitivity; (-) No effect/Not sensitive. *Study found after June 2021.

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
Pre-symbiotic phase					
Gigaspora margarita	Glyphosate	Germ tube length	15	+	Malty et al., 2006
	Sodium hypochlorite solution 5% free chlorine (concentration 0.5%)	Spore germination	14	-	Colozzi-Filho et al., 1994
	Sodium hypochlorite solution 5% free chlorine (concentration 1%)	Spore germination	14	+	Colozzi-Filho et al., 1994
	Chloramine-T	Spore germination	14	-	Colozzi-Filho et al., 1994
	Formaldehyde solution	Spore germination	14	-	Colozzi-Filho et al., 1994
	Mercury chloride	Spore germination	14	-	Colozzi-Filho et al., 1994
	Streptomycin (Streptomycin sulfate)	Spore germination	14	-	Colozzi-Filho et al., 1994
	Gentamicyn (injectable Garamicyn 30mg)	Spore germination	14	-	Colozzi-Filho et al., 1994
	Pimaricin (Pimaracin) suspension 1.5%	Spore germination	14	-	Colozzi-Filho et al., 1994
	Chloramphenicol (injectable "Quemicetina")	Spore germination	14	-	Colozzi-Filho et al., 1994
	Glyphosate	Spore germination	15	+	Malty et al., 2006
Rhizophagus (*Glomus*) *intraradices*	Triclosan	Spore germination	21	+	Twanabasu et al., 2013

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Triclosan	Hyphal lenght*	21	+	Twanabasu et al., 2013
	Triclosan	Hyphal branching	21	+	Twanabasu et al., 2013
	Fenpropimorph	Spore germination	27 d for non-germinated spores; 15 d for pre-germinated spores	+	Zocco et al., 2008
	Fenhexamid	Spore germination	27 d for non-germinated spores; 15 d for pre-germinated spores	+	Zocco et al., 2008
	Fenpropimorph	Germ tube length	27 d for non-germinated spores; 15 d for pre-germinated spores	+	Zocco et al., 2008
	Fenhexamid	Germ tube length	27 d for non-germinated spores; 15 d for pre-germinated spores	+	Zocco et al., 2008
Funneliformis (*Glomus*) *mosseae*	Alachlor	Spore germination	30	-	Pasaribu et al., 2011
	Glyphosate	Spore germination	30	-	Pasaribu et al., 2011
	Alachlor	Hyphal length	30	-	Pasaribu et al., 2011
	Glyphosate	Hyphal length	30	-	Pasaribu et al., 2011
	Mancozeb (Crittox MZ 80)	Spore germination	10	+	Giovannetti et al., 2006
	Ziram (Pomarsol Z Wg)	Spore germination	10	+	Giovannetti et al., 2006

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Copper Hydroxide (Ramid 30 Pb)	Spore germination	10	+	Giovannetti et al., 2006
	Oxycarboxin (Plantvax 20 E)	Spore germination	10	- (caused a dose-response effect, but germination was never completely inhibited)	Giovannetti et al., 2006
	Iprodione (Rovral)	Spore germination	10	- (caused a dose-response effect, but germination was never completely inhibited)	Giovannetti et al., 2006
	Fosetyl Al (Aliette)	Spore germination	10	-	Giovannetti et al., 2006
	Metalaxyl (Ridomil 5 G)	Spore germination	10	-	Giovannetti et al., 2006
	Terbuthylazine (Basel FL)	Spore germination	10	-	Giovannetti et al., 2006
	Glyphosate (Risolutiv)	Spore germination	10	-	Giovannetti et al., 2006
	MCPA (Agritox Dry 800)	Spore germination	10	-	Giovannetti et al., 2006
	Pendimethalin (Disetalin L)	Spore germination	10	-	Giovannetti et al., 2006
	Dicamba (Aric 480 L.S.)	Spore germination	10	-	Giovannetti et al., 2006
	Delfin (6.4% *Bacillus thuringiensis* Sa 11)	Spore germination	10	-	Giovannetti et al., 2006
	Biofox C (10^5 propagules /mg *Fusarium oxysporum* 251/2RB)	Spore germination	10	-	Giovannetti et al., 2006
	MCPA (Agritox Dry 800)	Hyphal length	10	-	Giovannetti et al., 2006
	Ziram (Pomarsol Z Wg)	Hyphal length	10	+	Giovannetti et al., 2006

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Iprodione (Rovral)	Hyphal length	10	+	Giovannetti et al., 2006
	Fosetyl Al (Aliette)	Hyphal length	10	+	Giovannetti et al., 2006
	Terbuthylazine (Basel FL)	Hyphal length	10	+	Giovannetti et al., 2006
	Pendimethalin (Disetalin L)	Hyphal length	10	+	Giovannetti et al., 2006
	Glyphosate (Risolutiv)	Hyphal length	10	+	Giovannetti et al., 2006
	Delfin (6.4% *Bacillus thuringiensis* Sa 11)	Hyphal length	10	-	Giovannetti et al., 2006
	Biofox C (10^5 propagules /mg *Fusarium oxysporum* 251/2RB)	Hyphal length	10	-	Giovannetti et al., 2006
	Difenzoquat methyl sulphate (Avenge 630)	Spore germination	20	+	Dodd & Jeffries, 1989a
	Bifenox (Ceridor)	Spore germination	20	+ (low concentrations) - (high concentrations)	Dodd & Jeffries, 1989a
	Chlorotoluron (Dicurane 500 L)	Spore germination	20	+	Dodd & Jeffries, 1989a
	Mecoprop+ioxynil+clopyralid (Harrier)	Spore germination	20	+ (low concentrations) - (high concentrations)	Dodd & Jeffries, 1989a
	Carbendazim (Bavistin FL)	Spore germination	20	+	Dodd & Jeffries, 1989b
	Tridemorph (Calixin)	Spore germination	20	-	Dodd & Jeffries, 1989b
	Propiconazole and tridemorph (Tilt Turbo 375)	Spore germination	20	+	Dodd & Jeffries, 1989b

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
Funneliformis (*Glomus*) *mosseae* BEG46	Benomyl	Spore germination	14	+	Schreiner & Bethlenfalvay, 1997
	Pentachloronitrobenzene (PCNB)	Spore germination	14	+	Schreiner & Bethlenfalvay, 1997
	Captan	Spore germination	14	+	Schreiner & Bethlenfalvay, 1997
	Benomyl	Hyphal length (early hyphal growth of spores)	14	+	Schreiner & Bethlenfalvay, 1997
	Pentachloronitrobenzene (PCNB)	Hyphal length (early hyphal growth of spores)	14	+	Schreiner & Bethlenfalvay, 1997
	Captan	Hyphal length (early hyphal growth of spores)	14	-	Schreiner & Bethlenfalvay, 1997
Rhizophagus clarus RJN102A	Glyphosate	Spore germination	14	+	Malfatti et al., 2021
	Diuron+Paraquat	Spore germination	14	+	Malfatti et al., 2021
	Chlorothalonil	Spore germination	14	+	Mallmann et al., 2018
	Mancozeb	Spore germination	14	+	Mallmann et al., 2018
	Metalaxyl-M	Spore germination	14	+	Mallmann et al., 2018
Rhizophagus irregularis MUCL 41833	Azoxystrobin	Spore germination	3, 6, 14, 28	+	Buysens et al., 2015
	Flutolanil	Spore germination	3, 6, 14, 28	+	Buysens et al., 2015
	Pencycuron	Spore germination	3, 6, 14, 28	+	Buysens et al., 2015

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
Native AMF spores	Tebuthiuron	Spore germination		-	Allen & West, 1993
Symbiotic phase					
Funneliformis mosseae	Mancozeb (Dithane DG NT)	Intra-radicular total colonization	39	- (lower concentrations) + (high concentrations)	Jakobsen et al., 2021*
	Mancozeb (Dithane DG NT)	Extra-mycelial production	39	-	Jakobsen et al., 2021*
	Carbendazim (Derosal fl.)	Intra-radicular total colonization	39	- (lower concentrations) + (high concentrations)	Jakobsen et al., 2021*
	Carbendazim (Derosal fl.)	Extra-mycelial production	39	-	Jakobsen et al., 2021*
Gigaspora margarita	Glyphosate	Intra-radicular total colonization	17, 31, 45	-	Malty et al., 2006
Rhizophagus (*Glomus*) *clarum*	Fenhexamid	Anastomosis frequency (probability of fusion between two lineages paired)	21	-	Cardenas-Flores et al., 2011
	Fenhexamid	Spore production	70	No significant difference was observed between the lineages within a same generation.	Cardenas-Flores et al., 2011
Rhizophagus (*Glomus*) *intraradices*	17- α -ethynyl estradiol	Spore production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Atorvastatin	Spore production	14, 21, 28	-	Hillis et al., 2008

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Carbamazepine	Spore production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Chlortetracycline	Spore production	14, 21, 28	-	Hillis et al., 2008
	Doxycycline	Spore production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Irgasan	Spore production	14, 21, 28	-	Hillis et al., 2008
	Levofloxacin	Spore production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Monensin	Spore production	14, 21, 28	-	Hillis et al., 2008
	Oxytetracycline	Spore production	14, 21, 28	-	Hillis et al., 2008
	Sulfamethoxazole	Spore production	14, 21, 28	+ (at 14, 21, and 28 days)	Hillis et al., 2008
	Tetracycline	Spore production	14, 21, 28	-	Hillis et al., 2008
	Tylosin	Spore production	14, 21, 28	-	Hillis et al., 2008
	17- α -ethynyl estradiol	Extra-mycelial production	14, 21, 28	-	Hillis et al., 2008
	Atorvastatin	Extra-mycelial production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Carbamazepine	Extra-mycelial production	14, 21, 28	-	Hillis et al., 2008
	Chlortetracycline	Extra-mycelial production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Doxycycline	Extra-mycelial production	14, 21, 28	+ (at 21 and 28 days)	Hillis et al., 2008
	Irgasan	Extra-mycelial production	14, 21, 28	-	Hillis et al., 2008
	Levofloxacin	Extra-mycelial production	14, 21, 28	+ (at 28 days)	Hillis et al., 2008
	Monensin	Extra-mycelial production	14, 21, 28	-	Hillis et al., 2008
	Oxytetracycline	Extra-mycelial production	14, 21, 28	-	Hillis et al., 2008

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Sulfamethoxazole	Extra-mycelial production	14, 21, 28	+ (at 21 and 28 days)	Hillis et al., 2008
	Tetracycline	Extra-mycelial production	14, 21, 28	+ (at 21 and 28 days)	Hillis et al., 2008
	Tylosin	Extra-mycelial production	14, 21, 28	-	Hillis et al., 2008
Rhizophagus (*Glomus*) *irregulares*	Propiconazole	Extra-mycelial production	42	+	Calonne et al., 2012
	Propiconazole	Spore production	42	+	Calonne et al., 2012
Funneliformis (*Glomus*) *mosseae*	Difenzoquat methyl sulphate (Avenge 630)	Intra-radicular total colonization	146	-	Dodd & Jeffries, 1989a
	Difenzoquat methyl sulphate (Avenge 630)	Spore production	146	-	Dodd & Jeffries, 1989a
	Bifenox (Ceridor)	Intra-radicular total colonization	146	-	Dodd & Jeffries, 1989a
	Bifenox (Ceridor)	Spore production	146	-	Dodd & Jeffries, 1989a
	Chlorotoluron (Dicurane 500 L)	Intra-radicular total colonization	146	-	Dodd & Jeffries, 1989a
	Chlorotoluron (Dicurane 500 L)	Spore production	146	-	Dodd & Jeffries, 1989a
	Mecoprop+ioxynil+clopyralid (Harrier)	Intra-radicular total colonization	146	-	Dodd & Jeffries, 1989a
	Mecoprop+ioxynil+clopyralid (Harrier)	Spore production	146	-	Dodd & Jeffries, 1989a
	Difenzoquat methyl sulphate (Avenge 630)	Intra-radicular total colonization	98	-	Dodd & Jeffries, 1989a
	Difenzoquat methyl sulphate (Avenge 630)	Spore production	98	-	Dodd & Jeffries, 1989a

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Bifenox (Ceridor)	Intra-radicular total colonization	98	-	Dodd & Jeffries, 1989a
	Bifenox (Ceridor)	Spore production	98	-	Dodd & Jeffries, 1989a
	Chlorotoluron (Dicurane 500 L)	Intra-radicular total colonization	98	-	Dodd & Jeffries, 1989a
	Chlorotoluron (Dicurane 500 L)	Spore production	98	-	Dodd & Jeffries, 1989a
	Mecoprop+ioxynil+clopyralid (Harrier)	Intra-radicular total colonization	98	-	Dodd & Jeffries, 1989a
	Mecoprop+ioxynil+clopyralid (Harrier)	Spore production	98	-	Dodd & Jeffries, 1989a
	Carbendazim (Bavistin FL)	Intra-radicular total colonization	38, 75, 104	- - +	Dodd & Jeffries, 1989b
	Ethirimol (Milgo E)	Intra-radicular total colonization	38, 75, 104	- - -	Dodd & Jeffries, 1989b
	Prochloraz (Sportak)	Intra-radicular total colonization	38, 75, 104	- - +	Dodd & Jeffries, 1989b
	Carbendazim (Bavistin FL)+Ethirimol (Milgo E)+Prochloraz (Sportak)	Intra-radicular total colonization	38, 75, 104	- - +	Dodd & Jeffries, 1989b
	Tridemorph (Calixin)	Intra-radicular total colonization	26, 56, 74, 88	+ (26 and 56 days) - (74 and 88 days)	Dodd & Jeffries, 1989b
	Carbendazim (Bavistin FL)	Intra-radicular total colonization	26, 56, 74, 88	+ (26 and 56 days) - (74 and 88 days)	Dodd & Jeffries, 1989b

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Propiconazole and tridemorph (Tilt Turbo 375)	Intra-radicular total colonization	26, 56, 74, 88	+ (26 and 56 days) - (74 and 88 days) + (88 days)	Dodd & Jeffries, 1989b
	Fenpropimorph	Spore production	42	+	Zocco et al., 2008
	Fenpropimorph	Intra-radicular total colonization	42	-	Zocco et al., 2008
	Fenpropimorph	Extra-radical mycelium architecture	42	+	Zocco et al., 2008
	Fenhexamid	Spore production	42	+	Zocco et al., 2008
	Fenhexamid	Intra-radicular total colonization	42	-	Zocco et al., 2008
	Fenhexamid	Extra-radical mycelium architecture	42	+	Zocco et al., 2008
	Glyphosate	Extra-mycelial production	28	-	Pasaribu et al., 2011
	Alachlor	Extra-mycelial production	28	-	Pasaribu et al., 2011
Rhizophagus irregularis MUCL 41833	Azoxystrobin	Intra-radicular total colonization	14, 63	+	Buysens et al., 2015
	Flutolanil	Intra-radicular total colonization	14, 63	-	Buysens et al., 2015
	Pencycuron	Intra-radicular total colonization	14, 63	+	Buysens et al., 2015
	Azoxystrobin	Spore production	14, 63	-	Buysens et al., 2015
	Flutolanil	Spore production	14, 63	-	Buysens et al., 2015
	Pencycuron	Spore production	14, 63	+	Buysens et al., 2015
	Azoxystrobin	Extra-mycelial production	14, 63	-	Buysens et al., 2015

AMF Species	Chemical	Parameter	Test duration (days)	Species sensitivity	Reference
	Flutolanil	Extra-mycelial production	14, 63	+	Buysens et al., 2015
	Pencycuron	Extra-mycelial production	14, 63	+	Buysens et al., 2015

* Growth of hyphae originated from spores.

1.3.3 Effects on the pre-symbiotic phase

1.3.3.1 Effects of PPPs

Information regarding effects of PPPs on pre-symbiotic parameters are often contradictory. Giovannetti et al. (2006) developed one on the first studies to evaluate the effect of PPPs on the pre-symbiotic phase on an AMF species. These authors investigated the effect of formulations having as a.i. the fungicides Mancozeb, Ziram, Copper Hydroxide, Oxycarboxin, Iprodione, Fosetyl Al, Metalaxyl, and *Fusarium oxysporum* 251/2RB and the herbicides Terbuthylazine, Glyphosate, MCPA, Pendimethalin, Dicamba, *Bacillus thuringiensis* Sa 11 in the pre-symbiotic phase of *Glomus Funneliformis* (*Glomus*) *mosseae*. Regarding fungicides, spore germination of *Funneliformis* (*Glomus*) *mosseae* was strongly inhibited by Mancozeb even at the lowest doses tested (400 mg a.i. L^{-1}), and by Ziram from 36 mg a.i. L^{-1} upwards. Oxycarboxin and Iprodione affected germination but without completely inhibiting it. On the other hand, Fosetyl Al and Metalaxyl did not show any effects towards spore germination. Regarding the herbicides, none of them affected spore germination, but affected the mycelium growth. This data showed that even PPPs of the same group have different effects for AMF and there is clearly a dose-effect response between AMF species and pesticides tested.

Malty et al. (2006) investigated the effects of increasing concentrations of Roundup (360 g L^{-1}, Glyphosate) (0 to 454 µmol L^{-1}) on *Gigaspora margarita*, *Claroideoglomus* (*Glomus*) *etunicatum,* and *Scutellospora heterogama* mycorrhization of soybean. The addition of glyphosate stimulated the germination of *Gigaspora margarita* spores (up to 111.75 µM concentration), while the germination of *Scutellospora heterogama* and *Claroideoglomus* (*Glomus*) *etunicatum* spores was inhibited linearly with Glyphosate concentrations. The growth of the spore germ tube was inhibited in the three AMF species tested.

Druille et al. (2013) also investigated the effect of Glyphosate on native AMF spores and on root colonization. The authors used *Paspalum dilatatum* and *Lotus tenuis* as host plants, exposed to 0, 0.8 and 3 L ha^{-1} doses of Glyphosate, with separate applications directly in the soil (Mollic Gleyic Solonetz/FAO Soil Taxonomy) and in plant foliage. Spore viability was reduced already at the lowest dose (0.8 L ha^{-1}) when Glyphosate was applied on the soil.

The effect of the herbicides Prometryn and Acetochlor was evaluated by Li et al. (2013) who exposed *Claroideoglomus* (*Glomus*) *etunicatum* to the doses of 0.1, 1 and 10 mg L^{-1}. No significant effects were found in spore germination at the presence of Acetochlor in all three concentrations tested, but a significant reduction in spore germination was observed at the highest concentration of Prometryn. Both herbicides had negative effects on mycorrhizal symbiosis with increasing concentrations and Prometryn was the most toxic.

The impact of the herbicide Nicosulfuron on the mycorrhizal colonization potential of a native community of AMF species of a field from Serbia was tested in two stages by Karpouzas et al. (2014). First an extreme exposure by repeated applications (five culture cycles of *Zea mays*) of a range of doses (0, 10, 100, 1000 times the recommended dose) was tested and, lastly, a realistic exposure scenario by exposing AMF community to concentrations based on the recommended dose (0, 1, 2, 5 times the recommended dose) was tested. In the extreme doses of 100 and 1000 times, there was a significant reduction in plant biomass, mycorrhizal colonization (hyphae, arbuscules, and vesicles) and fungal richness (determined by DGGE), which followed the progressive accumulation of the herbicide in the soil. In contrast, no significant effects were observed on the AMFs of those doses of the realistic exposure scenario.

Other studies have shown that AMF can also be threatened by the application of fungicides used to control pathogenic fungi. Buysens et al. (2015) investigated the impact of Azoxystrobin (a

broad-spectrum fungicide), Flutholanil (a fungicide for systemic-specific Basidiomycota) and Pencycuron (a specific contact fungicide for *Rhizoctonia*) on the growth and development of the AMF *Rhizophagus irregularis* MUCL 41833 under *in vitro* conditions. The results revealed that Azoxystrobin did not affect spore germination and colonization of potato roots by *Rhizophagus irregularis,* considering the recommended values (inhibitory concentration - IC, IC_{50} ≤0.1 mg L^{-1} a.i.) for controlling *Rhizoctonia solani*, while the development of extra-radical mycelium and spore production was reduced 10 times. Flutholanil did not affect spore germination or development of extra-radical mycelium, but it decreased root colonization and arbuscule formation. In the case of Pencycuron, there were no effects on spore germination and on intra or extra-radical development of *Rhizophagus irregularis*.

Recently, Mallmann et al. (2018) proposed modifications to the ISO 10832 protocol (ISO, 2009) to evaluate the effect of fungicides in the pre-symbiotic phase of AMFs. These modifications showed the adequacy of these types of tests to evaluate the risk of fungicides to spore germination using *Gigaspora albida* and *Rhizophagus clarus* as AMF species representative of subtropical soils. These authors found that *Gigaspora albida* was more sensitive to fungicides Chlorotalonil (IC50=12.14), Mancozeb (IC50=5.66), and Metalaxyl-M (IC50=3.50) than *Rhizophagus clarus*. On the other hand, Malfatti et al. (2021) found a higher sensitivity of *Rhizophagus clarus* to herbicides Glyphosate and Diuron combined with Paraquat than *Gigaspora albida*.

1.3.3.2 Effects of veterinary pharmaceuticals

No published scientific articles were found about the effects of these chemicals on AMF species in pre-symbiotic phase.

1.3.3.3 Effects of biocides

Biocides products have not been studied very well with regard to AMF. The first study reported was carried out by Colozzi-Filho et al. (1994) in Brazil. The authors evaluated the effect of Sodium hypochlorite, Formaldehyde, Mercury chloride, and antibiotics (Streptomycin sulfate, Gentamicyn, Pimaricin, Chloramphenicol), on spore germination of *Gigaspora margarita*. They found that Formaldehyde, Mercury chloride, and all of the antibiotics significantly reduced spore germination.

Twanabasu et al. (2013a) and Twanabasu et al. (2013b) reported the impacts of Triclosan (biocides used as a preservative and antimicrobial) on AMF. Spore germination, hyphal growth, and hyphal branching of *Rhizophagus (Glomus) intraradices* were all significantly reduced compared to control, in both doses tested (0.4 and 4.0 mg L^{-1}). Spore germination was reduced from 86.9% in the control, to 43% at the lowest dose tested (Twanabasu et al., 2013b). Similarly, triclosan caused significant decline in *Rhizophagus (Glomus) intraradices* hyphal and arbuscular root colonization of various wetland plant species (Twanabasu et al., 2013a).

A single study with *Fusarium oxysporum* and *Bacillus thuringiensis*-based products did not show any effects on spore germination and mycelium growth of *Funnelliformis* (*Glomus*) *mosseae.* The experimental setup consisted of a spore "sandwich system", placed in Petri dishes filled with quartz and incubated in the dark at 25°C, in the presence of tested products (Giovannetti et al., 2006).

1.3.4 Effects on the symbiotic phase

The vast majority of research involving the symbiotic phase of AMF has been carried out considering mainly the benefits for the host plants. In agricultural systems, AMF and the symbiosis, are exposed to various chemicals that may pose a risk to the events that precede the establishment of the symbiosis, in later events (consolidation and functionality of the symbiosis) and in the continuity of these fungi in the field through the production of propagules (Hage-Ahmed et al., 2019). Furthermore, considering that spores are not the main promoter of soil infectivity in some conditions (Smith and Read, 2008), and that exposure of AMF symbiotic structures may occur in a different way than pre-symbiotic, it is important to also evaluate the effects of PPPs when the symbiosis has already established.

The characteristic obligatory biotrophism of AMF is one of the greatest challenges for studying these organisms because there is a need to consider the presence of a host. The effect of chemical substances such as fertilizers, residues and contaminants on mycorrhizal symbiosis and their functionality are commonly carried out through experiments in pots with host plants (*in vivo* system), such as the works carried out by Ipsilantis et al. (2012) and Karpouzas et al. (2014), which evaluated the impact of the studied substances on mycorrhizal colonization.

AMF and their mutualistic association with plants can be affected when exposed to chemicals via contact or systemic uptake. Effects can occur directly by root or hypha contact and/or uptake, and indirectly when pesticide application promotes changes in plant metabolism. The impact of chemicals on several AMF species have been reviewed (Hage-Ahmed et al., 2018), tested (Giovannetti et al., 2006; Mallmann, 2020) and they reported the effects of PPPs on numerous endpoints, mainly related to fungal spore production, germination, mycelium growth and root colonization.

1.3.4.1 Effects of PPPs

Glyphosate is the most studied a.i. among PPPs. Zaller et al. (2014) found a significant decrease in root mycorrhization, soil AMF spore biomass, vesicles and propagules of *Funneliformis mosseae*. However, other authors found that, for the same AMF species – but probably different isolate – mycorrhizal sporulation and hyphal infectivity were significantly affected by Alachlor, but not by Glyphosate (Pasaribu et al,. 2013). Novais et al. (2019a) reported that the herbicides Dicamba and Glufosinate negatively affect extra-radical mycelium (ERM) viability and structural traits of *Funneliformis mosseae* isolate BEG 116, even more significantly than fungicides. Although these findings suggest that several factors are influencing these negative effects (e.g., such as AMF isolate, evaluated endpoint, choice of pesticide) it is not possible to make comparisons, as experimental setups are notably different, especially regarding the soil/matrix used.

Rivera-Becerril et al. (2017) reported that the abundance (measured by nested and real-time qPCR on purified DNA) of the Glomeromycota taxa decreased in both a vineyard and an arable soil due to the application of a pesticide cocktail composed of Fenhexamid, Folpel, and Deltamethrin, even at the recommended field dose. Some taxa (*Funneliformis mosseae* and *Claroideoglomus*), however, decreased only in arable soil, which indicates that rather than reducing global abundance, pesticides alter the community structure. As a result, ecosystem services provided by these fungi may be affected (Rivera-Becerril et al., 2017).

Campos et al. (2015) evaluated the effect of fungicides currently used to control diseases in common bean using native AMF as bioindicators. These authors evaluated the effects of various fungicides on mycorrhizal colonization, the number of spores in soil, leaf N, P and K contents, dry

mass of the root, shoot and beans. The following treatments were used: water (control), Azoxystrobin (120 g ha^{-1}), Pyraclostrobin (75 g ha^{-1}), Tebuconazole+Trifloxystrobin (0.7 L ha^{-1}), Trifloxystrobin (225 g ha^{-1}), Oxide cuprous (1.5 Kg ha^{-1}) and Mancozebe (2.5 Kg ha^{-1}). Azoxystrobin, Tebuconazole+Trifloxystrobin in a combi product and Trifloxystrobin applied as monoformulation did not cause a significant difference in the root colonization with native AMF, in the number of spores in the soil or in the leaf N, P and K contents in relation to the control. Pyraclostrobin, Cuprous oxide, and Mancozebe inhibited mycorrhizal colonization when compared to the control. These findings suggested that Azoxystrobin, Tebuconazole+Trifloxystrobin or Trifloxystrobin are the most adequate fungicides to control bean diseases without compromising the symbiotic performance of the native AMF.

Another study reported that high doses of Nicosulfuron (x100 and x1000 the recommended dose) significantly reduced root mycorrhizal colonization, AM fungal richness, and host plant biomass (*Zea mays*). The authors hypothesised that these effects might be related to the accumulation of the herbicide in soil (Karpouzas et al., 2014). Also, high doses of pesticides can remain in the soil for long periods, constituting a risk to AMFs. Pánková et al. (2018), found that negative effects of the fungicide Carbendazim on plant species composition, diversity, AMF infectivity still persisted five years after the last fungicide application.

However, according to Rabab & Reda (2019), a correct use (i.e., recommended dose) of Ridomil and Bavistin may have stimulatory effect on AM fungal colonization in the host plant *Cucumis sativus*. Indeed, these authors reported increased mycorrhizal colonization after application of both pesticides (250 ml a.i. L^{-1} of Ridomil [Metalaxyl-M 8% and Mancozeb 64%] and 500 a.i. L^{-1} of Bavistin [Carbendazin]) when compared to treatment without pesticide. This may constitute per se a way for the farmer to stimulate mycorrhizal symbiosis.

1.3.4.2 Effects of veterinary and human-use pharmaceuticals

Pharmaceuticals and personal care products show a potential risk on beneficial soil microbe populations, including AMF species. Nevertheless, until this moment there is a gap of knowledge about this topic for AMF. The only work found that investigated the effects of pharmaceutical products was a study conducted by Hillis et al. (2008). The authors investigated the effects of veterinary and human-use pharmaceuticals and PCPs in the symbiotic phase of *Rhizophagus* (*Glomus*) *intraradices* (in transformed carrot roots). Among the 12 products evaluated, the EC_{50} values for *Rhizophagus* (*Glomus*) *intraradices* hyphal length was 45 µg L^{-1} for Doxycycline and Sulfamethoxazole, and 65 µg L^{-1} for Atorvastatin, while Carbamazepine and 17-α-ethynyl estradiol targeted spore production with EC_{50} values of 113 and 116 µg L^{-1}, respectively. This study provided important information regarding the minimum time period of exposure needed for certain AMF endpoints. For example, for hyphal length, at least 21 days of exposure is needed, while spore production requires 28 days, otherwise no significant differences could be detected (Hillis et al., 2008). Similarly to pesticides, these products showed a dose-response curve that changed according to the product and the endpoint measured.

1.3.4.3 Effects of biocides

Personal care products, as Triclosan (TCS) and Triclocarban (TCC), are compounds frequently detected at high concentrations in biosolids. Considering their antimicrobial properties and being relatively persistent in the soil, the mode of action of TCS and TCC could adversely affect AMF (Prosser et al., 2015). However, Prosser et al., (2015) demonstrated that these TCS and TCC-amended biosolids do not inhibit the colonization of corn roots by a mix of AMF (*Glomus*

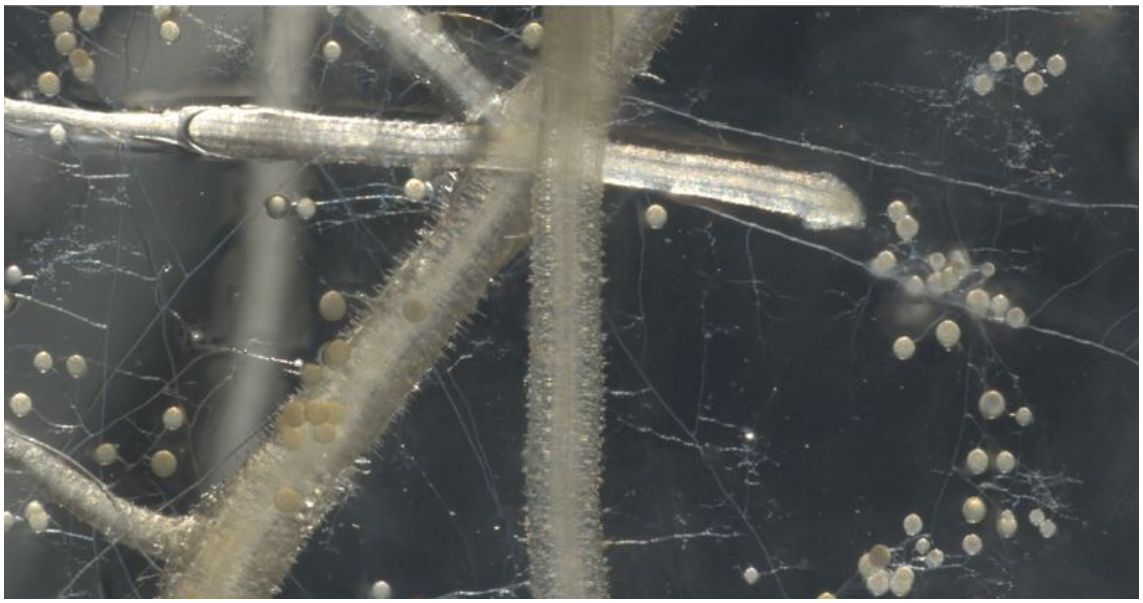
aggregatum, *Claroideoglomus* (*Glomus*) *etunicatum*, *Glomus intraradices*, *Funneliformis* (*Glomus*) *mosseae*, *Rhizophagus clarus* (*Glomus clarum*), *Glomus monosporus*, *Gigaspora margarita*, and *Paraglomus brasilianum*), and that they have a significant and positive effect on the colonization of lettuce roots, improving root growth. With the same mix of AMF, Shahmohamdloo et al. (2017) showed that TCS present in four different types of biosolids poses minimal risks in the establishment of the symbiotic phase of AMF and host plants (*Zea mays*, *Glycine max*, *Triticum aestivum*), since TCS typical concentrations (found in municipal biosolids in Canada and United States) caused no significant effects for root colonization.

In organic agriculture, the use of biological products or biological pesticides to control pests and diseases is widespread. These pesticides are generally considered environmentally safe (Isman, 2006). However, as well as many other products, there is limited information on their effects on non-target soil organisms like AMF. Giovannetti et al. (2006) has shown effects for the pre-symbiotic phase, as previously reported. One of the first studies published about the effects of biological products on the diversity of AMF (Ipsilantis et al., 2012), showed that Spinosad, Pyrethrum, and Terpenes did not affect the colonization ability and the structure of an AM fungal community. On the other hand, in a pot experiment, Azadirachtin selectively inhibited *Glomus etunicatum*, and promoted significant and persistent shifts in the AM fungal community.

1.4 Suitability of different *in vitro* (AM fungi only or AM fungi with plant roots) and *in vivo* test designs (AM fungi plus host plants) for the reliable detection of different endpoints

The *in vitro* system is based on an association between AMF and root explants, cultivated in Petri dishes at low levels of nutrients, and is the only system that allows real time observation of a mycorrhizal association (Bécard and Fortin, 1988; Cranenbrouck et al., 2005). This system became a valuable tool to study characteristics that cannot be observed in any other cultivation system and, therefore, this is an important tool to better understand AMF dynamics. Such aspects include non-destructive monitoring of sporulation (Declerck et al., 2001), as well as spore color and wall thickness (Pawlowska et al., 1999). Mycelium-healing mechanisms, hyphal length, and anastomosis patterns can also be monitored throughout different stages of the symbiosis (de la Providencia et al., 2005). In this cultivation system, a transformed plant root is colonized by an AMF, and extraradical hyphae and spores are spread through a medium with very low nutrient concentrations, supplied with sucrose (Bécard and Fortin, 1988; Cranenbrouck et al., 2005). Throughout the past years, most research work with this system has been focused on massive spore production for inoculum production purposes (Costa et al., 2013; Ellatif et al., 2019). However, many characteristics of this system can be directed to study endpoints in toxicological research, given its remarkable traits. First, the *in vitro* cultures allow observation of both roots and mycelia growing simultaneously (Figure 13). Hence, evaluations of hyphal production, as well as spore number and spatial distribution can be easily assessed.

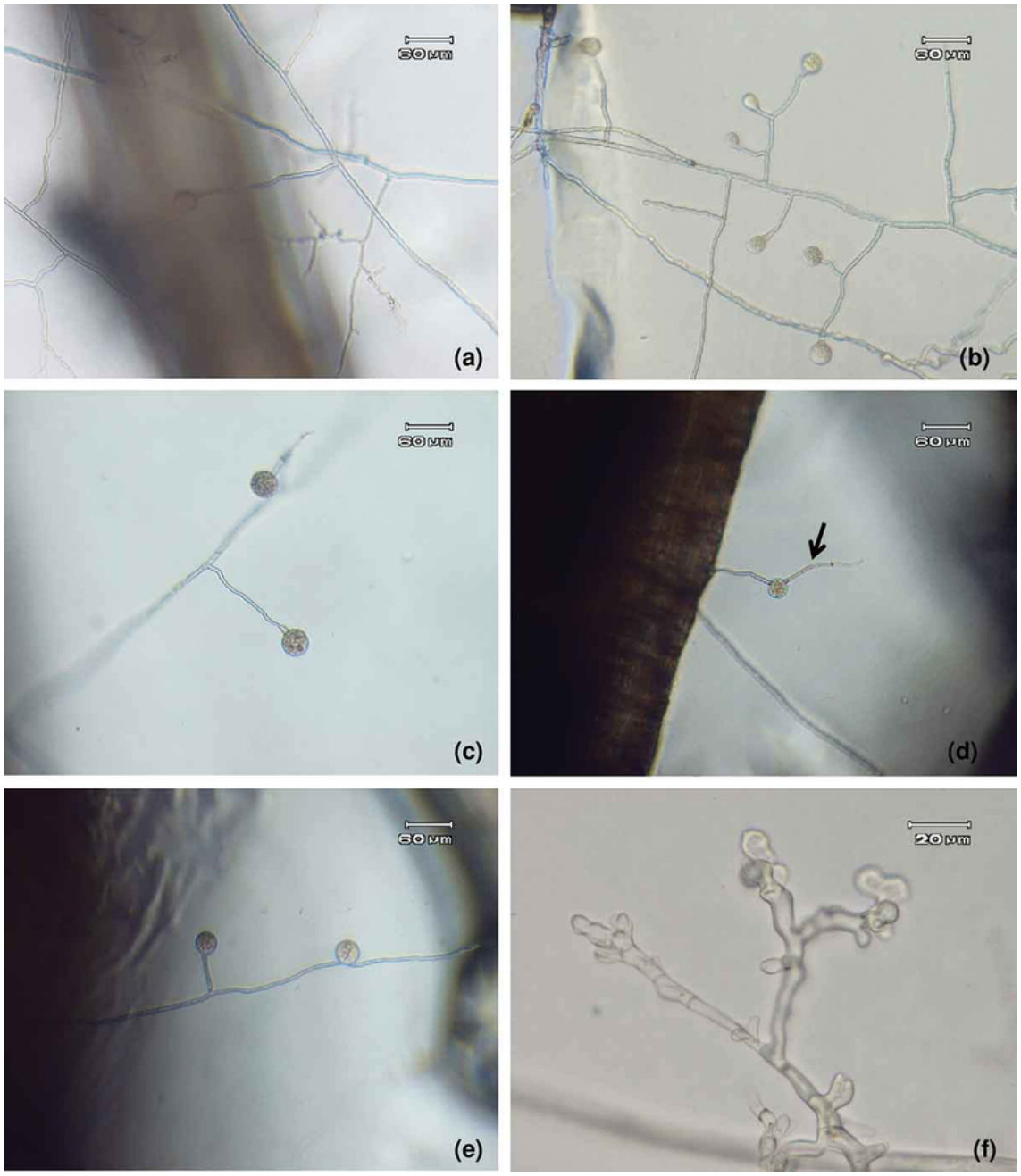
Figure 13: ***In vitro*** **coculture of** ***Rhizophagus irregularis*** **with carrot roots showing extraradical hyphae and spores.**



Source: Tisserant et al. (2013).

Aspects of spore development, as well as hyphae architecture, can be also evaluated with *in vitro* cultures. Figure 14 (a-f) details those aspects in root organ cultures.

Figure 14: Development of extra-radical mycelium of *Funneliformis mosseae* and *in vitro* spore formation (a–f).



Source: Rodrigues & Rodrigues (2015).

Given all these features, AMF *in vitro* cultures, when applied to ecotoxicological studies, have the potential to provide information regarding:

- Extra-radical mycelium production
- Spore number, morphology, color, and distribution
- Intra-radical root colonization
- Hyphae morphology, branching and thickness

However, it is important to highlight that few studies based on *in vitro* cultures (Hillis et al., 2008; Costa et al., 2013; Li et al., 2013; Novais et al., 2019a, 2019b) have addressed these aspects in an ecotoxicological perspective, and this project aims to explore the potential of endpoints measured from roots in a real scenario. Although *in vitro* tests can bring important information related to AMF symbiosis, *in vivo* tests can bring greater reality to the tests, allowing the evaluation of the effects of chemicals in AMF based on the exposure of AMF to chemicals when submitted to the influence of environmental factors like soil properties.

1.5 Conclusions of the literature review

The current review gives an overview of AMF ecological relevance and their life cycle. It also brings together information about the experiments conducted with the target organic chemicals of this project (i.e., PPPs, biocides and veterinary pharmaceuticals) to test their effects on AMF species and on the symbiosis with the host plants. Aspects dealing with tested species, test conditions, and parameters measured (and their sensitivity) are described below.

Given that species *Gigaspora margarita*, *Rhizophagus irregularis*, *Rhizophagus intraradices*, *Rhizophagus clarus*, and *Funneliformis mosseae* showed a high frequency of occurrence or global distribution, these species seem to be the most promising candidates to be selected as test species for the laboratory tests to be developed in WP2 of the project. Moreover, these species being among the ones mostly used to assess the effects of chemicals on AMF (together with *Claroideoglomus* (*Glomus*) *etunicatum* and *Glomus aggregatum*) highlights their adequacy as potential candidates.

Besides the selection of the AMF species, aspects like the temperature and time of inoculum storage, especially spores, must be considered, as they can affect their performance. Depending on the species to be used, spores can remain in storage for five years without losing germination viability. This must be considered when buying inoculum from different suppliers.

Regarding host plants, a wide range of both monocot and dicot species can be used. So, in WP2 the selection of the host plants for testing (at least one moncot and one dicot) should take into account the duration of the plant life cycle (fast growing species should be selected in order not to prolong the test duration), but also the experience of the different partners, i.e., the most frequently used species in their laboratories should be prioritized.

About test conditions, considering soil moisture, AMF can endure a wide range of moisture contents, and during tests, the optimal moisture level for plant growth should be considered. Regarding temperature, the range adequate for the development of symbiosis in **most species studied is between 25 and 28 °C. Therefore, a temperature around this range should be selected for testing.**

Regarding soils, besides the artificial OECD soil, natural soils can be used in WP2. Although in most tests reported in literature, the soils presented a **pH between 6.0 and 7.0, soils with lower pH can also be used.** So, the influence of different soil parameters should be tested in WP2, by using a wide range of soils with different properties, including an evaluation of the robustness of the test itself.

When looking at effects of chemicals and the selection of the test parameters according to their sensitivity, a separation of measured effects during the pre-symbiotic phase (spore germination and mycelium growth) and assessed effects at the symbiotic phase (e.g., intra-radicular colonization, percentage of arbuscules in the roots, extra-radicular mycelium and spore production) should be considered in the evaluation.

Spore germination has been the most studied parameter but not always the most sensitive when compared to mycelium growth, with sensitivity of both parameters being species and substance dependent. From the 13 herbicides tested, 6 showed toxicity to the parameter spore germination, while from the 6 herbicides tested through the measurement of mycelium growth, 4 showed toxicity for this parameter. Regarding fungicides, from the 21 substances assessed through spore germination, 15 showed toxicity to this parameter, while 7 out of 8 fungicides significantly impaired the mycelium growth. Data on biocides is more consistent, with most studied substances inducing a reduction in spore germination. No information is available on effects of veterinary pharmaceuticals. This aspect should be taken into account when improving the existing protocol for assessing effects on the pre-symbiotic phase, although the easiness of evaluating **spore germination** when compared to mycelium growth, makes this parameter much more attractive to the main parameter in **a pre-symbiotic** protocol.

On the symbiotic phase, **extra-radicular mycelium** has been reported as the most sensitive parameter, followed by spore production and root colonization (considering several fungicides tested). Curiously, until this moment, there are no toxicity data reported to these parameters when testing herbicides. On the other hand, several veterinary and human pharmaceuticals induced a toxic response both on extra-radicular mycelium growth and spore production. **So, as expected, the sensitivity of these parameters can be species and substance dependent**. When developing a standard protocol for the symbiotic phase, it is important to take these aspects into account, but adopting a compromise solution regarding the feasibility of measuring some of the parameters and the duration of the test to observe them (e.g., to observe effects on spore germination, usually 14 days are necessary while to observe effects on root colonization and extra-radicular mycelium production, 56 days are needed).

2 Section 2 – WP2: Performance of ecotoxicological tests with AMF – development phase

The strategy defined for WP2 was based on the outcome of the review presented in Section 1 of this Report and on the experience of the consortium members of the project.

WP2 was divided into two phases (i.e. two clusters of experiments), and both phases were dedicated to the symbiotic phase tests, as these experiments were expected to be more time consuming and required a more significant amount of work for standardization given the fact that it comprised a new method about which no international standards existed (despite the existence of the guidance document from AFNOR).

In the first phase, no chemicals were used, and the aim was to evaluate the performance/suitability of selected AMF and plant host species in selected soils under specific test environmental conditions. The most relevant AMF parameters were assessed to evaluate the operational aspects of their measurement and the intra-specific variability also to define adequate validity criteria for combinations of AMF species/measured parameters. Initial drafts of the SOPs were prepared in this phase to guide the experiments conducted on the second phase of WP2.

The second phase included the use of test chemicals, and the aim was to assess how the different AMF species and parameters measured respond to the selected chemicals. Tests with artificial soil were prioritized to facilitate comparability of results between different laboratories, followed by the tests in natural soils. The SOPs were improved during the performance of these tests.

The results obtained in WP2 guided the preparation of the symbiotic and pre-symbiotic ring tests (e.g., selection of methods to apply, substances to use).

The three laboratories composing the ERAMYC consortium conducted the experiments of WP2:

- UDESC laboratory from Brazil;
- University of Tartu laboratory from Estonia;
- CloverStrategy and University of Coimbra laboratory from Portugal.

2.1 Selection of test soils

A modified artificial soil (composed of 2.5% peat, 20% kaolin clay and 77.5% sand, hereon designated as OECD 2.5%), plus 4 natural soils, 3 from Europe and 1 from Brazil, were selected for the execution of the tests. The 3 natural soils from Europe comprised 1 natural soil from Portugal, 1 from Estonia and 1 from Germany.

The selection of the natural soils was based on two principles: (i) the result of a Principal Coordinate Analysis performed with all the natural soils possible to collect by the different laboratories in the different countries (Portugal, Estonia, Germany and Brazil) based on pre-existing knowledge of soil characterization, and (ii) the availability of the soils to be collected.

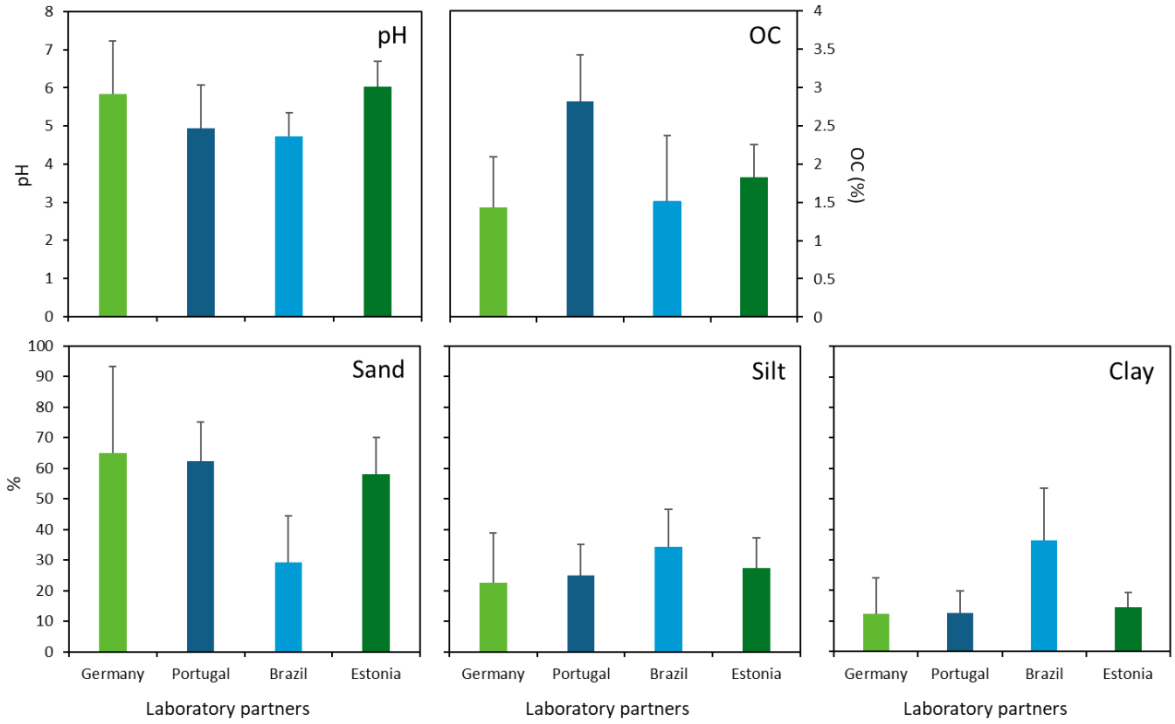
Moreover, the natural soils should be locally representative and should reflect (as well as the artificial soil) the reality of the natural European soil properties. As reported by Xu et al. (2019),

European agricultural soils are acidic or weakly acidic and the organic carbon content is on average of 1.73-1.80%. This agrees with the PERSAM/JRC database and Soil Atlas of Europe (EC 2005), according to which most European soils are typically from humid temperate climate with organic carbon content between 1 and 2.5% and pH values predominantly of 4.1-4.7, 5.0-5.2 and 6.4-6.7 (EFSA PPR Panel 2021). Beyond the relatively low organic carbon content (≤ 2.5%), a low fine sand and a low clay content are also relatively common in European agricultural soils (EFSA Scientific report, 2010). Despite that, preference was also given to soils with low clay content to ensure adequate colonization of AMF species in laboratory tests as data from literature have shown greater AMF root colonization in sandy soils (e.g. Zaller et al. 2011).

The main properties taken into consideration for the selection of the natural soils were pH, organic carbon content (OC) and percentages of sand, silt and clay. The list of natural soils considered in the multivariate analysis is presented in Appendix A.10.

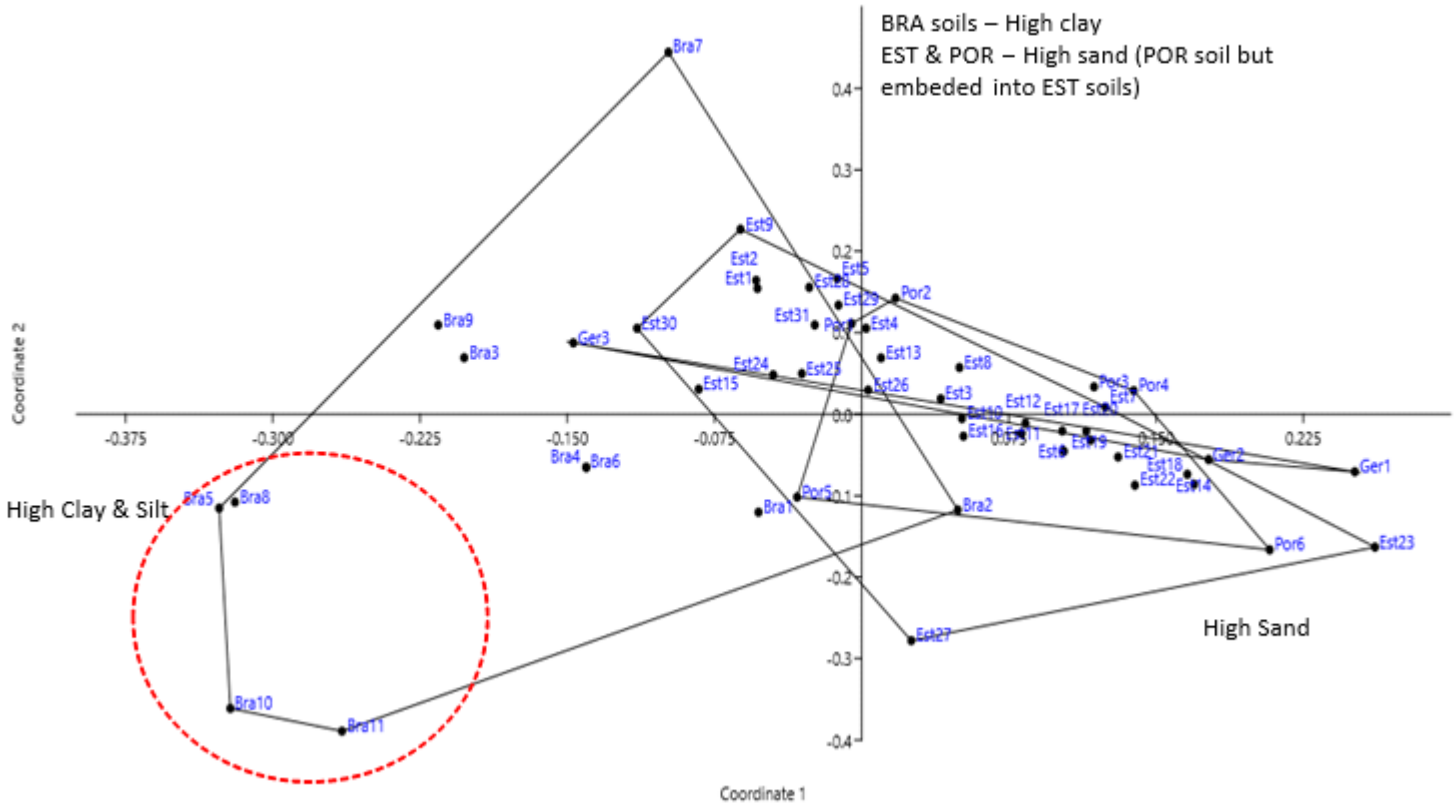
Soil pH does not differ considerably between the natural soils of the different countries, while Portuguese soils tend to be richer in organic carbon content compared to the natural soils of the other countries. On the other hand, Brazilian soils are richer in silt and clay content, while the European soils have the highest content of sand (Figures 15 and 16).

Figure 15: Average (± standard deviation) of soil pH, organic carbon content (OC) and sand, silt, and clay percentages of natural soils of Germany, Portugal, Brazil and Estonia listed in Appendix A.10.



Source: own illustration, CloverStrategy.

Figure 16: Principal Coordinate Analysis based on the similarity of soil properties (through Bray-Curtis indices) of natural soils from Germany (Ger), Portugal (Por), Brazil (Bra) and Estonia (Est; listed in Appendix A.10).



Source: own illustration, University of Coimbra.

According to a SIMPER analysis using Bray-Curtis indices, texture is the major soil property, discriminating soils from both continents (Figure 17).

Figure 17: Output of the SIMPER analysis performed using the Bray-Curtis indices to estimate the average dissimilarities when comparing Portuguese with Brazilian soils and Brazilian with Estonian soils.

Groups POR & BRA
Average dissimilarity = *35,58*

	Group POR	Group BRA				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sand	62,40	29,20	15,81	1,95	44,43	44,43
Clay	12,72	36,45	11,84	1,70	33,28	77,70
Silt	24,88	34,35	6,46	1,25	18,15	95,86

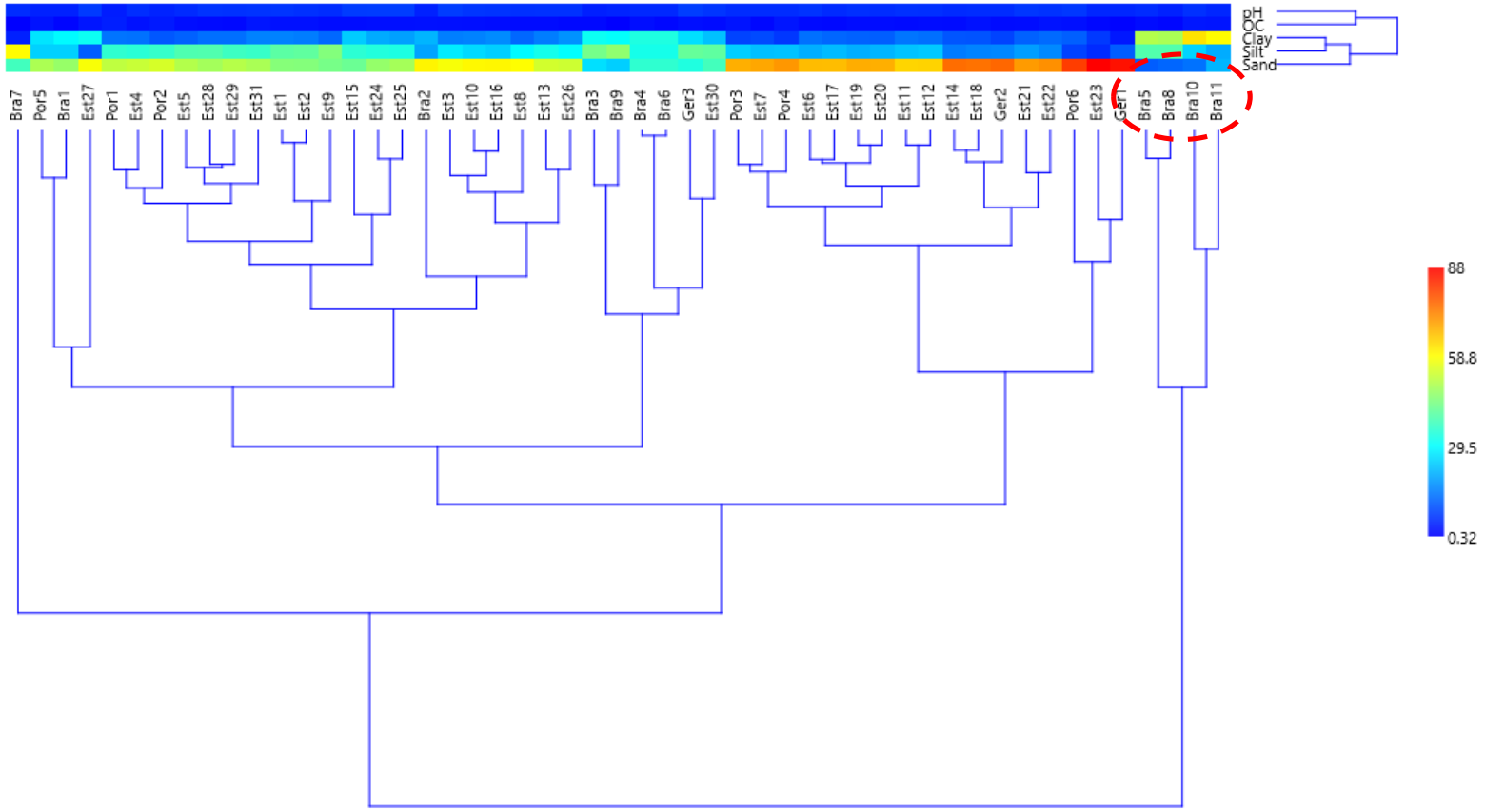
Groups BRA & EST
Average dissimilarity = *32,49*

	Group BRA	Group EST				
Species	Av.Abund	Av.Abund	Av.Diss	Diss/SD	Contrib%	Cum.%
Sand	29,20	58,16	14,09	1,80	43,36	43,36
Clay	36,45	14,44	11,20	1,72	34,47	77,82
Silt	34,35	27,40	6,19	1,29	19,06	96,89

Source: own illustration, University of Coimbra.

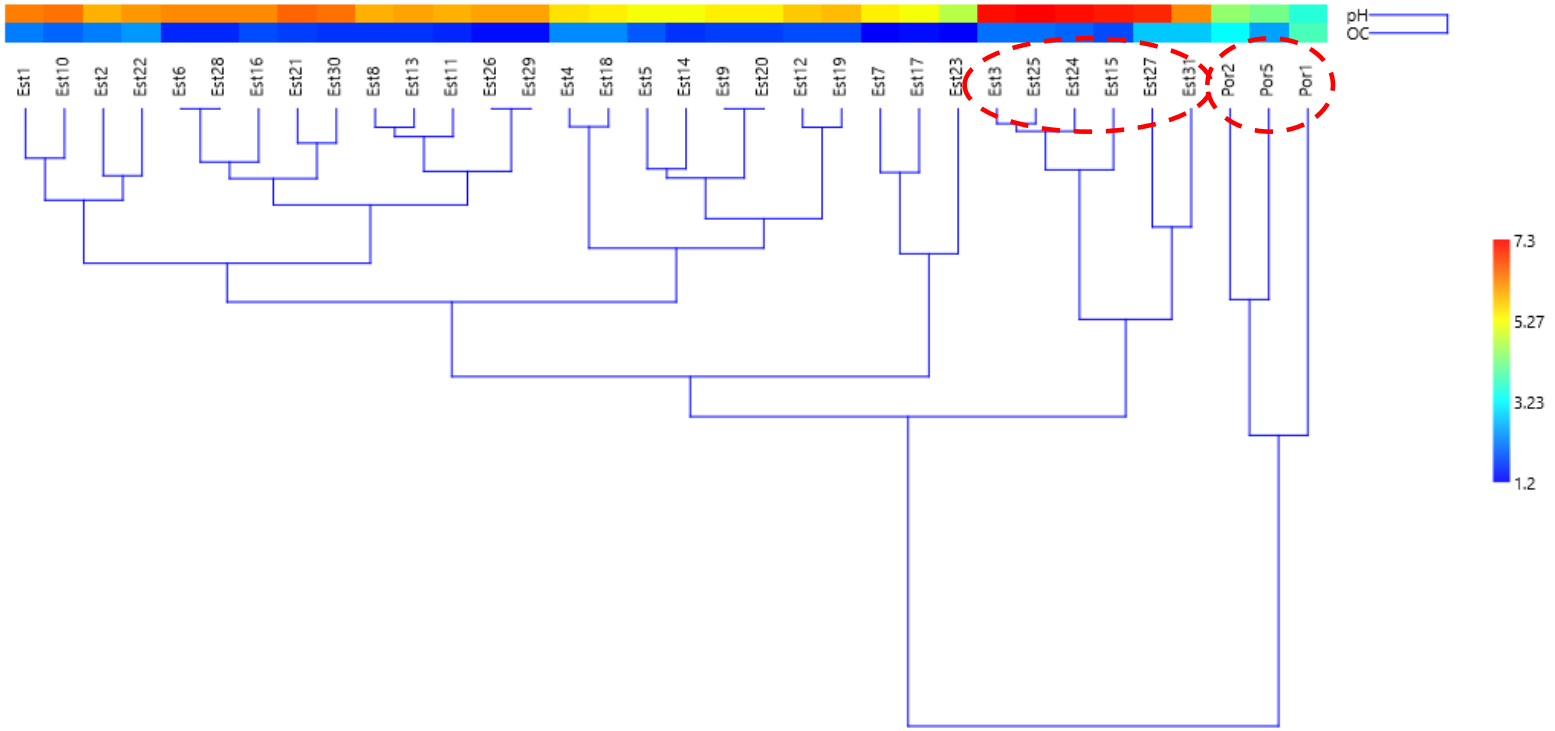
Cluster analysis based on Bray-Curtis dissimilarity indices discriminated 4 Brazilian soils (Bra5, Bra8, Bra10, Bra11) taking into consideration pH, organic carbon content and clay, silt and sand percentages (Figure 18), while 3 Portuguese soils (Por2, Por5, Por1) and 6 Estonian soils (Est3, Est25, Est24, Est15, Est27, Est31) were discriminated when considering pH and organic carbon content of soils (Figure 19).

Figure 18: **Cluster analysis based on the Bray-Curtis indices of dissimilarities considering the soil pH, organic carbon content (OC) and clay, silt and sand percentages of natural soils from different countries. Dashed red circle highlights the Brazilian soils. Soil properties of natural soils are presented in Appendix A.10.**



Source: own illustration, University of Coimbra.

Figure 19: **Cluster analysis based on the Bray-Curtis indices of dissimilarities considering the soil pH and organic carbon content (OC) of natural soils from different countries excluding the Brazilian natural soils and some of the Portuguese soils. Dashed red circles highlight the most extreme Estonian and Portuguese soils. Soil properties of natural soils are presented in Appendix A.10.**



Source: own illustration, University of Coimbra.

The selection of natural soils was based on the analysis described above. The soils selected were Lufa 2.1, Por3, Bra8 and Est32. The natural Estonian soil selected for the experiments was a soil that presented higher probability to have lower residue of PPPs than the soils from the list used for the multivariate analysis (Appendix A.10), but of which previous analysis have shown that soil pH and organic carbon and clay content are in the same range as the other Estonian soils (i.e. Est3, Est15, Est24, Est25, Est27 and Est31). The natural Portuguese soil selected, Por3, presented a higher sand content, was easier to collect and was safer in terms of PPPs residues than other soils selected on the Bray-Curtis analysis (Figure 19) as it comes from an experimental field without historical use of PPPs for the last 10 years.

After soils selection, additional analyses were performed to evaluate the soil properties of the 4 natural soils selected (Table 10), including: soil pH (ISO, 2021), organic matter content (ISO, 1995), cation exchange capacity (ISO, 2018), electric conductivity (ISO, 1994b), total nitrogen (ISO, 1998) and soil texture (ISO, 2020). Exchangeable phosphorous (P) was quantified through the Olsen method: P was extracted from soil using 0.5 M sodium bicarbonate and determinated by molecular elemental analysis (EA) (ISO, 1994a). Extractable potassium (K) was quantified following the Égner-Riehm method by the extraction of K from soil with acetate/ammonium lactate (at pH = 3.6) and determination by atomic EA (Egnér et al., 1960). Extractable aluminium (Al) was determinated by titration, after extraction with 1M KCl (Thomas, 1960). Finally, the quantification of iron and manganese (Fe and Mn, respectively) was conducted according to the Lakanen method, using ammonium acetate-EDTA (AFNOR, 2024) for extraction followed by determination through ICP-OES.

By the end of WP2, a great difference between the pH generally described for Lufa 2.1 soil (pH= 5.9) and the pH measured in the Lufa 2.1 soil used on the tests (pH= 7.59) was realized. Actually, the soil supplier (i.e. Lufa Speyer company) confirmed to have sent a mistaken soil instead of the ordered Lufa 2.1 and provided information about that soil. The characterization of the "mistaken Lufa 2.1 soil" is presented in Table 10. Hereon, a # will be added to the soil code (i.e., #Lufa 2.1 soil) throughout the report so that the mistaken soil can be correctly identified.

Table 10: Chemical and physical characterization of the natural soils selected for the experiments of WP2 of ERAMYC.

Parameter	#Lufa 2.1	Por3	Bra8	Est32
Country	Germany	Portugal	Brazil	Estonia
Origin	Lufa 2.1	FM	Ferralsol (FAO)	KIK069T
pH (H_2O)	7.8	5.7	5.3	6.6
pH (KCl)	7.6	5.0	4.2	5.6
Organic matter (%)	0.65	1.92	7.34	4.13
Extractable P (mg P kg^{-1})	23.3	14.0	4.7	21.7
Extractable K (mg K_2O kg^{-1})	70	275	208	310
Exchangeable Al^{3+} (cmolc kg^{-1})	0.0	0.04	0.93	0.0
Extractable Fe^{2+} (mg Fe kg^{-1})	121.7	54.7	148.7	286.0
Extractable Mn^{2+} (mg Mn kg^{-1})	56.8	59.3	263	150.3
Cation Exchange Capacity (cmolc kg^{-1})	4.4	6.81	7.92	12.44

Parameter	#Lufa 2.1	Por3	Bra8	Est32
Electric conductivity (dS m^{-1})	0.123	0.121	0.253	0.101
Total N (g N kg^{-1})	0.58	1.92	7.34	4.13
Ratio (C/N)	11.2	5.8	5.8	5.8
Clay (%)	3.5	15.6	52.6	19.0
Silt (%)	12.7	9.0	25.4	14.4
Sand (%)	83.8	75.4	22.0	66.6
Soil type	Loamy sand	Sandy loam	Clay	Sandy loam

Multi-residue analyses were performed with the 4 natural soils to evaluate the presence of pesticide residues in the soil. The list of active ingredients and the respective quantification limits considered in these analyses are presented in Appendix A.11. No active ingredients above the detection limit were found in Por3, Bra8 and Est32 soils. In #Lufa 2.1 soil, the active ingredients Anthraquinone, p,p'-DDE and p,p'-DDT were found but in concentrations that were considered residual and not sufficiently high to influence the outcome of the laboratory experiments (i.e. 0.0091, 0.0074 and 0.0083 mg/kg, respectively).

2.2 Selection of AMF species to be used in the experiments

AMF species were selected based on the outcome of the review from WP1, on the availability in culture collections and considering the top AMF species recorded globally. Considering these aspects, the following species were selected to be used in the laboratory experiments:

- *Funneliformis mosseae* (from the Swiss AGROSCOPE bank – contact person Marcel van der Heijden)
- *Rhizophagus irregularis* (from the Swiss AGROSCOPE bank – contact person Marcel van der Heijden)
- *Rhizophagus clarus* (isolated from a tropical soil of Brazil - used only by the Brazilian laboratory)
- *Gigaspora margarita / albida* (isolated from a tropical soil of Brazil - used only by the Brazilian laboratory)

This selection was also based on the availability of enough inoculant from a supplier for WP2 and for the ring test of WP3. The project consortium considered that the propagation of a sufficient amount of inoculant for all laboratories was unfeasible within the project timeline.

Therefore, inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* (in a sterile medium) were provided (free of charge) by AGROSCOPE to all laboratories partners of the project to be used in the experiments of WP2.

2.3 Selection of the host plant species

The selection of plant species for the experiments was based on the consortium members previous experiences and some criteria set by discussions. The plant host had to be a mycorrhizal species with suitable growth characteristics to be used in both growth chambers and greenhouses. Nitrogen-fixing species were excluded due to the added complexity of having an additional symbiont in the experiments. The plant species diaspores also had to be readily available, meaning that most natural plants were excluded, and mostly agricultural cultivars were considered. Finally, the plant species had to be sufficiently easy to work with – i.e. have roots that are easy to process for staining.

The monocot species *Allium ampeloprasum* (leek – variety Carentan) and the dicot species *Solanum lycopersicum* (tomato – variety Beef heart tomato) were selected as host species for the experiments of the first phase of WP2. These species are widely used in ecological studies with AMF and there is a long-term experience of using these plants in the laboratories of the consortium members. All laboratories used the same batches of commercial seeds to reduce variability of data between experiments within the same host plant species.

2.4 Experiments to refine test protocols for the symbiotic phase (1st phase of WP2)

The first phase of the WP2 was composed of laboratory experiments for:

- Testing and refining test protocols for the symbiotic phase experiments in artificial soil (with 2.5% of OM) and natural soils;
- Preparation of Standard Operating Procedures (SOP) for the critical phases of the test;
- Evaluate the suitability of the selected AMF species and hosts to the selected soils;
- Evaluate the suitability of the selected endpoint parameters.

2.4.1 Objective 1: Define the most adequate type of inoculum to obtain a suitable percentage of AMF colonization for symbiotic phase laboratory tests

Definitions:

Spore inoculum –	Inoculum consisting exclusively of spores of the AMF tested
Mixed inoculum –	Inoculum consisting of a mixture of different types of AMF propagules, such as fungal hyphae, spores and colonized roots
Inoculant –	Mixture of the mixed inoculum with a soil (usually a sandy soil)

Aiming to obtain an optimal root colonization rate in the test replicates, experiments were performed following two different approaches:

1. Spore inoculum approach - Using a gradient of increasing number of counted spores (spore inoculum) in each replicate
2. Mixed inoculum approach - Using a gradient of increasing weights of inoculant in the replicates

A method based on wet sieving was adopted to extract the spores from the inoculant to obtain the spore inoculum (method described in Annex A of Appendix A.17). Once extracted, the spores were counted, using a binocular microscope, and transferred to the test containers to obtain replicates with specific numbers of spores. Each treatment was composed of 4 replicates each one with 200 g of soil (OECD 2.5% artificial soil or a natural soil). The percentage of colonization obtained in these replicates was compared with the percentage of colonization obtained in replicates where specific weights of inoculant (instead of spores) were used. In both approaches, the percentage of total colonization was evaluated after a test period of 4 weeks by the observation of the presence of intra-radicular hyphae, arbuscules, vesicles and spores in the root fragments following the procedures described in Appendix A.15. These observations were carried out after staining the roots of host plants following the procedures described in Appendix A.14, which were defined based on the methods routinely adopted in UDESC and UT and on some preliminary experiments conducted in UDESC and UC laboratories to optimize the staining method.

Brazilian partner (UDESC) performed both approaches (i.e. pre-counted spores and increasing weights of inoculant) using the Brazilian natural soil (Bra8 without correcting the pH) for tomato (*Solanum lycopersicum*) and leek (*Allium ampeloprasum*) and using the AMF species *F. mosseae* BEG240 (isolated from a temperate soil of Germany) and *R. clarus* RJN102A (isolated from a tropical soil of Brazil). The test soil was previously autoclaved through one cycle of 60 min at 120 °C and under 1.5 atm. After autoclaving, the soil was kept at room temperature for 5-7 days before being used in the tests. Treatments of the spores inoculum approach were 0, 25, 50, 75 and 100 spores per replicate, while the approach based on specific weights of inoculant was composed of the following treatments: 0, 15, 30, 45 and 60 g of inoculant per replicate for *F. mosseae* and 0, 10, 20, 30 and 40 g of inoculant per replicate for *R. clarus*. For the spore inoculum approach, in each replicate, 200 g of soil previously moistened with distilled water to 50% of its water holding capacity (WHC) was used. Then, three seeds (tomato or leek) and an appropriated number of spores (*F. mosseae* or *R. clarus*) were buried in the soil at a maximum depth of 1 cm from the soil surface. For the mixed inoculum approach, in each replicate 130 g of soil previously moistened with distilled water to 50% of its WHC was introduced in the replicates. Then, at the top of this soil, 70 g of a mixture of soil (moistened with 50% of its WHC) + inoculant (dry) following the gradient of proportions presented in Table 11 was introduced. Afterwards, three seeds were buried in the soil in the same way as performed in the spore inoculum approach.

Table 11: **Weights of soil (fresh weight equivalent) and inoculant (with mixed inoculum of *Funneliformis mosseae* or *Rhizophagus clarus*) used in the 70 g mixtures introduced in the top of each replicate of the mixed inoculum approach treatments.**

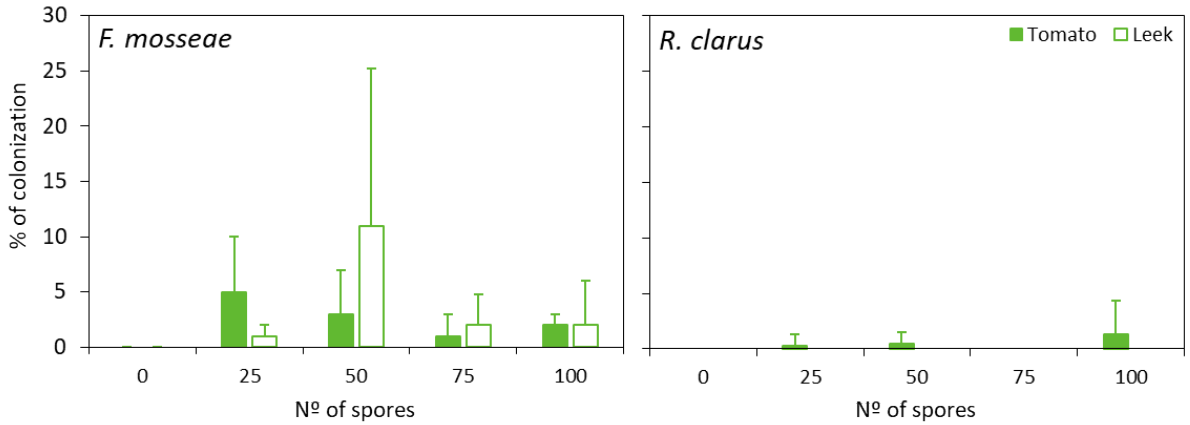
Treatment	Soil (g)	Inoculant (g)
F. mosseae		
0	70	0
15	55	15
30	40	30
45	25	45
60	10	60
R. clarus		
0	70	0
10	60	10
20	50	20
30	40	30
40	30	40

In both approaches and for both host plants, seven days after seeds germination, one plant per replicate was left and the test period lasted 28 days. Over the experiments, replicates were randomly distributed within the space used for the test and their positions were changed once a week at the growth chamber. Test vessels were irrigated twice a week with distilled water according to the weight losses during the test. At the end of the test period, the percentage of intra-radicular total colonization was determined following the procedures described in Appendixes A.14 and A.15. Raw data from the experiments conducted for both approaches are presented on Appendix A.12.

There was no AMF colonization in any of the control replicates independently of plant or AMF species and approach, which means that the experiments were valid and there were no active propagules coming from the soil used. Results obtained in the spore inoculum approach evidenced no clear relationship between the number of spores initially used in the replicates and the percentage of AMF colonization for both AMF species and host plants (Figure 20).

Figure 20: **Percentage of total colonization (average + standard deviation; *n*=4) of *Funneliformis mosseae* and *Rhizophagus clarus* in replicates with tomato (*Solanum lycopersicum*) and leek (*Allium ampeloprasum*) and using the natural soil Bra8,**

when submitted to an increasing number of pre-counted arbuscular mycorrhiza fungi spores. Tests performed by UDESC, Brazil.

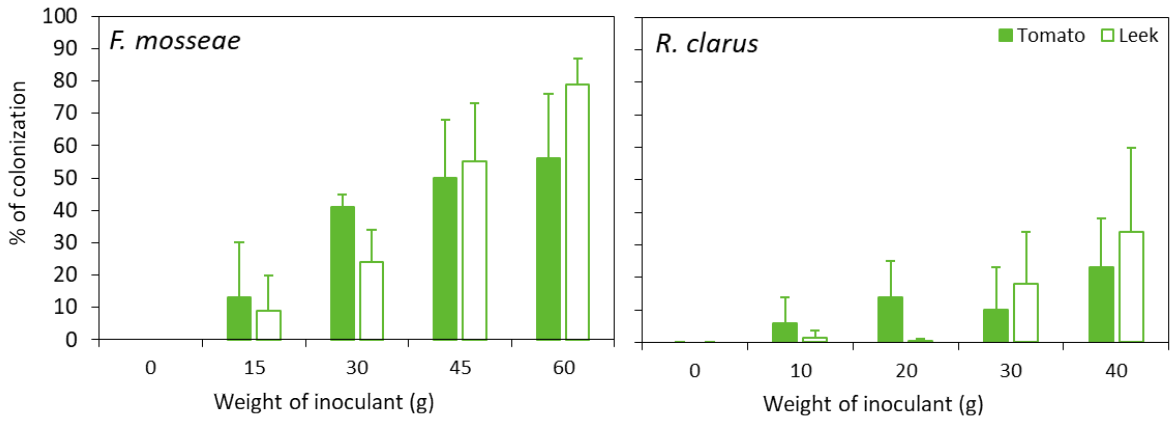


Source: own illustration, CloverStrategy.

On the other hand, in the mixed inoculum approach, the percentage of AMF colonization in the roots was highly dependent on the weight of inoculant used (Figure 21). In both approaches, the percentage of total colonization of *F. mosseae* was generally higher than that of *R. clarus* for both host plants.

Over the experiments, the weight loss due to water evaporation was high, especially in the soil surface. This fact may have delayed seed germination and reduced spores' germination resulting in a lower percentage of colonization in both approaches. Because of that, it is recommended the use of a thin layer of a coarse sand on the soil surface of replicates to reduce water losses through evaporation, keeping the soil moisture stable over the experiment.

Figure 21: **Percentage of total colonization (average + standard deviation; *n*=4) of *Funneliformis mosseae* and *Rhizophagus clarus* in replicates with seeds of tomato (*Solanum lycopersicum*) and leek (*Allium ampeloprasum*) and using the natural soil Bra8 with increasing weights of inoculant. Tests performed by UDESC, Brazil.**



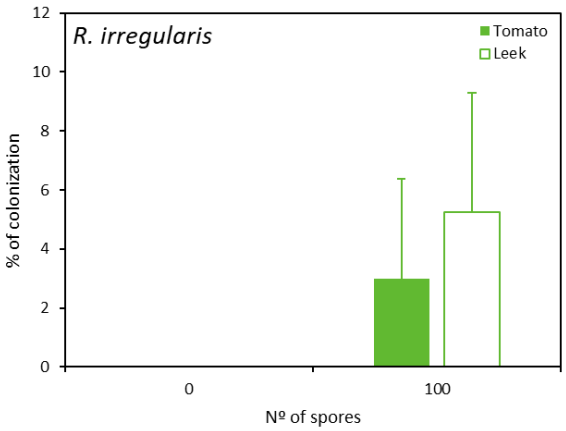
Source: own illustration, CloverStrategy.

The Portuguese partner (UC) also performed laboratory tests following both approaches (i.e. spore inoculum approach and mixed inoculum approach). In these tests, the AMF species *F.*

mosseae SAF 11 and *R. irregularis* SAF 22 (using the inoculant provided by Swiss AGROSCOPE bank) were used. Tomato (*S. lycopersicum*) or leek (*A. ampeloprasum*) in artificial soil with 2.5% of peat were used in the replicates. After preliminary tests, it was decided to use seedlings instead of seeds in the replicates. This decision was taken to reduce the loss of replicates due to ungerminated seeds. Seedlings were prepared before the beginning of the test by introducing pre-disinfected seeds in wet filter paper inside of Petri dishes during 3 to 5 days. Germinated seedlings were transferred to the replicates once they reached a size no higher than 1 cm. One seedling per replicate was used for both plants. The tests had a duration of 28 days after which the percentage of total colonization was determined following the procedures describe in Appendixes A.14 and A.15.

As these experiments started after knowing the results of the experiments conducted in the laboratory of UDESC (experiments described above), in the spores inoculum approach it was decided to determine the percentage of total colonization initially only in replicates of the treatments with 0 (control) and 100 spores and the determination of the percentage of total colonization in the other treatments would be determined only if high percentages of AMF colonization were found in the replicates of the 100 spores treatment. In control replicates no AMF colonization was observed in any plant roots independently of the AMF and plant species. In replicates with 100 spores of *F. mosseae*, no colonization was observed in roots of both tomato and leek (raw data in Appendix A.12), while in replicates with spores of *R. irregularis*, a total colonization of 3.0 ± 3.4 and 5.3 ± 4.0 % was observed, on average (± standard deviation; *n=4*), in tomato and leek roots, respectively (Figure 22). The percentage of colonization in replicates of the other treatments of the spore inoculum approach was not determined as the AMF colonization in replicates of the 100 spores treatment was low to absent (Appendix A.12).

Figure 22: **Percentage of total colonization (average + standard deviation; *n*=4) of *Rhizophagus irregularis* in replicates with tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) seedlings and using standard artificial soil with 2.5% of peat as substrate with increasing numbers of pre-counted AMF spores. Tests performed by UC, Portugal.**



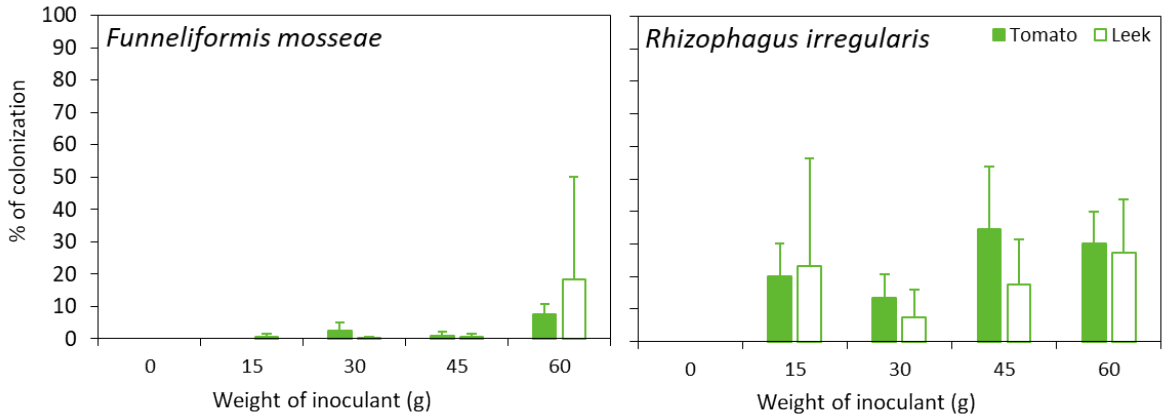
Source: own illustration, CloverStrategy.

In the mixed inoculum approach, control replicates did not show any AMF colonization, in any of the AMF and plant species. Replicates with *F. mosseae* inoculant had the highest percentages of total colonization when using 60 g of inoculant per replicate. A total colonization of 7.5 and 18.25% was found in replicates with tomato and leek, respectively. In the treatments with less than 60 g of inoculant, the *F. mosseae* colonization was residual to inexistent in roots of both plant species. In replicates with *R. irregularis* inoculant, the highest percentage of total

colonization was generally observed in replicates of 45 and 60 g treatments. Replicates with tomato had total colonization of 34.5 and 30.25%, while replicates with leek showed total colonization of 17.5 and 27.3% in 45 and 60 g treatments, respectively (Figure 23). Raw data from the experiments conducted on UC following the mixed inoculum approach are presented on Appendix A.12.

The percentages of total colonization found in the experiments performed in UC were generally lower than those observed in the experiments performed in UDESC. This difference could be related to the fact that in UDESC the experiments started with seeds while in UC the experiments started with seedlings with approximately 1 cm of length. In fact, the importance of the initial stage of seeds germination for the AMF colonization has been reported in previous studies (Singla and Garg, 2017), which suggests that the use of over-developed seedlings in the experiments may reduce the AMF colonization plant roots. Because of that, it was decided in the next experiments to use germlings (i.e. freshly sprouted seeds) or pre-moistened seeds (e.g. seeds previously soaked in water for 3 to 5 days but not germinated) in the test instead of seedlings.

Figure 23: **Percentage of total colonization (average + standard deviation; *n*=4) of *Funneliformis mosseae* and *Rhizophagus irregularis* in replicates with tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) seedlings and using standard artificial soil with 2.5% of peat as substrate with increasing weights of inoculant. Tests performed by UC, Portugal.**



Source: own illustration, CloverStrategy.

Conclusion of objective 1:

i) The use of mixed inoculum is the most adequate inoculum type to ensure a suitable percentage of AMF colonization in symbiotic phase laboratory tests.

2.4.2 Objective 2: Define the most adequate weight of inoculant of different AMF species (dependent on the inoculum potential of each mixed inoculum) to ensure a suitable percentage of AMF colonization in leek and tomato

Definition:

Inoculum potential - capacity of a certain weight of inoculant to colonize (through its propagules) a host plant's root.

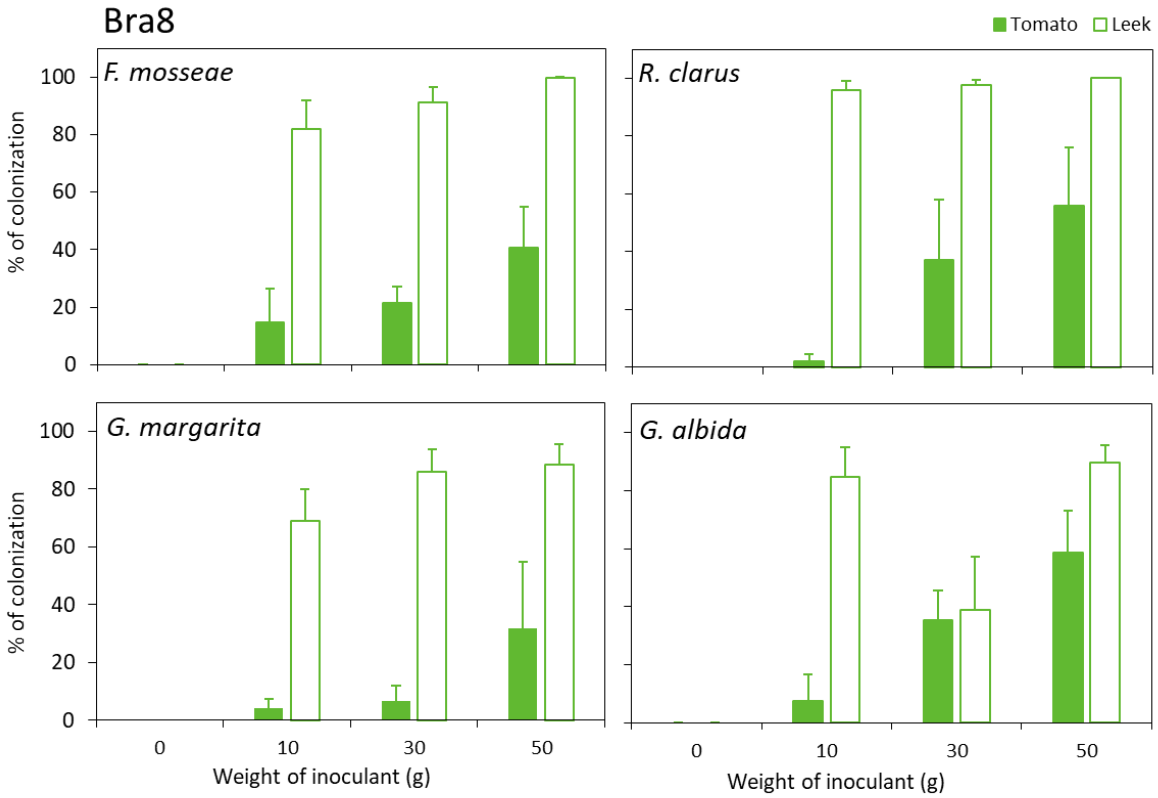
Additional experiments were performed in UDESC laboratory following the mixed inoculum approach. In these experiments, beyond the inoculants of the two AMF species used in the previous experiments (i.e. *F. mosseae* and *R. clarus*), inoculants of AMF *Gigaspora margarita* SCT077A and *Gigaspora albida* SCT200A (isolated from tropical soils of Brazil) were used. Experiments were conducted in artificial standard soil with 2.5% of *Sphagnum* peat and Bra8 natural soil previously autoclaved (following the same procedures adopted in the previous experiments) with tomato and leek. The irrigation frequency over the experiments was similar to that used in the previous tests (i.e. twice a week) with the difference that 10 mL of a Long Ashton fertilizer solution (Appendix A.13) was added per replicate once at the beginning of the experiment (i.e. 7 days after seeds germination). The test treatments considered were 0, 10, 30 and 50 g of inoculant/replicate for both soils and all AMF species, except for the inoculant of *G. albida* that was used only in the Bra8 natural soil. The procedures adopted in the preparation of the replicates was similar to that used in the previous experiments. The proportions of soil and inoculant in each replicate varied with the test treatment as presented in Table 12. As in the previous experiments, a test period of 28 days starting 7 days after seeds germination was considered. Three seeds per replicate were used, being only one left (after 7 days of germination) until the end of the experiment.

Table 12: Weights of soil (fresh weight equivalent) and inoculant of *Funneliformis mosseae*, *Rhizophagus clarus, Gigaspora margarita* or *Gigaspora albida* used in the 70 g mixtures introduced in the top of each replicate of mixed inoculum approach treatments.

Treatment	Soil (g)	Inoculant (g)
0	70	0
10	60	10
30	40	30
50	20	50

For all inoculants and host plants, results evidenced that the higher the inoculant weight used in the replicates the higher the percentage of AMF colonization (raw data are presented on Appendix A.12). In Bra8 soil, AMF root colonization was generally higher in leek than in tomato, independently of the inoculant used. In replicates with leek, total colonization was generally higher than 80% in treatments with 30 g or more inoculant. The exception was the inoculant of *G. albida* that resulted in a colonization of about 40% in the 30 g treatment. In treatments using 50 g of inoculant the colonization of leek roots was always close to 100%; the highest AMF root colonization in tomato was always found in 50 g treatments generally presenting a total colonization of about 40% (Figure 24).

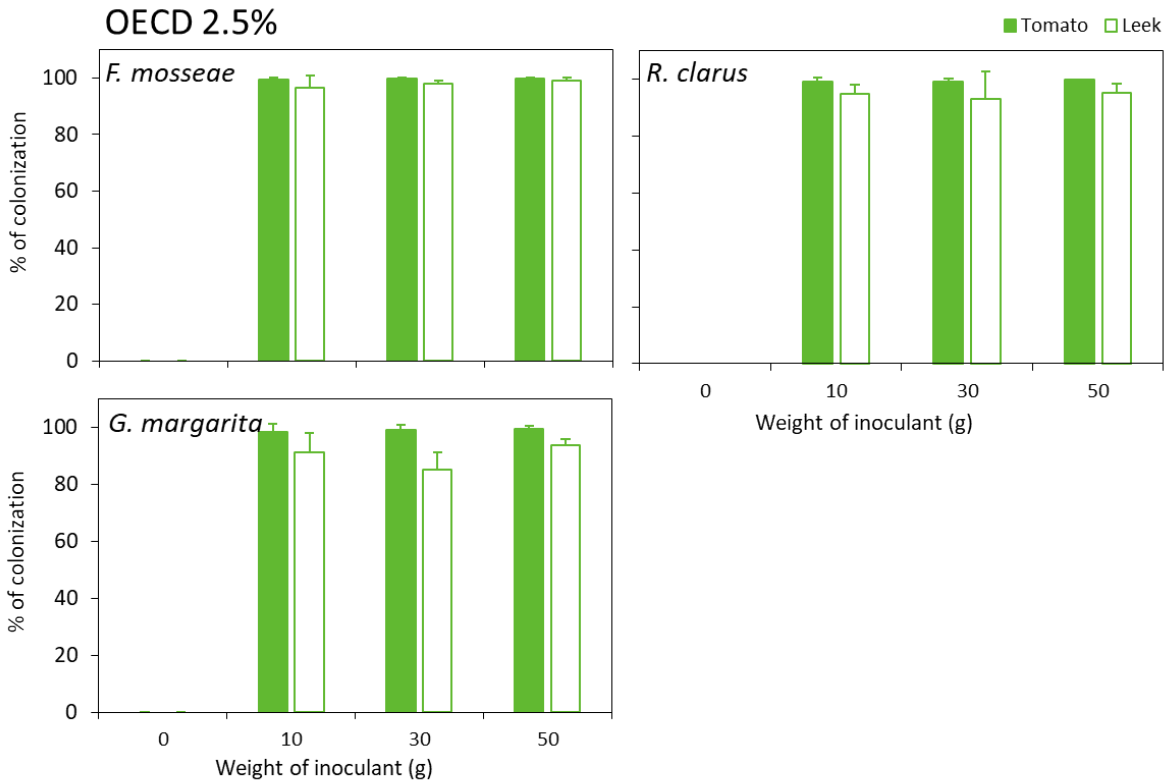
Figure 24: **Percentage of total colonization (average + standard deviation; *n*=4) of *Funneliformis mosseae*, *Rhizophagus clarus, Gigaspora margarita* and *Gigaspora albida* in replicates with tomato (*Solanum lycopersicum*) and leek (*Allium ampeloprasum*) and using the Bra8 natural soil as substrate with increasing weights of inoculant. Tests performed by UDESC, Brazil.**



Source: own illustration, CloverStrategy.

In replicates with OECD 2.5% soil, the percentage of colonization was always close to 100% independently of the treatment (i.e. inoculant weight), mixed inoculum and host plant (Figure 25).

Figure 25: **Percentage of total colonization (average + standard deviation; *n*=4) of *Funneliformis mosseae*, *Rhizophagus clarus* and *Gigaspora margarita*) in replicates with tomato (*Solanum lycopersicum*) and leek (*Allium ampeloprasum*) and using standard artificial soil with 2.5% of peat with increasing weights of inoculant. Tests perfomed by UDESC, Brazil.**



Source: own illustration, CloverStrategy.

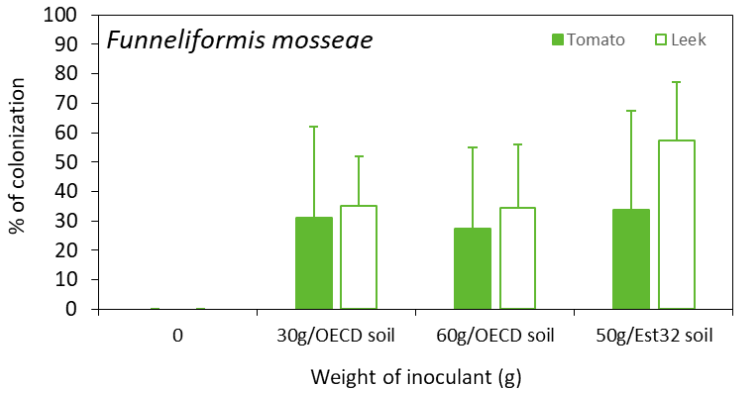
Estonian partner (UT) performed an experiment using the mixed inoculum approach for the AMF species *F. mosseae* SAF 11 (supplied by Swiss AGROSCOPE bank) to confirm the adequacy of this inoculant. Note that this was the only inoculant used in the experiments performed in UC that showed a percentage of total colonization always lower than 30% in all treatments (Figure 22). Experiments were conducted using 250 g of soil or mixture of soil and inoculant per replicate in 300 ml vessels and 4 replicates per treatment were used as in the previous experiments. In replicates with mixed inoculum, specific weights of inoculant were mixed with OECD 2.5% soil to make 250 g of substrate in total that were then transferred to the test vessels. Three previously disinfected seeds (in sodium hypochlorite solution 1%) of tomato or leek were sown per pot. Test treatments consisted of OECD 2.5% artificial soil with 0, 30 and 60 g of *F. mosseae* inoculant. Additional replicates with the Est32 natural soil were prepared using 50 g of *F. mosseae* inoculant. A test period of 60 days (starting on the sowing day) was used. Replicates were irrigated each 2 days by compensating replicate weight losses with the addition of distilled water without any nutrient solution. A thin layer of coarse sand was added in the soil surface after seeds germination to reduce water losses over the experiment.

After the test period, plant shoots were cut at soil surface and discarded. Plant roots were separated from soil by sieving (1 mm) and hand-sorting. Roots were stored in paper bags and air-dried at 50 ºC for 24h for root colonization assessment. The methods adopted for staining

AMF structures inside the collected roots followed the procedures described in Appendix A.14 and the assessment of the percentage of total AMF colonization followed the procedures described in Appendix A.15.

Results obtained (Appendix A.12) evidenced that, in OECD 2.5% with tomato and leek there is no difference in the percentage of AMF colonization whether using 30 or 60 g of *F. mosseae* inoculant. In these treatments, both plants exhibited suitable colonization (30 to 40% on average) for further symbiotic phase laboratory experiments. In replicates with the Est32 natural soil, tomato presented a percentage of total colonization similar to that found in treatments with 30 and 60 g of inoculant with OECD 2.5%. Leek showed the highest percentage of colonization (on average) in replicates with Est32 soil and 50 g of inoculant (Figure 26).

Figure 26: **Percentage of total root colonization (average ± standard deviation; *n*=4) of *Funneliformis mosseae* after 60 days in replicates with tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) and using artificial soil (OECD 2.5% soil) mixed with 30 and 60 g of inoculant and an Estonian natural soil (Est32 soil) mixed with 50 g of inoculant as substrate. Tests performed in UT, Estonia.**



Source: own illustration, CloverStrategy.

Conclusions of objective 2:

i) The use of an inoculant weight equivalent of about 10% of the soil weight per replicate allows a suitable percentage of total root colonization of the generality of AMF species;

ii) The use of a nutrient solution promotes growth of tomato and leek, which can result in increasing percentages of AMF total root colonization.

2.4.3 Objective 3: Define acceptable outcomes for test validation in symbiotic phase tests and select the most suitable plant (either tomato or leek) to be used in experiments of the 2nd phase of WP2 and ring test

Definition:

Hyphae length – length of extra-radicular hyphae produced in AMF colonized plant roots.

Experiments performed in UDESC, used OECD 2.5% artificial soil and the Brazilian natural soil Bra8 as test substrate. These soils were previously prepared and managed in a similar way as performed in the previous experiments. AMF symbiotic phase tests were conducted using inoculants of *Funneliformis mosseae* BEG 240, *Rhizophagus clarus* RJN102A and *Gigaspora margarita* SCT077A and tomato (*Solanum licopersicum*) and leek (*Allium ampeloprasum*) as host plants (seeds of the same batch used in the previous experiments). Three groups of treatment types were considered: 1) the NCt treatments were composed of replicates with the test soil (i.e. OECD 2.5% or Bra8), without host plant but with autoclaved AMF inoculum and aimed to quantify and control the existing extra-radicular hyphae natural from the soil at the end of the experiments; 2) the Ct treatments were composed of replicates with test soil (i.e. OECD 2.5% or Bra8) and host plant (i.e. tomato or leek) but without AMF inoculant and aimed to confirm that no AMF colonization occurs in the roots of the host plants in the absence of the AMF inoculant in the replicates (i.e. evidencing the inexistence of active propagules in the soil); 3) Ti treatments were composed of replicates with test soil (i.e. OECD 2.5% or Bra8), host plant (i.e. tomato or leek) and 50 g of inoculant (i.e. about 10% of the soil weight per replicate) of *Funneliformis mosseae*, *Rhizophagus clarus* or *Gigaspora margarita* and aimed to evaluate the outcome of the measured endpoints related to the AMF roots colonization. A factorial design was used for each treatment type (i.e. NCt, Ct and Ti) in order to test all possible combinations. More specifically, there were 2 NCt treatments (i.e. 2 test soils), 4 Ct treatments (i.e. 2 test soils x 2 host plants) and 12 Ti treatments (i.e. 2 test soils x 2 host plants x 3 AMF sp. inoculants). The treatment codes and respective components are described in Table 13.

Table 13: List of treatments and respective codes, test soils (Bra8 natural soil or OECD 2.5% artificial soil), host plant (tomato - *Solanum licopersicum* or leek - *Allium ampeloprasum*) and AMF inoculant (of Fm - *Funneliformis mosseae*, Rc - *Rhizophagus clarus or Gm - Gigaspora margarita*) used in the experiments of the objective 3 of WP2. Tests performed in UDESC, Brazil.

Treatment code	Test soil	Host plant	Inoculant
NCtNat	Bra8	-	-
NCtO	OECD 2.5%	-	-
CtNatT	Bra8	Tomato	-
CtNatL	Bra8	Leek	-
CtOT	OECD 2.5%	Tomato	-

Treatment code	Test soil	Host plant	Inoculant
CtOL	OECD 2.5%	Leek	-
TiNatTFm	Bra8	Tomato	Fm
TiNatLFm	Bra8	Leek	Fm
TiOTFm	OECD 2.5%	Tomato	Fm
TiOLFm	OECD 2.5%	Leek	Fm
TiNatTRc	Bra8	Tomato	Rc
TiNatLRc	Bra8	Leek	Rc
TiOTRc	OECD 2.5%	Tomato	Rc
TiOLRc	OECD 2.5%	Leek	Rc
TiNatTGm	Bra8	Tomato	Gm
TiNatLGm	Bra8	Leek	Gm
TiOTGm	OECD 2.5%	Tomato	Gm
TiOLGm	OECD 2.5%	Leek	Gm

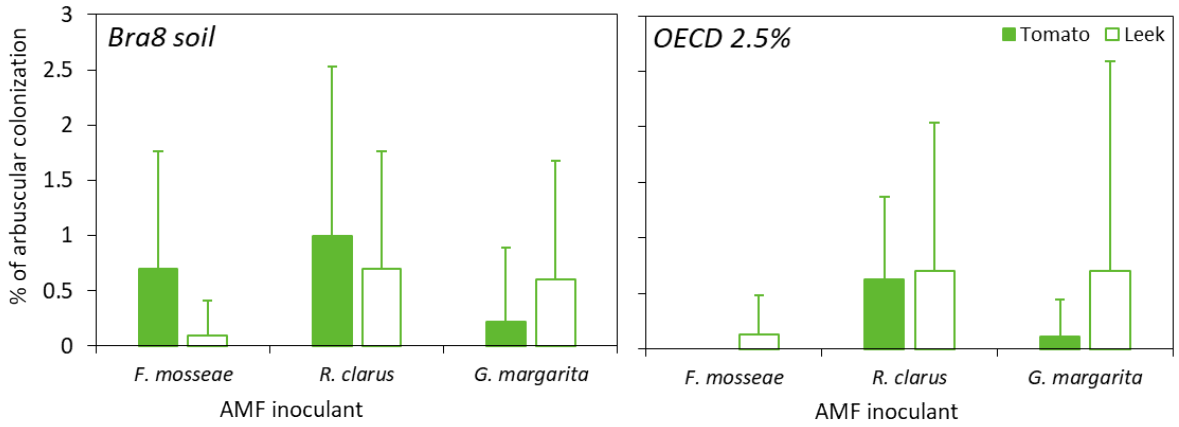
Ten replicates were used per treatment and each replicate consisted of a 500 ml plastic vessel with 250 g DW of test soil (Bra8 or OECD 2.5% artificial soil) previously moistened with 50% of its WHC in the bottom and a mixture (prepared separately) of 200 g of pre-moistened soil with 50 g of inoculant (making a total of 250 g of soil + inoculant) in the top. Each replicate was perforated in the bottom and connected to a vessel filled with deionized water by a glass fibre wick to ensure a suitable moisture of soil over the experiment. Replicates were left for 24 h to allow an equal distribution of the substrate moisture, before sowing the plant seeds. Seeds were previously kept in moist paper for five days to allow the seeds to absorb water before being buried in the soil (three seeds per replicate) at a maximum depth of 1 cm. Seven days after seeds germination one plant was left per replicate and growth was allowed for 56 days. Five millilitres of a Long Ashton fertilizer solution (Appendix A.13) was provided in each replicate 7 days after germination and 10 ml every 10 days.

After a test period of 56 days, the following parameters were measured: percentage of total intra-radicular and arbuscular colonization following the procedures described in Appendixes A.14 and A.15 and extra-mycelial production (hyphal length in m g^{-1} soil dry weight) following

the procedures described in Appendix A.16. Raw data of these experiments are presented on Appendix A.12.

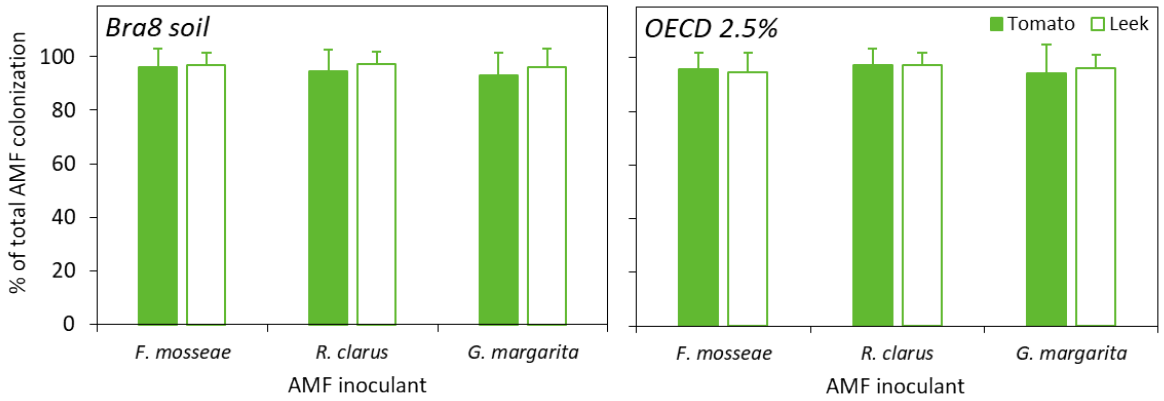
Since, at the end of the test, roots of the host plants of replicates of the Ct treatments (i.e. CtNatT, CtNatL, CtOT and CtOL) did not have any AMF colonization, the test was valid. In replicates with mixed inoculum, although the number of arbuscles per replicate were lower than 1% in most of the replicates (Figure 27), the percentage of total colonization was always higher than 90%, independently of the test soil, host plant and AMF species (Figure 28). Extra-radicular hyphae length measurements showed high variability between treatments (Figure 29). For tomato, using *F. mosseae* and *R. clarus* inoculants, the hyphae length was higher in replicates with OECD 2.5% soil compared to replicates with Bra8 soil, while replicates with *G. margarita* inoculant showed similar hyphae length in both soils. For leek, although the influence of soil type in the hyphae length appeared to be lower than in tomato roots, extra-radicular hyphae length tended to be higher in Bra8 soil than in OECD 2.5% soil. These data suggest that the hyphae length is dependent on soil type and host plant and, because of that, these two factors must be taken into account when establishing threshold values for test validations.

Figure 27: **Average percentage of arbuscular colonization (± standard deviation; *n*=10) in roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils Bra8 or OECD 2.5% and AMF inoculant of *Funneliformis mosseae, Rhizophagus clarus or Gigaspora margarita* after 56 days of exposure. Tests performed in UDESC, Brazil.**



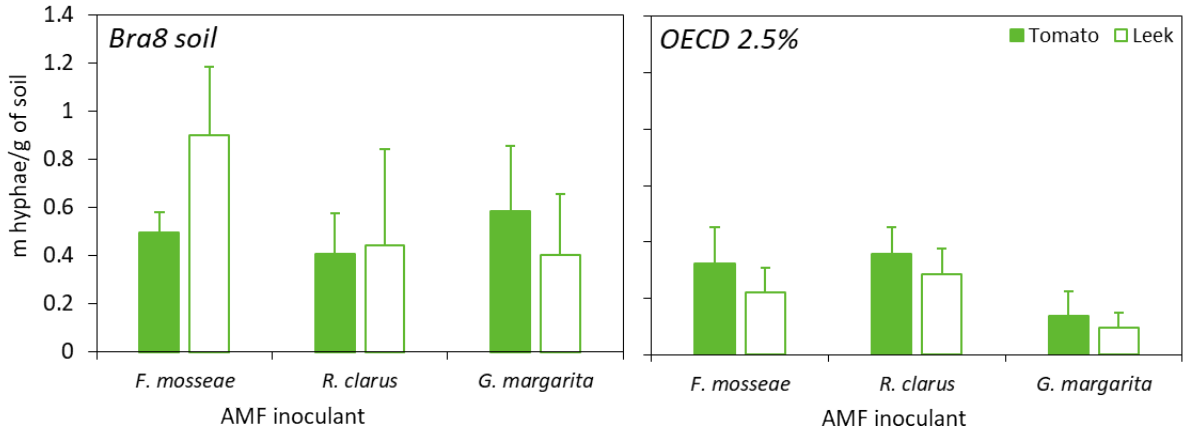
Source: own illustration, CloverStrategy.

Figure 28: **Average percentage of total AMF colonization (± standard deviation; *n*=10) in roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils Bra8 or OECD 2.5% and AMF inoculant of *Funneliformis mosseae, Rhizophagus clarus or Gigaspora margarita* after 56 days of exposure. Tests performed in UDESC, Brazil.**



Source: own illustration, CloverStrategy.

Figure 29: **Average extra-radicular hyphae length (± standard deviation; *n*=10) found in the roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils Bra8 or OECD 2.5% and AMF inoculant of *Funneliformis mosseae, Rhizophagus clarus or Gigaspora margarita* after 56 days of exposure. Hyphae length is expressed in m of hyphae per g of soil. Tests performed in UDESC, Brazil.**



Source: own illustration, CloverStrategy.

In the UC, a strategy similar to that used by UDESC was followed. NCt, CT and Ti treatment types were prepared using OECD 2.5% artificial soil, #Lufa 2.1 and Por3 natural soils as test substrate, tomato (*S. lycopersicum*) and leek (*A. ampeloprasum*) as host plants and inoculants of the AMF species *R. irregularis* SAF 22. Additionally, replicates with inoculant of *F. mosseae* SAF 11 using only leek as host plant were prepared. The test treatments considered for the experiments performed in UC are presented in Table 14.

Table 14: **List of treatments and respective codes, test soils (OECD 2.5% artificial soil, #Lufa 2.1 or Por3 natural soils), host plant (tomato - *Solanum licopersicum* or leek - *Allium ampeloprasum*) and AMF inoculant (of Fm - *Funneliformis mosseae* or Ri - *Rhizophagus irregularis*) used in the experiments of the objective 3 of WP2. Tests performed in UC, Portugal.**

Treatment code	Test soil	Host plant	AMF inoculant
NCtO	OECD 2.5%	-	-
NCtL21	#Lufa 2.1	-	-
NCtP	Por3	-	-
CtOT	OECD 2.5%	Tomato	-
CtOL	OECD 2.5%	Leek	-
CtL21T	#Lufa 2.1	Tomato	-
CtL21L	#Lufa 2.1	Leek	-
CtPT	Por3	Tomato	-
CtPL	Por3	Leek	-
TiOTRi	OECD 2.5%	Tomato	Ri
TiOLRi	OECD 2.5%	Leek	Ri
TiL21TRi	#Lufa 2.1	Tomato	Ri
TiL21LRi	#Lufa 2.1	Leek	Ri
TiPTRi	Por3	Tomato	Ri
TiPLRi	Por3	Leek	Ri
TiOLFm	OECD 2.5%	Leek	Fm
TiL21LFm	#Lufa 2.1	Leek	Fm
TiPLFm	Por3	Leek	Fm

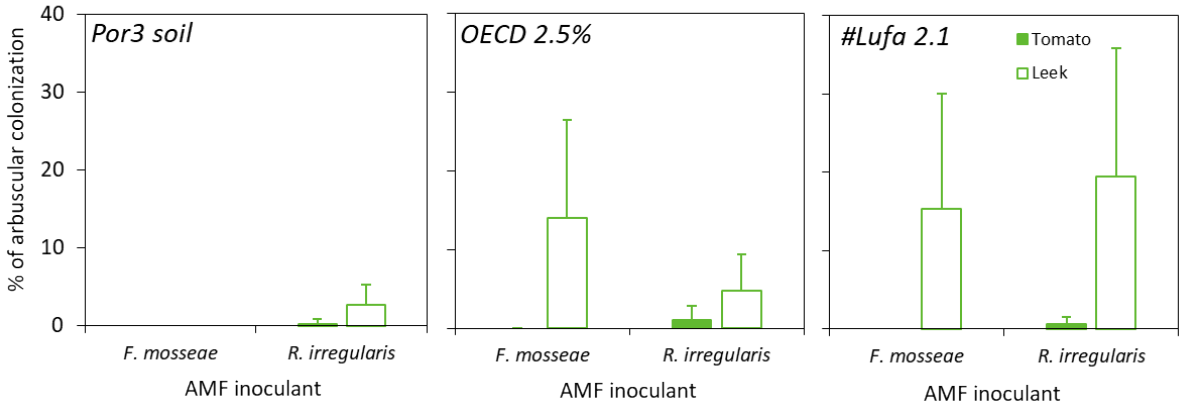
Treatment code	Test soil	Host plant	AMF inoculant
TiPTFm	Por3	Tomato	Fm

A thin layer of coarse sand was applied in the soil surface to reduce water losses over the experiments. Replicates were watered twice a week to compensate weight losses. Additionally, 12.5 mL of a Long Ashton fertilizer solution (Appendix A.13) was provided in each replicate once 5 to 7 days after the start of the test. For NCt replicates, only 5 mL of fertilizer solution were provided. One seedling per replicate was used in the first experiments (as at the beginning of these first experiments the decision had not been made to use germlings or seeds previously soaked in water) and three seeds previously soaked in water were sown per replicate in the last experiments. In these last replicates only one plant was left in the experiment after seeds germination.

The first experiments (i.e. using one seedling per replicate) consisted of replicates using inoculant of *R. irregularis*, in both host plants and in OECD 2.5%, #Lufa 2.1 and Por3 soils (i.e. TiOTRi, TiOLRi, TiL21Tri, TiL21Lri, TiPTRi, TiPLRi) and the respective controls (i.e. NCt and Ct treatments). Although in the replicates of Ct treatments no colonization was found, replicates of the Ti treatments especially with tomato in all soils and with tomato or leek in Por3 soil had a reduced percentage of colonization (i.e. ≤ 10%) (Appendix A.12). Apparently this low colonization was not directly related to the use of seedlings in the replicates (rather then of pre-soaked seeds) since pre-soaked seeds were used in the last experiments in OECD 2.5% artificial soil and in #Lufa 2.1 and Por3 natural soils, using leek and inoculant of *F. mosseae* and low or no AMF colonization was found, especially in the natural soils (Figure 30 and 31 for arbuscular and total colonization, respectively) (Appendix A.12). Extra-radicular hyphae length was measured only in tests where a total colonization ≥ 30% was found (Figure 32), i.e. in tests with leek only. In replicates with inoculant of *R. irregularis*, the hyphae length measured was generally proportional to the percentage of total colonization observed in the roots, while hyphae length of roots colonized by *F. mosseae* (i.e. in tests using inoculant of *F. mosseae*) tended to be lower than in roots colonized by *R. irregularis*. Extra-radicular hyphae measurements suggest that its length depends on the soil type.

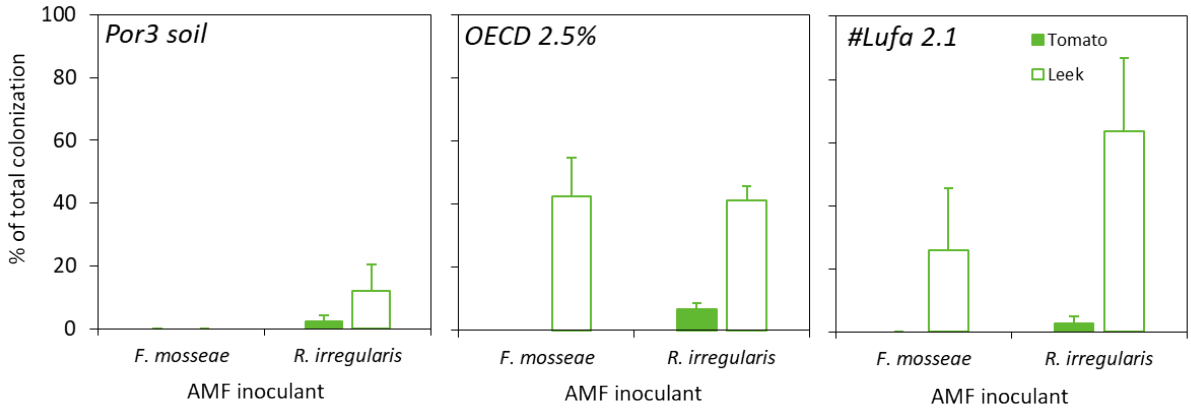
Figure 30: Average percentage of arbuscular colonization (± standard deviation; *n*=10) found in roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils OECD 2.5%, Por3 and #Lufa 2.1 and inoculants of

***Funneliformis mosseae* or *Rhizophagus irregularis* after 56 days of exposure. Tests performed in UC, Portugal.**



Source: own illustration, CloverStrategy.

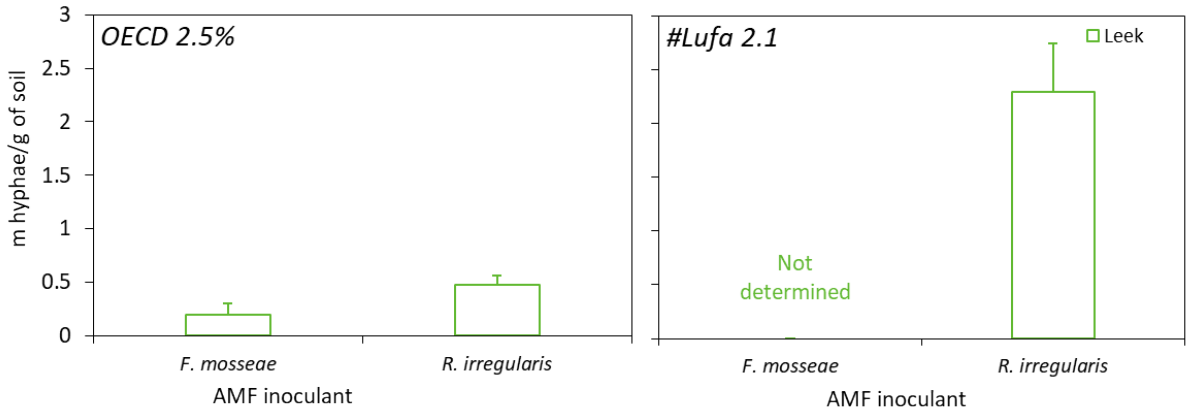
Figure 31: Average percentage of total AMF colonization (± standard deviation; *n*=10) found in roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils OECD 2.5%, Por3 and #Lufa 2.1 and inoculants of *Funneliformis mosseae* or *Rhizophagus irregularis* after 56 days of exposure. Tests performed in UC, Portugal.



Source: own illustration, CloverStrategy.

Figure 32: Average extra-radicular hyphae length (± standard deviation; *n*=10) found in roots of leek (*Allium ampeloprasum*) of replicates with the test soils OECD 2.5% and #Lufa 2.1 and inoculants of *Funneliformis mosseae* or *Rhizophagus irregularis* after

56 days of exposure. Hyphae length is expressed in m of hyphae per g of soil. Tests performed in UC, Portugal.



Source: own illustration, CloverStrategy.

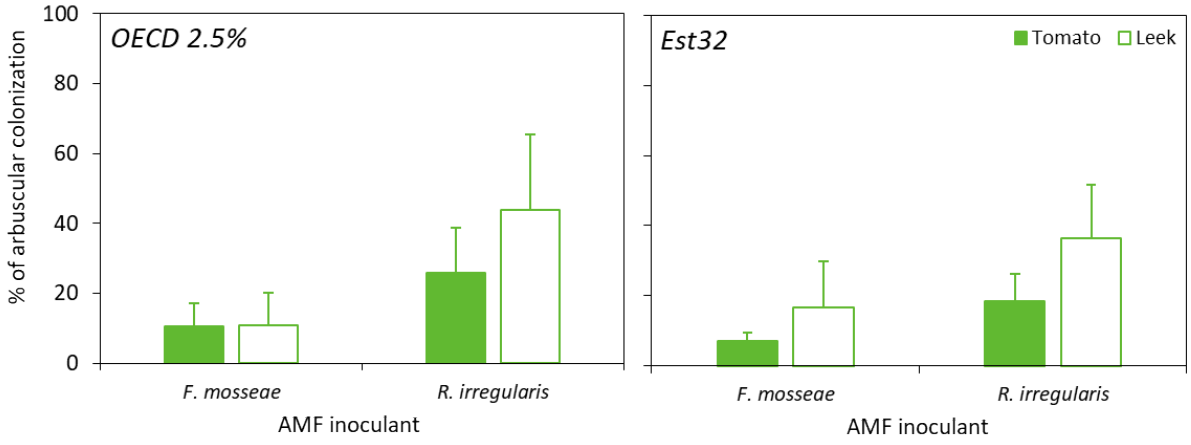
In the UT, additional experiments were performed following an approach similar to that followed in UDESC and UC for the objective 3. The experiments were performed in OECD 2.5% artificial soil and in the Est32 natural soil, using tomato and leek as host plant and inoculants of *R. irregularis* SAF 22 and *F. mosseae* SAF 11 (the same inoculants used in UC experiments) (Table 15). Ten replicates of 500 g of soil or a mixture of soil and inoculant per treatment were used. As in the previous experiments, three seeds per replicate were used and only one plant was left after seeds germination over a test period of 56 days. A thin layer of a coarse sand (previously autoclaved) was placed on the soil surface of replicates after seeds germination to reduce water loss by evaporation. Replicates were irrigated twice a week, and no fertilizer solution was added over the experiment. Arbuscular and total AMF colonization of roots measured in these experiments are presented in Figures 33 and 34, respectively. Extra-radicular hyphae length was not measured in any replicate. Percentage of arbuscular and total colonization of roots on average, showed a tendency to be higher in leek than in tomato in both inoculants. Raw data are presented on Appendix A.12.

Table 15: List of treatments and respective codes, test soils (OECD 2.5% artificial soil and Est32 natural soil), host plant (tomato - *Solanum licopersicum* or leek - *Allium ampeloprasum*) and AMF inoculant (of Fm - *Funneliformis mosseae* or Ri - *Rhizophagus irregularis*) used in the experiments of the objective 3. Tests performed in UT, Estonia.

Treatment code	Test soil	Host plant	AMF inoculant
NCtO	OECD 2.5%	-	-
NCtE32	Est32	-	-
CtOT	OECD 2.5%	Tomato	-

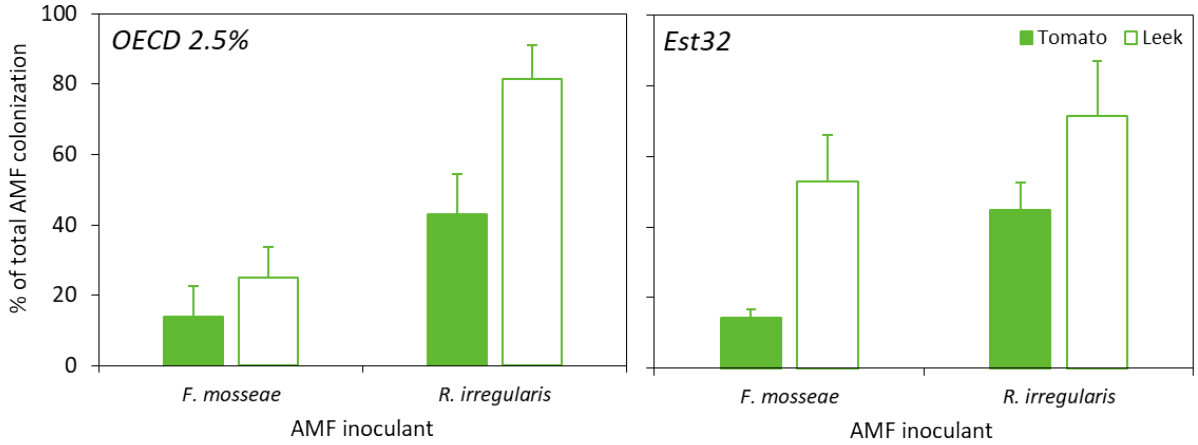
Treatment code	Test soil	Host plant	AMF inoculant
CtOL	OECD 2.5%	Leek	-
CtE32T	Est32	Tomato	-
CtE32L	Est32	Leek	-
TiOTRi	OECD 2.5%	Tomato	Ri
TiOLRi	OECD 2.5%	Leek	Ri
TiE32TRi	Est32	Tomato	Ri
TiE32LRi	Est32	Leek	Ri
TiOLFm	OECD 2.5%	Leek	Fm
TiOTFm	OECD 2.5%	Tomato	Fm
TiE32LFm	Est32	Leek	Fm
TiE32TFm	Est32	Tomato	Fm

Figure 33: **Average percentage of arbuscular colonization (± standard deviation; *n*=10) found in roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils OECD 2.5% and Est32 and inoculants of *Funneliformis mosseae* or *Rhizophagus irregularis* after 56 days of exposure. Tests performed in UT, Estonia.**



Source: own illustration, CloverStrategy.

Figure 34: **Average percentage of total AMF colonization (± standard deviation; *n*=10) found in roots of tomato (*Solanum lycopersicum*) or leek (*Allium ampeloprasum*) of replicates with the test soils OECD 2.5% and Est32 and inoculants of *Funneliformis mosseae* or *Rhizophagus irregularis* after 56 days of exposure. Tests performed in UT, Estonia.**



Source: own illustration, CloverStrategy.

Conclusions of objective 3:

i) 30% of total AMF intra-radicular colonization in control replicates (i.e. Ti treatments), on average, is the minimum AMF colonization required to validate a symbiotic phase test.

ii) The minimum acceptable extra-radicular hyphae length in control replicates (i.e. Ti treatments) could not be estimated and therefore, was not considered as a criterion for test validation. Measuring hyphal length is time-consuming, which makes this parameter more difficult to process.

iii) Leek was selected to be used as host plant in the experiments of the 2nd phase of WP2 and in the ring test as this plant generally had the highest average percentages of AMF root colonization and its roots are easier to process than those of tomato.

2.4.4 Conclusion

The results obtained in the first phase of WP2 helped to evaluate the performance of each AMF species in each soil and to select the best combination of AMF species/soil/host plant, test conditions and methods to apply on the subsequent experimental phases of the project.

More specifically:

- Inoculation of the replicates via mixed inoculum was selected over inoculation using exclusively AMF spores. This method induced higher colonization of the host plant's roots even when using low amounts of inoculant (approximately 10% of the weight of the

replicate is enough to induce a satisfactory percentage of root colonization). Besides, the use of mixed inoculum requires less laboratory labour and reduces the probability of technical errors when compared with the use of counted AMF spores.

- The use of a low P nutrient solution increased the growth of the host plants, which can be relevant in the promotion of the symbiosis AMF-host plant.
- Leek was selected as host plant to be used in the next phases because the percentages of root colonization observed on the experiments conducted with this plant are, generally, higher than the colonization observed in experiments conducted with tomato. Besides, since leek is a monocot, its root structure is less complex/branched, facilitating the processing of the roots (e.g. collection, selection and staining for AMF visualization).

Also, the feasibility of assessing different parameters, as well as their range of variation were assessed. These data supported the definition of the validity criteria to be considered for the next experimental phases.

More specifically:

- The feasibility of measuring extra-radicular hyphae length varies with the soil and host plant. This parameter is a more time-consuming and more difficult to assess than intra-radicular colonization. Since it was not possible to define a minimum acceptable extra-radicular hyphae length in control replicates, this aspect was not considered as a criterion for test validation.
- Total colonization > 30% in control replicates was achievable in the generality of the tests performed using different combinations of AMF species/soil/host plant. Therefore, this parameter was established as criteria for test validation.

2.5 Standard Operating Procedures developed

Based on the experiments described above, the Standard Operating Procedures (SOPs) previously prepared were refined and improved. The preparation of the SOPs was led by UDESC team and counted with the review and support of all partners of the consortium. These SOPs were followed in the experiments conducted in the 2nd phase of the WP2, where experiments with chemicals were carried out. More specifically, the following SOPs were considered for the subsequent experiments:

- SOP 1.1 – Extraction of Arbuscular Mycorrhizal Fungi (AMF) spores from an inoculant (Appendix A.17, Annex A). This SOP describes a method to extract and determine the number of spores in an inoculant. The method is based on a wet sieving technique, followed by a sucrose centrifugation, after which, spores are separated and counted.
- SOP 2.1 - Assessing the inoculum potential of Arbuscular Mycorrhizal Fungi (AMF) from an inoculant (Appendix A.13). This SOP describes a method to evaluate soil or substrate inoculum potential through an inoculant. The inoculum potential is intended as the capacity of a soil/substrate inoculant to promote root colonization (through the measurement of the percentage of colonized roots). The method is based on the use of an inoculant of the selected AMF species mixed with natural or artificial soil in different amounts in test containers with seeds of the host plant. After 28 days (4 weeks) of incubation, the

percentage of AMF colonization in the roots is determined by assessing the intra-radicular colonization.

- SOP 2.2 - Staining method for colonization structures of Arbuscular Mycorrhizal Fungi (AMF) in root tissues (Appendix A.14). This SOP describes a method for staining AMF colonization structures to allow its observation root tissues. Plant roots are first clarified and acidified and lastly submerged in an acid solution with ink to make visible the intra-radicular AMF structures.
- SOP 2.3 - Assessing the percentage of total and arbuscular colonization of Arbuscular Mycorrhizal Fungi (AMF) in roots (Appendix A.15). This SOP describes a method to determine the total and arbuscular colonization of AMF based on recording the presence or absence of fungal structures (i.e. spores, hyphae, vesicles and arbuscules) in previously stained root fragments.
- SOP 2.4 - Assessing the total extra-radicular mycelium length of Arbuscular Mycorrhizal Fungi (AMF) (Appendix A.16). This SOP describes a method to determine the length of extra-radicular hyphae in soil based on the extraction of hyphae from soil in a filter holder system where the hyphae retained in a cellulose nitrate membrane are measured through the grid line intersect method in the microscope.
- SOP Test 1 - Assessing effects of chemicals on the pre-symbiotic phase of Arbuscular Mycorrhizal Fungi (AMF) – AMF spore germination test (Appendix A.17). This protocol describes a method based on the procedures described in ISO 10832 (2009) and Mallmann et al. (2018) to evaluate the potential effect of chemicals in one of the key phases of AMF life cycle – spore germination. Spores of *Rhizophagus irregularis* are placed between two nitrocellulose membrane filters forming a sandwich, which is placed in a Petri dish containing the test substrate/soil. The percentage of germinated spores is estimated after 14 days, and the results allow to estimate the 10 and 50% inhibition concentration of the test substance for spore germination.
- SOP Test 2[16] - Assessing effects of chemicals on the symbiotic phase of Arbuscular Mycorrhizal Fungi (AMF) (Appendix A. 18). This protocol describes a method to evaluate the potential effect of chemicals in the symbiotic phase of the AMF *Rhizophagus irregularis*. Presoaked seeds of the host plant leek (*Allium ampeloprasum*) are planted in pots containing an AMF inoculant and soil spiked with increasing concentrations of the test substance. After 8 weeks of incubation, effects on the establishment of the AMF-plant symbiosis are assessed by measuring intra-radicular total and arbuscular colonization and extra-radicular mycelial production).

[16] SOP Test 2 presented in Appendix A.18 is the final version that includes improvements carried out after the performance of the symbiotic phase ring tests. For this reason, some of the methods applied in the experiments of the 2nd phase of WP2 slightly differed from the methods described in the Appendix (e.g. AMF species other than *R. irregularis* were used in the experiments).

2.6 Experiments with chemicals (2nd phase of WP2)

The second phase of WP2 comprised laboratory experiments to evaluate the sensitivity of the selected test parameters (defined in the first phase) in tests with selected chemicals to be used as reference substance in AMF symbiotic phase tests and in the ring tests of WP3.

In the experiments carried out in the 2nd phase of the WP2 (based on the methods described in SOP Test 2, Appendix A.18), at the end of the test period (i.e. 56 days), the host plants were harvested, and the shoot and/or root biomass production were measured. The percentage of total and arbuscular AMF colonization in roots and extra-radicular hyphae length were measured only on replicates where there was plant biomass production, as the presence of a plant is the necessary condition for the establishment of the symbiosis.

Only the experiments with a total colonization ≥ 30% in control replicates, on average, were considered valid and the measurement of extra-radicular hyphae length was not mandatory on invalid experiments.

The following chemicals were selected for testing:

- Boric acid;
- Copper sulphate;
- Tebuconazole;
- Terbuthylazine;
- Azoxystrobin;
- Fluazinam;
- Pendimethalin.

Table 16 summarizes the experiments carried out (with the respective soils and AMF inoculants used in each chemical tested) in the different laboratories of the consortium.

Table 16: Experiments performed by UDESC, UT and UC with Boric acid, Copper sulphate, Terbuthylazine, Tebuconazole, Azoxystrobin, Fluazinam and Pendimethalin using inoculants of *Funneliformis mosseae, Rhizophagus irregularis and R. clarus* and different soils (OECD 2.5% artificial soil, Bra8, Lufa 2.2, Est32, Por3 and #Lufa 2.1 natural soils).

		OECD 2.5%	Bra8	Lufa 2.2	Est32	Por3	#Lufa 2.1
Boric acid	*F. mosseae*	UDESC	UDESC	-	-	UC	UC
	R. clarus	-	-	-	-	-	-
	R. irregularis	UT	-	-	-	UC	UC
Copper sulphate	*F. mosseae*	UDESC	-	-	-	-	-
	R. clarus	-	-	-	-	-	-

		OECD 2.5%	Bra8	Lufa 2.2	Est32	Por3	#Lufa 2.1
	R. irregularis	UDESC	-	-	-	-	-
Terbuthylazine	*F. mosseae*	UDESC	UDESC	-	-	-	-
	R. clarus	UDESC	UDESC	-	-	-	-
	R. irregularis	-	-	-	-	-	-
Tebuconazole	*F. mosseae*	UDESC	UDESC	-	-	-	-
	R. clarus	UDESC	UDESC	-	-	-	-
	R. irregularis	-	-	-	-	-	-
Fluazinam	*F. mosseae*	UT	-	-	UT	-	-
	R. clarus	-	-	-	-	-	-
	R. irregularis	UT	UDESC	UC UDESC UT	UT	UC	
Azoxystrobin	*F. mosseae*	UC	-	-	-	-	UC
	R. clarus	-	-	-	-	-	-
	R. irregularis	UC	UDESC	UC UDESC UT	UC	UC	UC
Pendimethalin	*F. mosseae*	UT	-	-	UT	-	-
	R. clarus	-	-	-	-	-	-
	R. irregularis	UT	-	-	UT	-	-

At the beginning of the laboratory tests, one composite soil sample of about 50 g DW per treatment was collected and stored at -20°C for chemical analysis to measure the initial measured concentrations of the test chemicals in treatments of valid tests.

Replicates without plants were included on the statistical analysis of plant biomass parameters. However, for the analysis of total and arbuscular AMF colonization and of extra-radicular hyphae length, only replicates with plants were taken into consideration. The statistical analysis of plant biomass, AMF intra-radicular colonization and extra-radicular hyphae length was performed in R Studio (version 2023.09.1), using packages drc (v. 3.0-1), car (v. 3.1-2), dunn.test (v. 1.3.6), DescTools (v. 0.99.52) and PMCMRplus (v. 1.9.10).

Normality of data distribution and homogeneity of data variances were assessed through Shapiro and Bartlett tests, respectively. When these assumptions were confirmed, a one-way ANOVA was performed to assess differences among the treatments for each measured parameter (considering treatments as independent variable and plant biomass, AMF colonization, or hyphae length as dependent variables).

When the normality and/or homogeneity assumptions were not confirmed, data were log (i.e., log (x+1)) or square root (i.e., $\sqrt{x}$) transformed. If the transformed data complied with the assumptions, a one-way ANOVA was performed, following the methodology described above. If the transformed data still did not comply with the assumptions (also when there were treatments with ≤ 2 replicates with plant biomass), a non-parametric Kruskal-Wallis' test was performed, using the non-transformed data, to assess the differences among treatments.

When significant differences between treatments were found, differences between treatments and the respective control were assessed through post-hoc Dunnett test (when the parametric test one-way ANOVA was used) or post-hoc Dunn test (with Bonferroni correction; when the non-parametric Kruskal-Wallis' test was used) to estimate the No-Observed Effect Concentration (NOEC) and the Lowest-Observed Effect Concentration (LOEC). All statistical analyses considered a significance level of 5%.

When possible, Effect Concentration values for 10% and 50% reduction (EC10 and EC50, respectively) of total and arbuscular colonization and of extra-radicular hyphae length in relation to control (and the respective 95% confidence intervals) were estimated trough non-linear regression models (using logistic, exponential or Hormesis models, according to highest coefficient of regression and the best fit to data) based on nominal and initial measured concentrations of the tested substances. These estimations were performed on R Studio (version 2023.09.1), using packages drc (v. 3.0-1), tidyverse (v. 2.0.0), readxl (v. 1.4.3) and PMCMRplus (v. 1.9.10).

2.6.1 Experiments for the selection of a suitable chemical to be used as reference substance for AMF symbiotic phase laboratory tests and/or in the ring tests of WP3

2.6.1.1 Experiments with Boric acid (BA)

Boric acid (BA) was the first substance to be evaluated as possible reference substance for AMF testing (Table 17). BA has been used as reference substance for other ecotoxicological tests (Niemeyer et al. 2018) and is often use as fertilizer in soil.

Table 17: Physical and chemical characterization of boric acid.

Boric acid	
Pesticide type	Insecticide, Fungicide, Other substance
Substance group	Inorganic compound, Monobasic acid
IUPAC name	Boric acid
Empirical formula	H_3BO_3
CAS number	10043-35-3
Molecular mass	61.83 g/mol
Melting point (°C)	171
Solubility (water, 20°C)	57000 mg/L
Dissociation constant (pKa)(25°C)	9.24
Log Kow (20°C)	-0.757
Henry's Law constant	2.6×10^{-12} atm/m^3/mol

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

A total of seven experiments were performed with Boric acid. These experiments were carried out by the members of the consortium (UC, UDESC and UT) using two AMF species (*Funneliformis mosseae* and *Rhizophagus irregularis*), one host plant species (*Allium ampeloprasum*) and four soils (OECD 2.5%, Bra8, #Lufa 2.1 and Por3 soils). The experiments performed using boric acid are summarized in Table 18.

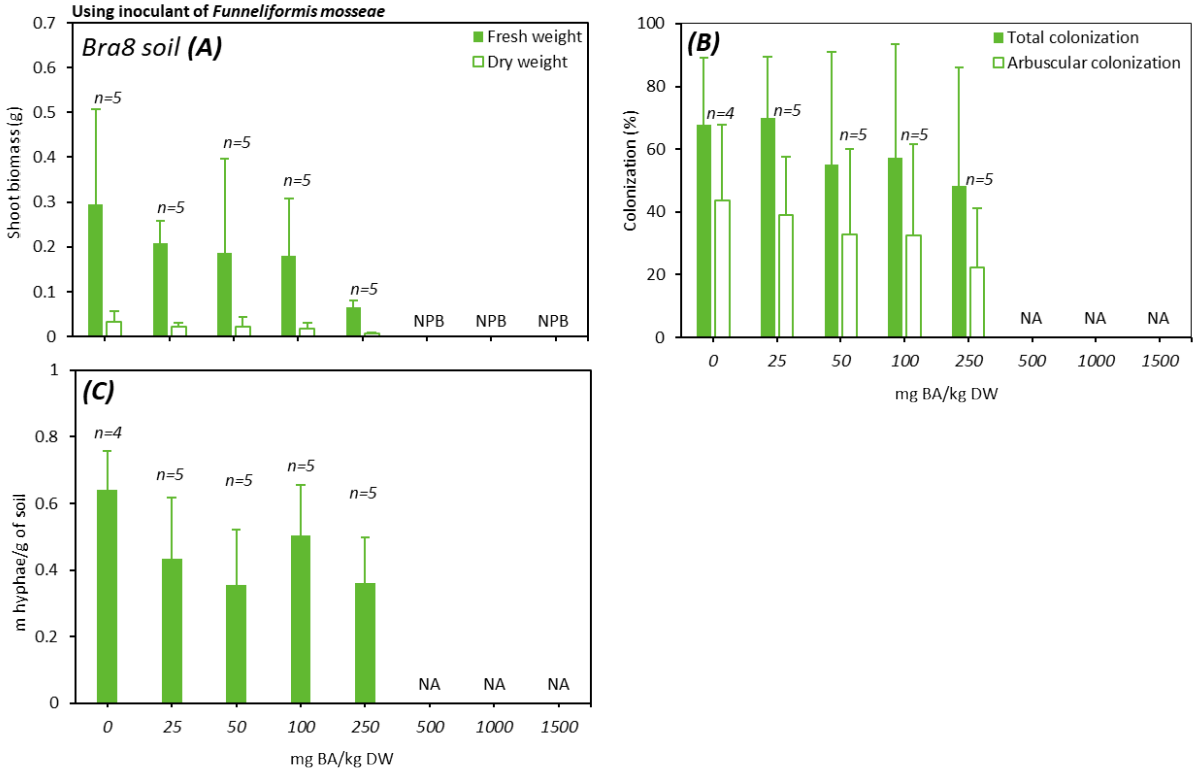
Table 18: Experiments performed by the consortium laboratories (UC, UDESC and UT) with boric acid using different soils (OECD 2.5%, Bra8, #Lufa 2.1 and Por3) and two AMF inoculants (*Funneliformis mosseae* and *Rhizophagus irregularis*).

	AMF species	**OECD 2.5%**	**Bra8**	**#Lufa 2.1**	**Por3**
Boric acid	*F. mosseae*	UDESC	UDESC	UC	UC
	R. irregularis	UT	-	UC	UC

The experiments were performed according to the procedures described in the SOP Test 2 (Appendix A.18) using the concentration gradient 0, 25, 50, 100, 250, 500, 1000 and 1500 mg Boric acid/kg of soil. The definition of the test concentrations took into consideration the doses of Boric acid usually recommended for soil fertilization indicated for leek in Brazil (15 kg/ha – 7.5 mg/kg dry soil) and the EC50 value reported in the literature for *F. mosseae* BEG 240 spore germination of 463.5 (327.7 – 599.3) mg/kg (Mallmann (2020)).

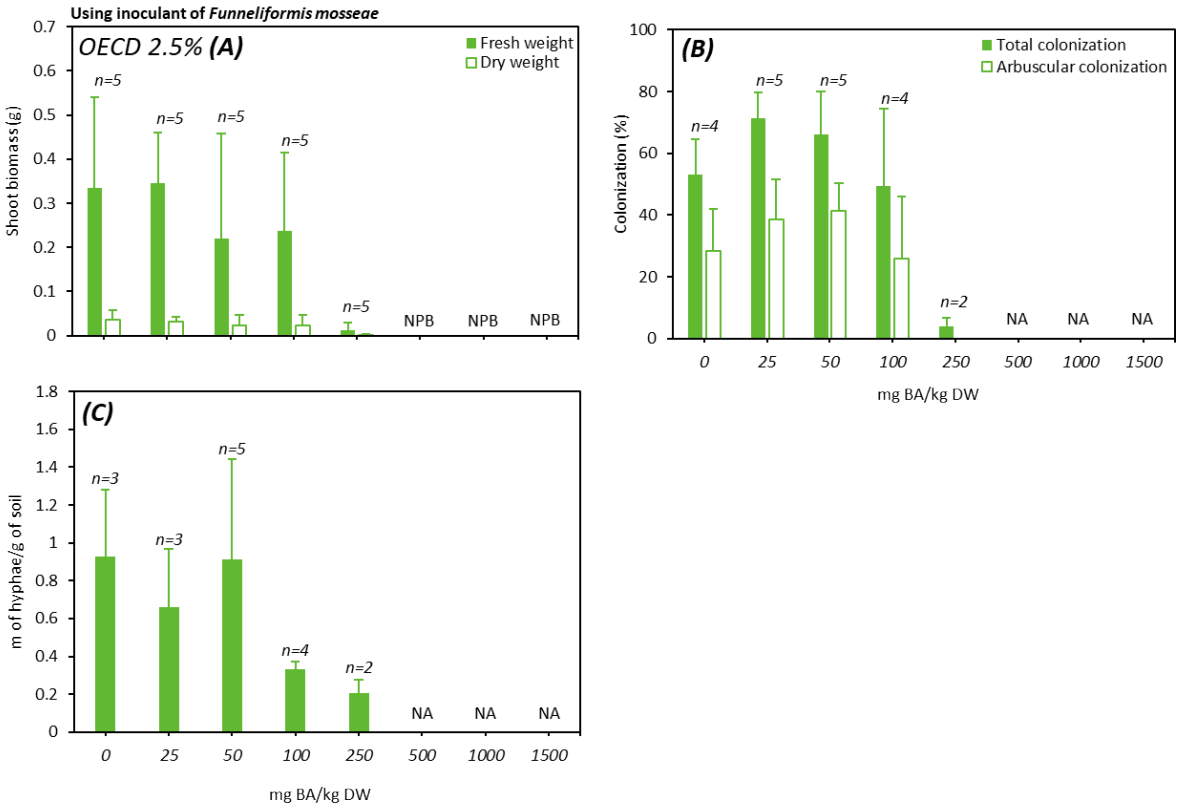
Results obtained by the three laboratories are presented in Figures 35 to 41 and raw data are presented in Appendix A.19.

Figure 35: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: Bra8; Test substance: Boric Acid; Performing lab: UDESC, Brazil.



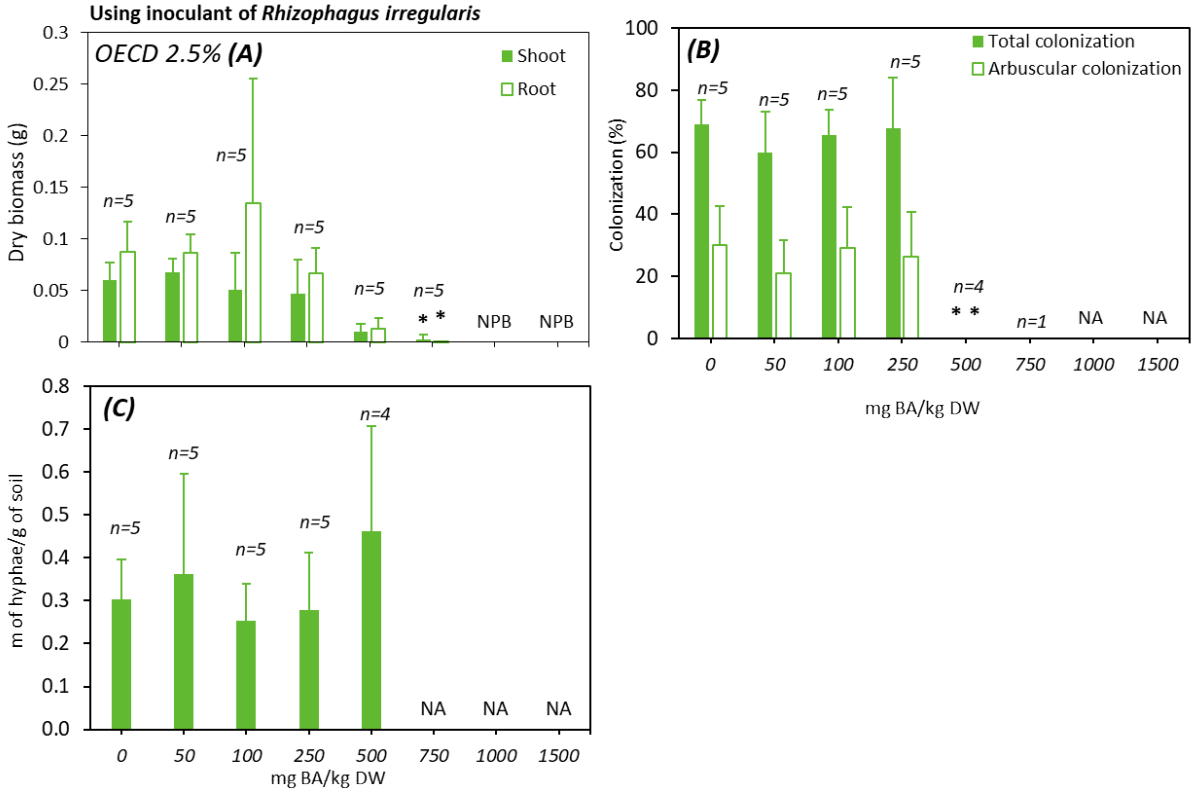
(A) Fresh and dry shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 36: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Boric Acid; Performing lab: UDESC, Brazil.



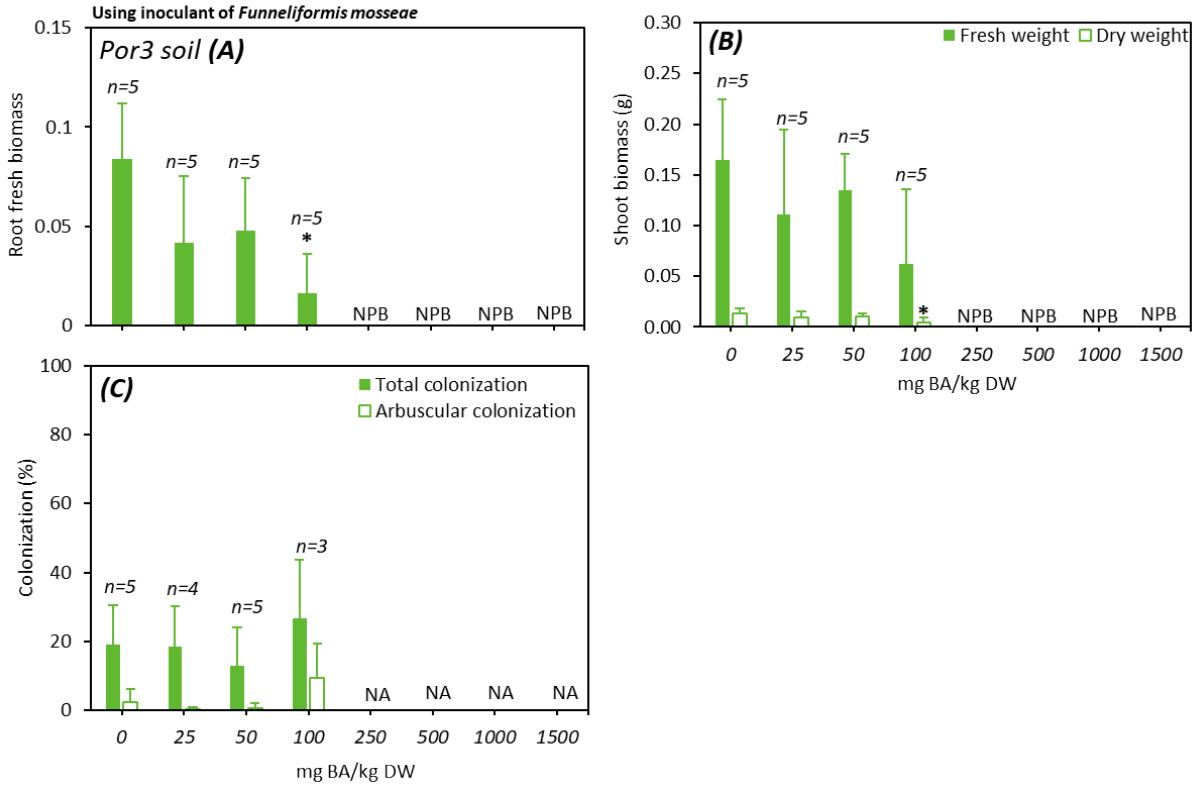
(A) Fresh and dry shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 37: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Boric Acid; Performing lab: UT, Estonia.



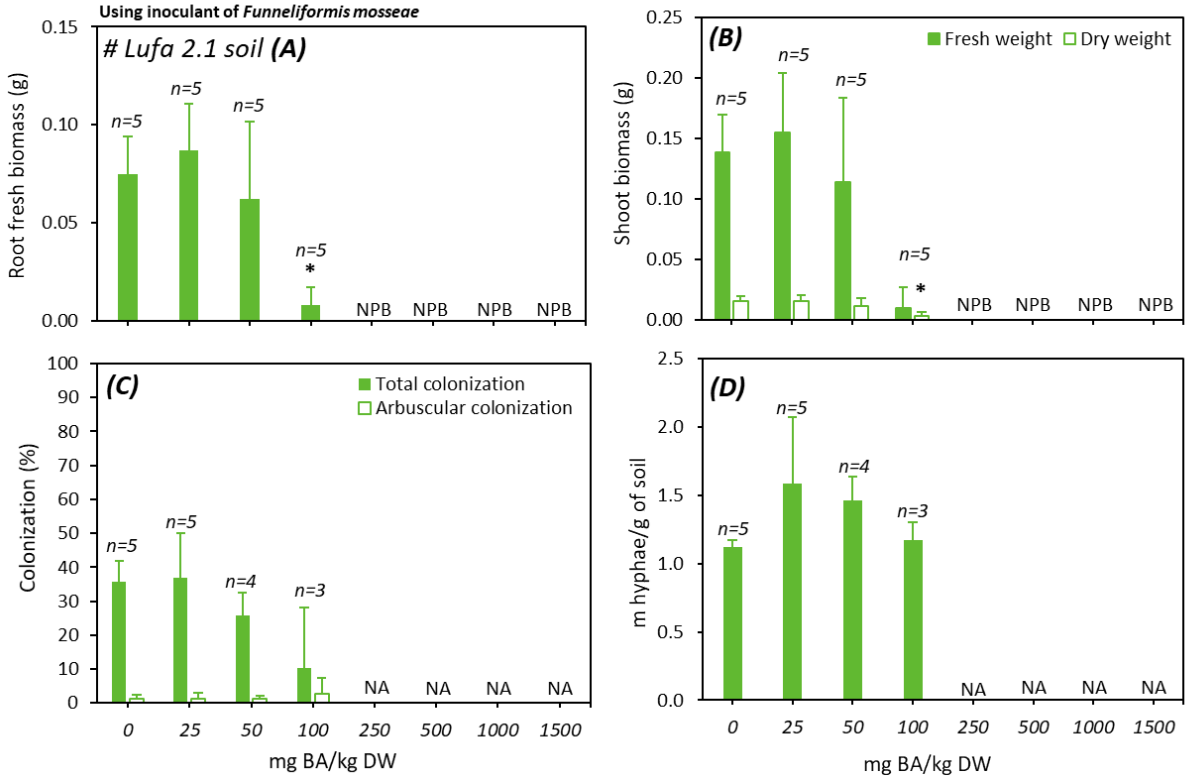
(A) Dry shoot and root biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 38: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: Por3; Test substance: Boric Acid; Performing lab: UC, Portugal.



(A) Fresh root biomass (average ± standard deviation), (B) fresh and dry shoot biomass (average ± standard deviation) and (C) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

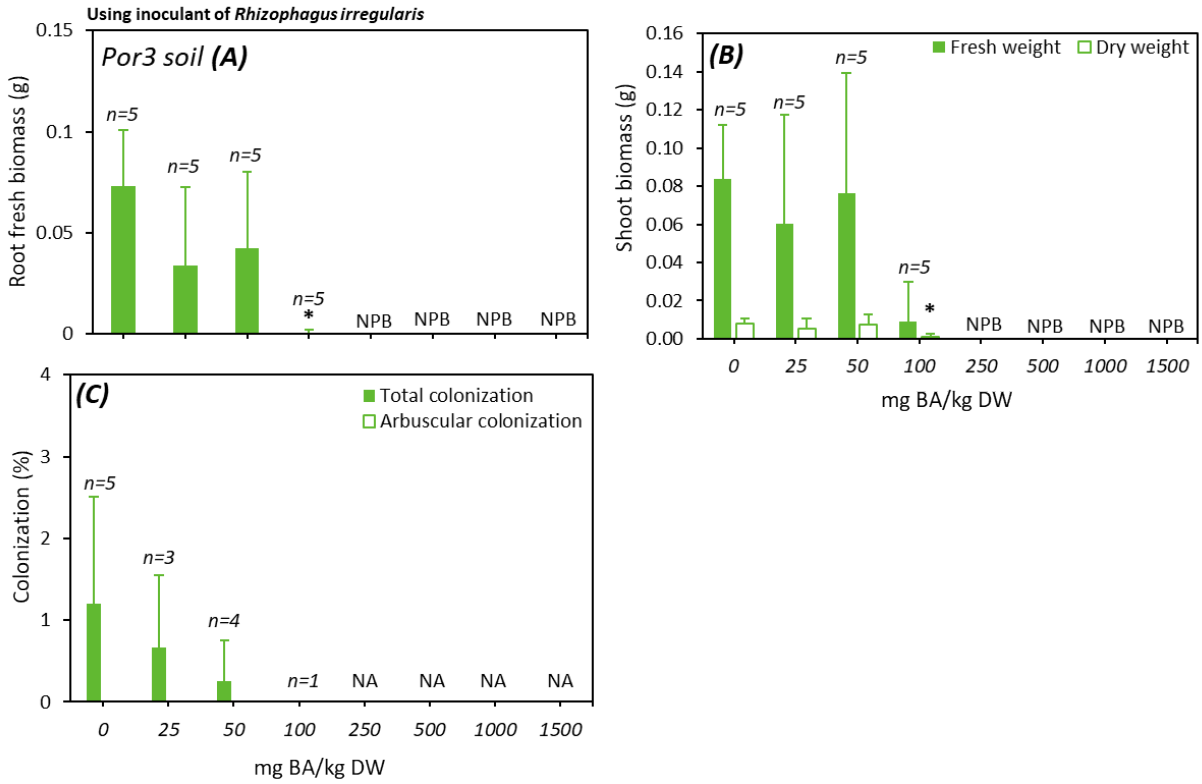
Figure 39: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: #Lufa 2.1; Test substance: Boric Acid; Performing lab: UC, Portugal.



(A) Fresh root biomass (average ± standard deviation), (B) fresh and dry shoot biomass (average ± standard deviation), (C) total and arbuscular colonization (average ± standard deviation) and (D) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.

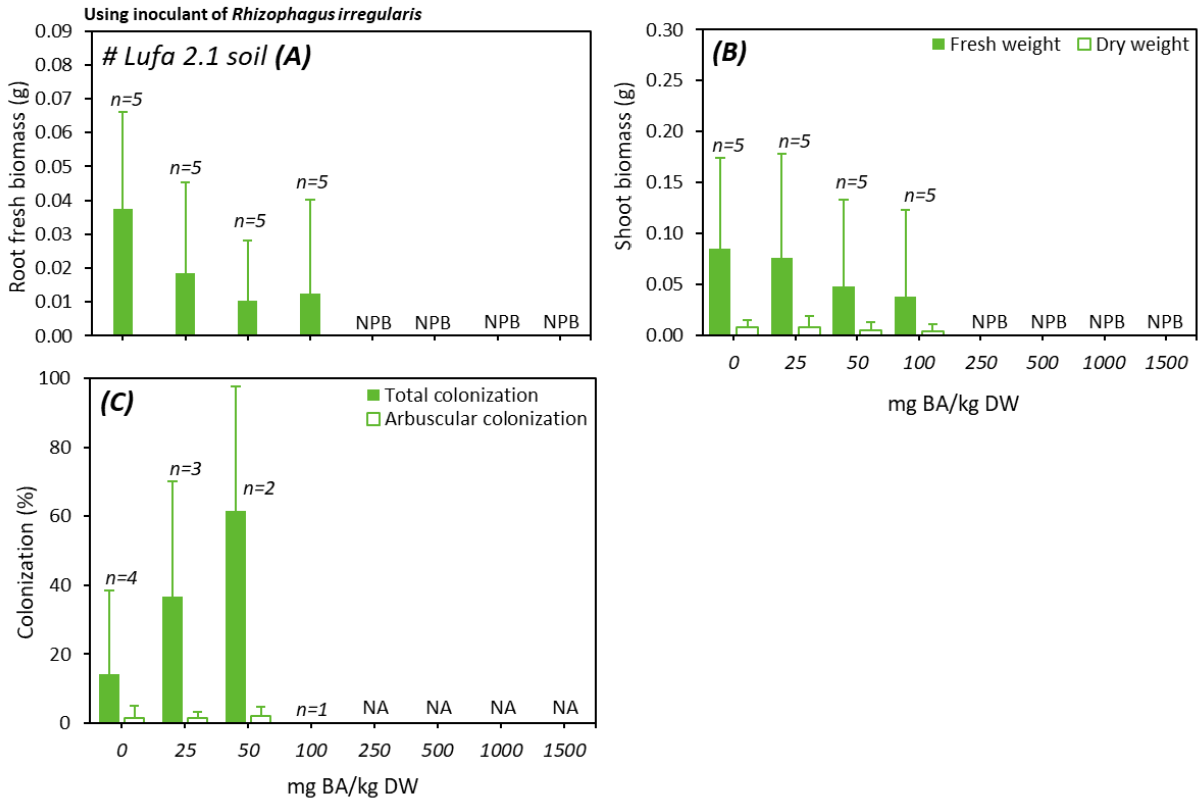
Source: own illustration, CloverStrategy.

Figure 40: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Por3; Test substance: Boric Acid; Performing lab: UC, Portugal.



(A) Fresh root biomass (average ± standard deviation), (B) fresh and dry shoot biomass (average ± standard deviation) and (C) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 41: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: #Lufa 2.1; Test substance: Boric Acid; Performing lab: UC, Portugal.



(A) Fresh root biomass (average ± standard deviation), (B) fresh and dry shoot biomass (average ± standard deviation) and (C) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Chemical analyses were performed to determine the actual contamination of boric acid on soil samples collected at the beginning of the experiments (samples were frozen at -20°C until chemical analysis). Chemical determination was only performed for valid experiments (i.e. total colonization ≥ 30% in negative control) and only for treatments where plant germination was observed. Due to a problem in the freezer where the soil samples were stored, no chemical analyses could be carried out for the experiments with Boric acid conducted in UT (Estonia).

The chemical determination method consisted of the extraction of boron, by digestion of a representative amount of soil sample (approximately 0.25 g), previously dried and sieved through a 2 mm mesh, in an oven with microwave technology, at high pressure and temperature and in an oxidizing acid medium (4 mL HNO_3:H_2O (1:1) + 1 mL H_2O_2). In this way, the matrix charge was eliminated, and the analyte was released in the resulting extract, which was made up to 25 mL with milliQ water and subsequently injected into ICP-MS. The instrumental analysis carried out consisted of the determination of elemental metals based on the mass charge ratio (m/z) of the ions under analysis. The samples were nebulized, forming an aerosol that was transported through argon to a torch in which a gas plasma was generated at a high

temperature, between 6 000 – 10 000 K. The ions thus produced were selected inside the equipment based on the m/z ratio and were subsequently quantified in the mass detector by comparing the signal obtained in the sample against that of a calibration line of known concentrations. Boron standards by Scharlab (BO00160100; 1003.7 ± 2.8 mg/L) were used.

Finally, the total boron measured on soil was used to estimate the initial measured concentration of boric acid in soil after extrapolation based on the molecular weight of boric acid (H_3BO_3; molecular weight of 61.833 g/mol). Measured concentrations of boric acid on the control treatment were lower than the detection limit (i.e., < 28.6 mg BA/kg DW). Measured concentrations on the treatments were generally higher than expected concentrations; however, an increase on the concentrations was maintained along the gradient (Table 19).

Table 19: Nominal and initial measured concentrations of Boric acid (BA) on soil samples collected from symbiotic experiments. Only valid experiments and treatments with plant growth were considered.

AMF species	Soil	Lab	Nominal concentration (mg BA/kg of soil DW)	Initial measured concentration (mg BA/kg of soil DW)	% nominal concentration
F. mosseae	#Lufa 2.1	UC	0	< 28.6	NA
			25	< 28.6	NA
			50	76.3	152.6
			100	84.8	84.8
F. mosseae	Bra8	UDESC	0	< 28.6	NA
			25	< 28.6	NA
			50	< 28.6	NA
			100	36.9	36.9
			250	96.2	38.5
F. mosseae	OECD 2.5%	UDESC	0	< 28.6	NA
			25	< 28.6	NA
			50	55.5	111.0
			100	106.7	106.7
			250	270.1	108.1

NA – not applicable

2.6.1.1.1 Conclusions of the experiments with Boric acid

The endpoints estimated (i.e. NOEC, LOEC, EC10 and EC50) as well as compliance of the results with the validity criterion (i.e., total colonization on average in the control treatment ≥ 30%) on the experiments with Boric acid are summarized in Table 20.

The host plant (leek) was sensitive to boric acid, which showed to have influenced the effects observed on AMF colonization and mycelium production. The significant effects in AMF colonization were generally associated to significant reductions of plant biomass production.

In experiments with OECD 2.5% artificial soil, EC10 and EC50 values of BA for *F. mosseae* were lower than those estimated for *R. irregularis*. However, this difference was likely influenced by the fact that in the experiment with *F. mosseae*, there was plant biomass production up to 250 mg BA/kg, while in experiment with *R. irregularis*, plant biomass production occurred up to concentration of 750 mg BA/kg.

Despite the apparent dependence between plant biomass production and AMF root colonization, the endpoints estimated for BA are below those reported in the literature for *F. mosseae* BEG 240 regarding spore germination, where an EC50 of 463.5 (327.7 – 599.3) mg/kg was reported in tropical artificial soil with 5% of OM by Mallmann (2020). On the other hand, the endpoints estimated for AMF are within the same order of magnitude of the data reported by Amorim et al. (2012) for two soil invertebrate species. These authors reported EC10 values of 31.7 and 39.3 mg BA/kg of soil for *Folsomia candida* and *Enchytraeus albidus*, respectively, in reproduction tests using Lufa 2.2 soil. However, among the data collected, other non-target soil organisms were estimated to be less sensitive to boric acid than *F. mosseae* and *R. irregularis* (data available in the existing literature presented in Appendix A.23).

Since all experiments conducted by the three consortium partners showed a strong relationship between reduction of the AMF root colonization or extra-radicular hyphae production and low plant biomass production, the effect of BA on the establishment of AMF-plant symbiosis could not be clearly evidenced. Because of that, BA was not considered a suitable chemical to be used as reference substance in AMF symbiotic phase tests.

Table 20: **Summary of the experiments performed in the consortium laboratories with Boric acid (BA) using two AMF species (*Rhizophagus irregularis* and *Funneliformis mosseae*), artificial soil (OECD 2.5%) and different natural soils (Bra8, Por3 and #Lufa 2.1). No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction (and respective 95% confidence intervals) on total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated on valid experiments only (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg BA/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg BA/kg DW)	Concentrations with plant biomass (mg BA/kg DW)	Laboratory	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	Bra8	0; 25; 50; 100; 250; 500; 1000; 1500	0; 25; 50; 100; 250	UDESC	Valid	TC	250	> 250	41.0*	> 250
						AC	250	> 250	19.9*	> 250
						EHL	25	50	ND	ND
F. mosseae	OECD 2.5%	0; 25; 50; 100; 250; 500; 1000; 1500	0; 25; 50; 100; 250	UDESC	Valid	TC	100	250	94.6 (58.9-130.4)	143.6 (78.9-208.4)
						AC	250	> 250	98.5 (59.1-138.0)	130.2 (43.6-216.8)
						EHL	250	> 250	83.9*	97.1 (36.5-157.7)
F. mosseae	Por3	0; 25; 50; 100; 250; 500; 1000; 1500	0; 25; 50; 100	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
F. mosseae	#Lufa 2.1		0; 25; 50; 100	UC	Valid	TC	50	100	36.8 (6.0-67.6)	69.0 (33.6-104.4)

AMF species	Soil	Concentrations (mg BA/kg DW)	Concentrations with plant biomass (mg BA/kg DW)	Laboratory	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
		0; 25; 50; 100; 250; 500; 1000; 1500				AC	100	> 100	ND	ND
						EHL	100	> 100	> 100	> 100
R. irregularis	Por3	0; 25; 50; 100; 250; 500; 1000; 1500	0; 25; 50; 100	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
R. irregularis	#Lufa 2.1	0; 25; 50; 100; 250; 500; 1000; 1500	0; 25; 50; 100	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
						EHL	-	-	-	-
R. irregularis	OECD 2.5%	0; 50; 100; 250; 500; 750; 1000; 1500	0; 50; 100; 250; 500; 750	UT	Valid	TC	250	500	346.9*	381.4*
						AC	250	500	289.6*	337.3*
						EHL	500	> 500	128.3*	> 500

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.6.1.2 Experiments with Copper sulphate

Copper sulphate ($CuSO_4$) is commonly used in organic farming practices as a fungicide (Tamm et al. 2022). Physical and chemical properties of copper sulphate are presented in Table 21.

Table 21: Physical and chemical properties of copper sulphate.

Copper sulphate	
Pesticide type	Fungicide, Algicide, Bactericide, Other substance
Substance group	Inorganic compound
IUPAC name	Copper II sulphate pentahydrate
Empirical formula	$CuSO_4$
CAS number	7758-98-7
Molecular mass	159.61 g/mol
Melting point (°C)	360
Solubility (water, 20°C)	3.42 mg/L
Dissociation constant (pKa)(25°C)	Not applicable
Log Kow (20°C)	0.44
Degradation point (°C)	360
Degradation in soil (aerobic)	
DT_{50} (lab at 20°C)	0.1 days
DT_{50} (field)	0.1 days

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

The two experiments performed using copper sulphate were carried out with inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and were conducted in the laboratory of the Brazilian partners from UDESC (Table 22).

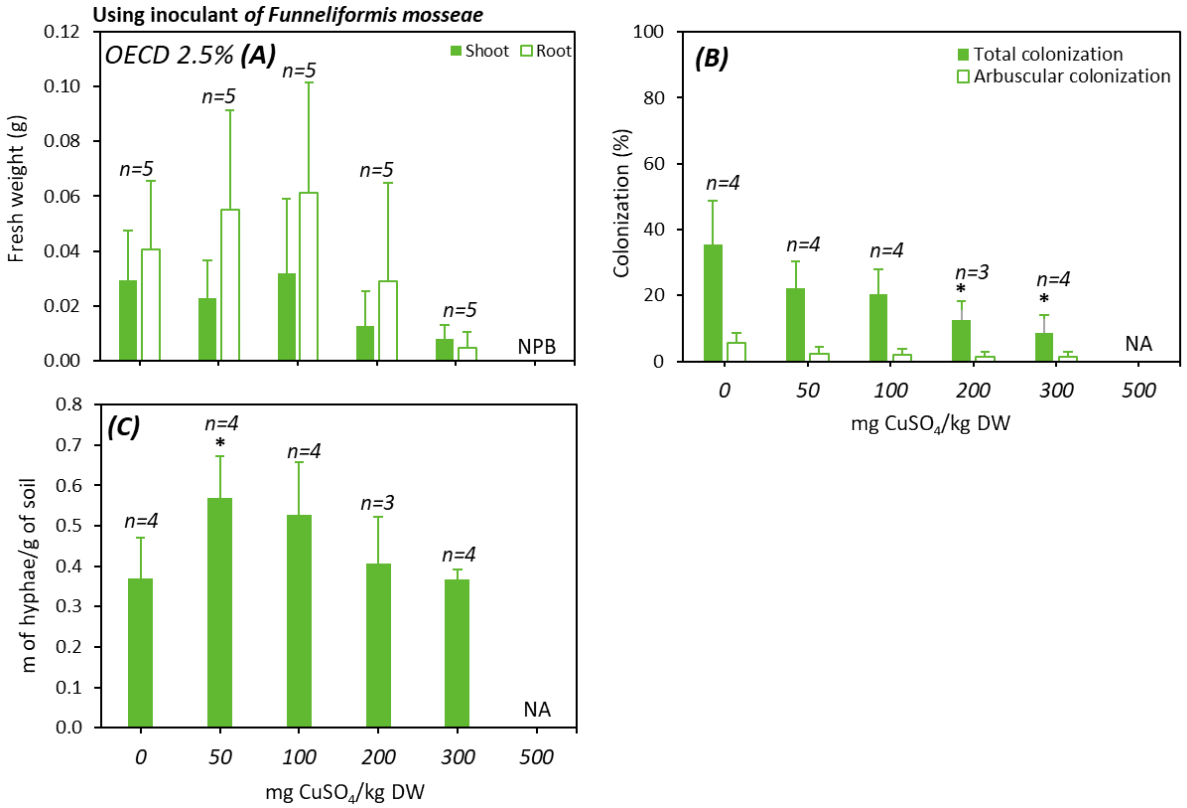
Table 22: Experiments performed in UDESC (Brazil) with copper sulphate using inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and OECD 2.5% artificial soil.

		OECD 2.5%
Copper sulphate	*F. mosseae*	UDESC
	R. irregularis	UDESC

The experiments were performed according to the procedures described in the protocol SOP Test 2 (Appendix A.18) and using a concentration gradient composed of the treatments: 0, 50, 100, 200, 300 and 500 mg $CuSO_4$/kg of soil.

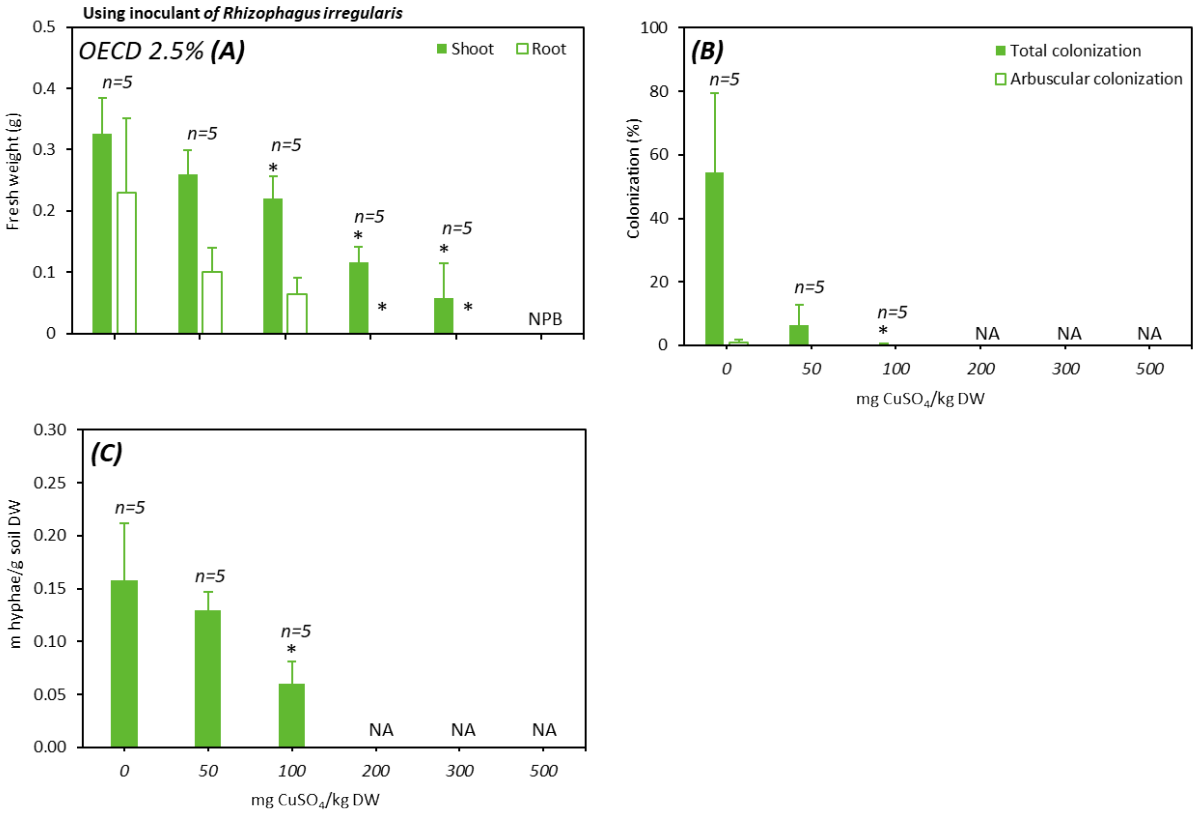
Results obtained are graphically shown in Figures 42 and 43 and raw data are presented in Appendix A.19.

Figure 42: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Copper sulphate; Performing lab: UDESC, Brazil.



(A) Fresh shoot and root biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e., total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 43: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Copper sulphate; Performing lab: UDESC, Brazil.



(A) Fresh shoot and root biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e., total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Chemical analyses to determine initial measured concentrations of copper sulphate were performed on soil samples from valid experiments only for treatments where there was plant biomass production (i.e., treatments up to 300 mg $CuSO_4$/kg of soil DW).

Total copper in soil was quantified following the methodology described by Tedesco et al. (1995). Copper was extracted from 10 g of air-dried soil per treatment using 40 mL of a HCl 0.1M solution. Quantification of copper in extracts was carried out by atomic absorption spectrometry by atomization in an air-acetylene flame. Five samples of reference soils from Brazil were used as standards to check the accuracy of the quantifications as advised by Rede Oficial de Laboratórios de Solos dos Estados do Rio Grande do Sul e de Santa Catarina (https://www.sbcs-nrs.org.br/rolas).

Total copper concentration measured in controls was subtracted to the copper concentrations measured in test treatments, that were then used to estimate initial measured concentrations of Copper sulphate in soil by extrapolation based on the $CuSO_4$ molecular weight of 159.61 g/mol. Initial measured concentrations confirmed that test treatments comprised a gradient of

increasing concentrations of Copper sulphate. However, initial measured concentrations were generally twice the nominal concentrations (Table 23).

Table 23: Nominal and initial measured concentrations of CuSO4 (in mg CuSO4/kg of soil DW), and the respective percentage of nominal concentrations measured in soil samples collected from AMF symbiotic phase tests using inoculants of *F. mosseae* or *R. irregularis* and OECD 2.5% artificial soil, carried out in UDESC (Brazil). Copper initial measured concentrations presented correspond to the measured values after subtracting the copper concentration measured in the control treatment (i.e., nominal concentration = 0 mg $CuSO_4$/kg of soil).

AMF species	Nominal concentration ($CuSO_4$ mg/kg)	Initial measured concentration ($CuSO_4$ mg/kg)	% of Nominal concentration
F. mosseae	0	3.0	NA
	50	115.8	231.6
	100	215.8	215.8
	200	636.0	318.0
	300	824.8	274.9
R. irregularis	0	9.5	NA
	50	93.7	187.4
	100	163.5	163.5
	200	363.7	181.8
	300	551.3	183.8

NA – not applicable.

2.6.1.2.1 Conclusions of the experiments with Copper Sulphate

Estimated endpoints and the validity of the experiments with copper sulphate based on the criterion defined in SOP Test 2, are summarized in Table 24.

The experiments performed showed clear dose-response patterns to most of the parameters measured. The exception was in the experiment with *F. mosseae* for plant biomass production and extra-radicular hyphae length as these parameters showed high variability (often appearing to be unrelated to increased Copper sulphate concentration) among test treatments lower or equal to 300 mg $CuSO_4$/kg. Due to this variation, it was not clear if the decrease in *F. mosseae* colonization was influenced not only by the presence of copper sulphate but also by the decrease in plant root biomass. In experiments with *R. irregularis*, results suggest that a refined concentration gradient of copper sulphate up to 100 mg/kg would show a clearer dose-response curve of the AMF colonization.

The effects observed on total colonization and extra-radicular hyphae of *R. irregularis* were higher than those estimated to *F. mosseae*, which suggests that *R. irregularis* is more sensitive to Copper sulphate than *F. mosseae*. The AMF EC10 and EC50 estimated for total colonization and extra-radicular hyphae length are in the same range of concentrations of the endpoints

reported in the literature for in-soil non-target invertebrates such as *Folsomia candida, Folsomia fimetaria* and *Eisenia andrei* (see Appendix A.23).

Although Copper sulphate has shown to be a potentially toxic substance for AMF, further testing is advised to confirm the suitability of this chemical to be used as reference substance in AMF symbiotic phase tests.

Table 24: **Summary of the experiments performed in UDESC laboratory with Copper sulphate ($CuSO_4$) using two AMF species (*Rhizophagus irregularis* and *Funneliformis mosseae*) and artificial soil (OECD 2.5%). No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction (and respective 95% confidence intervals) on total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg $CuSO_4$/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg $CuSO_4$/kg DW)	Concentrations with plant biomass (mg $CuSO_4$/kg DW)	Laboratory	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	OECD 2.5%	0; 50; 100; 200; 300; 500	0; 50; 100; 200; 300	UDESC	Valid	TC	100	200	20.9 (8.8-33.0)	137.6 (58.0-217.1)
						AC	300	> 300	0.00004*	2.66*
						EHL	300	> 300	207.2 (38.8-375.5)	> 300
R. irregularis	OECD 2.5%	0; 50; 100; 200; 300; 500	0; 50; 100; 200; 300	UDESC	Valid	TC	50	100	2.4 (0.01-4.9)	16.1 (0.05-32.0)
						AC	100	> 100	ND	ND
						EHL	50	100	38.4*	90.8 (56.7-125.0)

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.6.1.3 Experiments with Tebuconazole (TBC)

Tebuconazole has been widely used in agriculture due to its high efficiency as a fungicide. Its effect on the inhibition of spores' germination and fungal growth has been reported on literature (Dong 2024). Physical and chemical properties of Tebuconazole are described in Table 25.

Table 25: Physical and chemical properties of Tebuconazole.

Tebuconazole	
Pesticide type	Fungicide
Substance group	Triazole fungicide, Conazole fungicide
IUPAC name	1-(4-chlorophenyl)-4,4-dimethyl-3-(1,2,4-triazol-1-ylmethyl)pentan-3-ol
Empirical formula	$C_{16}H_{22}ClN_3O$
CAS number	107534-96-3
Molecular mass	307.82 g/mol
Melting point (°C)	105
Solubility (water, 20°C)	36 mg/L
Dissociation constant (pKa) (25°C)	5.0
Log Kow (20°C)	3.7
Henry's Law constant	1.0 x 10^{-5} Pa/m^3/mol
Degradation point (°C)	350
Degradation in soil (aerobic)	
DT_{50} (lab at 20°C)	365 days
DT_{50} (field)	47.1 days

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

Four experiments exposing AMF inoculants of *Rhizophagus clarus* and *Funneliformis mosseae* to Tebuconazole in OECD 2.5% artificial soil and Bra8 natural soil, using leek as host plant, were carried out in UDESC laboratory (Brazil) (Table 26).

Table 26: Experiments performed in UDESC (Brazil) with Tebuconazole using inoculants of *Funneliformis mosseae* and *Rhizophagus clarus* in OECD 2.5% artificial soil and the natural Brazilian soil Bra8.

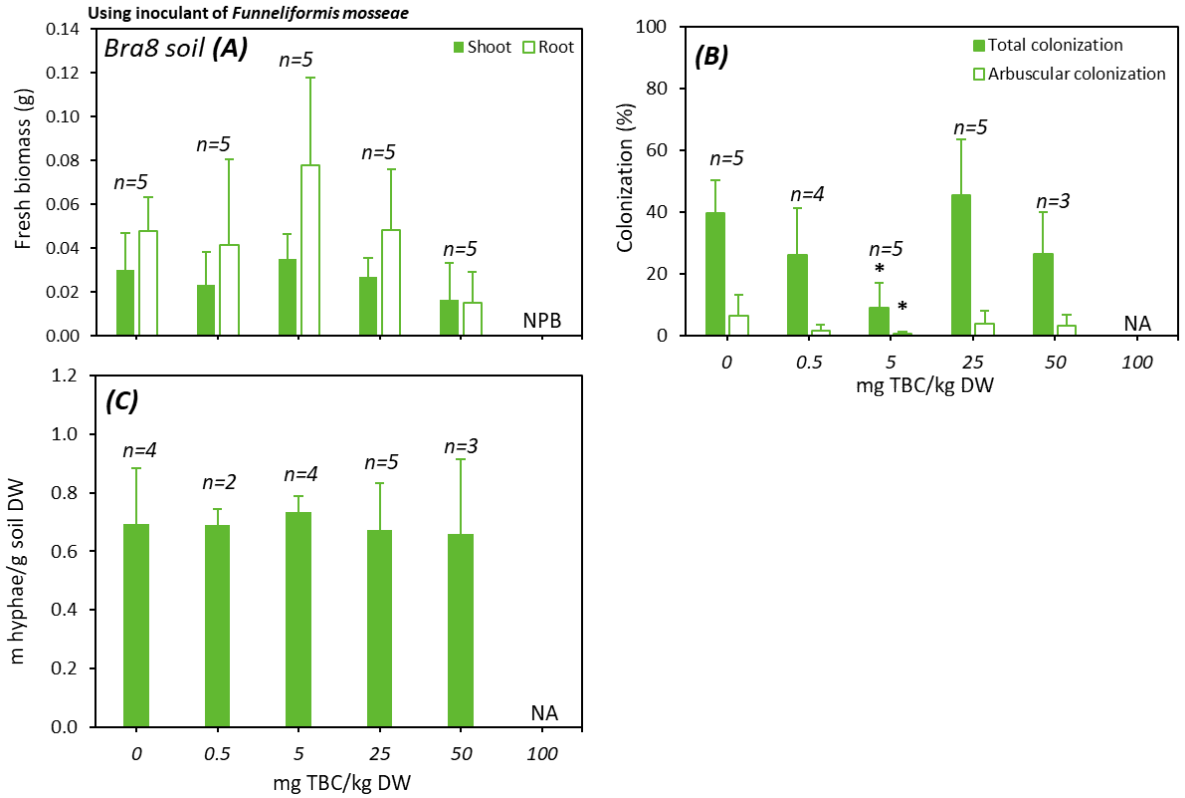
		OECD 2.5%	**Bra8**
Tebuconazole	*F. mosseae*	UDESC	UDESC

		OECD 2.5%	**Bra8**
	R. clarus	UDESC	UDESC

The experiments were performed according to the procedures described in the SOP Test 2 (Appendix A.18) and using concentration gradients composed of the treatments 0, 0.5, 5, 25, 50, and 100 mg TBC/kg of soil. Test concentrations used in the experiments aimed to define EC10 and EC50 values and not to cover realistic application doses.

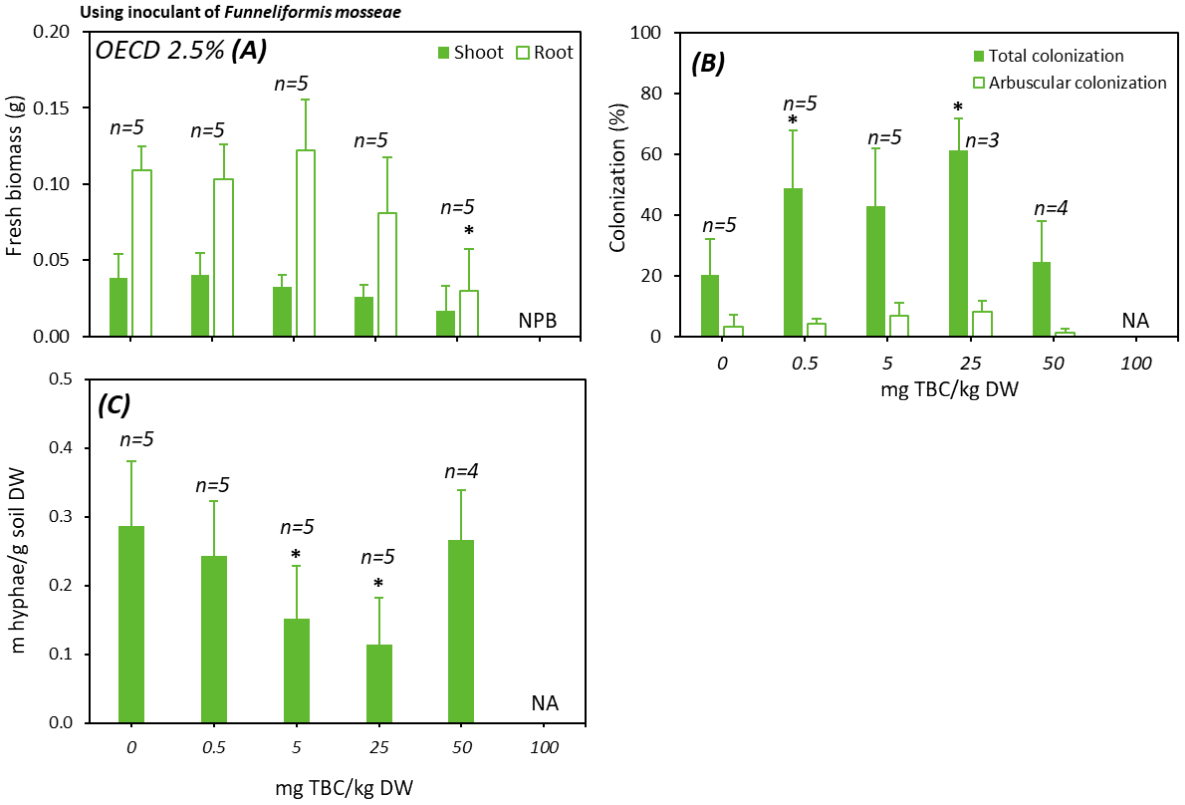
Results obtained in the experiments are graphically presented in Figures 44 to 47 and raw data are shown in Appendix A.19.

Figure 44: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: Bra8; Test substance: Tebuconazole; Performing lab: UDESC, Brazil.



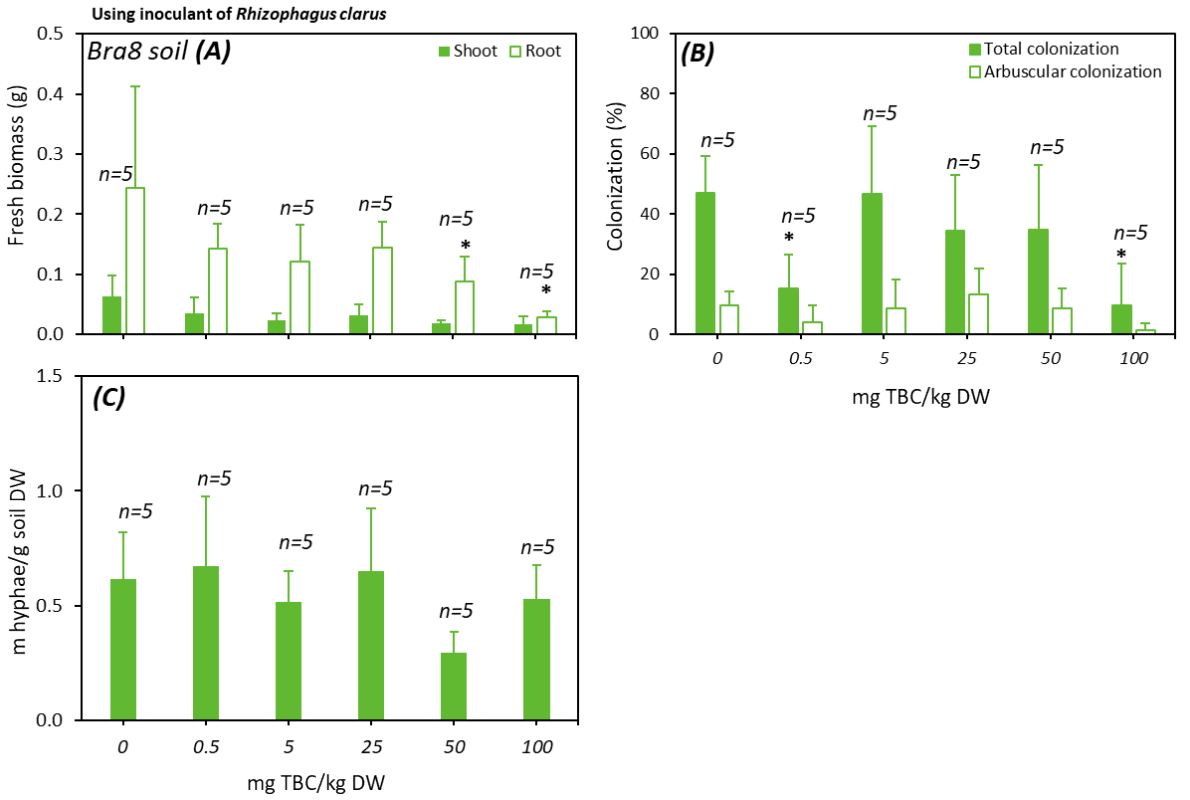
(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 45: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Tebuconazole; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

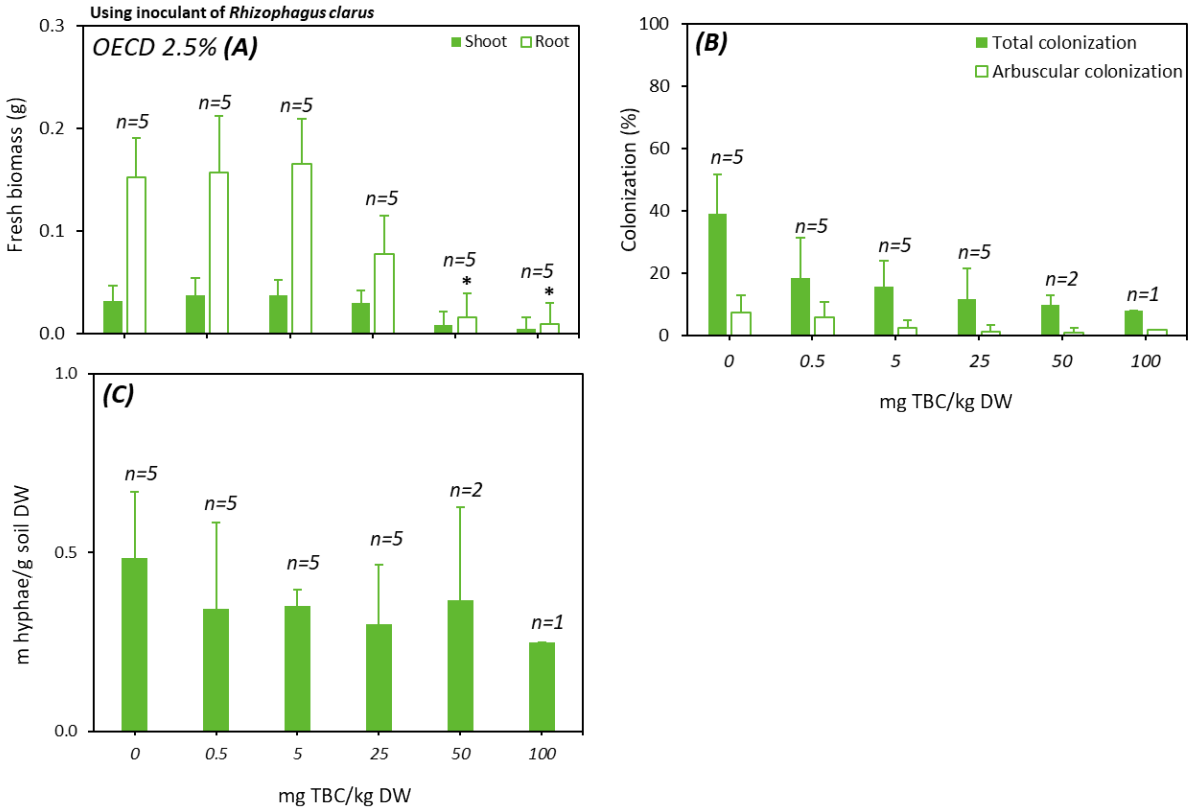
Figure 46: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus clarus*; Test soil: Bra8; Test substance: Tebuconazole; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.

Source: own illustration, CloverStrategy.

Figure 47: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus clarus*; Test soil: OECD 2.5%; Test substance: Tebuconazole; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Chemical analyses were performed to determine the actual contamination of Tebuconazole in soil collected at the beginning of the experiments (Table 27).

These analyses followed the modified QuEChERS methodology (Perestrelo et al., 2019), where aliquots of each treatment and control were prepared by mixing 5 g of soil with 10 mL of ultra-pure water and 10 mL of acetonitrile. After shaking the mixture for 2 min, 1 g of citrate dibasic, 0.5 g of disodium citrate, 1 g of sodium chloride and 4 g of magnesium sulphate were added. The later mixture was shaken for 2 min, centrifuged at 4500 rpm for 10 min and injected with 10 µL of acetonitrile. Aliquots were analyzed by UPLC-MS/MS. Calibration was performed on matrix (soil blank) with internal standard. Tebuconazole standard by Dr Ehrenstorfer (CAS 107534-96-3; purity 98.57%) was used to assess the quality of the analysis. The percentage of recovery of Tebuconazole was 99% and the quantification limit was 0.005 mg TBC/kg.

Table 27: Nominal and initial measured concentrations of Tebuconazole (in mg TBC/kg of soil DW), and the respective percentage of nominal concentrations measured in soil samples collected from AMF symbiotic phase tests using inoculants of *F. mosseae* or *R. clarus* and test soils Bra8 and OECD 2.5% artificial soil, carried out in laboratory of UDESC (Brazil).

AMF species	Soil	Lab	Nominal concentration (mg TBC/kg of soil DW)	Measured concentration (mg TBC/kg of soil DW)	% nominal concentration
F. mosseae	Bra8	UDESC	0	0.018	NA
			0.5	0.035	7.0
			5	0.0079	0.158
			25	0.018	0.072
			50	0.072	0.144
R. clarus	Bra8	UDESC	0	0.018	NA
			0.5	0.78	156.0
			5	3.7	74.0
			25	18.5	74.0
			50	40.0	80.0
			100	94.8	94.8
R. clarus	OECD 2.5%	UDESC	0	0.076	NA
			0.5	0.92	184.0
			5	4.1	82.0
			25	20.5*	NA
			50	32.3	64.6
			100	79.8	79.8

NA – not applicable; * - Lost sample –value estimated by multiplying the measured concentration of the previous treatment by the dilution factor between the two treatments (i.e. 5).

2.6.1.3.1 Conclusions of the experiments with Tebuconazole

Results obtained in the experiments with Tebuconazole (Table 28) did not allow to observe a coherent relationship between the AMF parameters assessed (i.e. total and arbuscular colonization or extra-radicular hyphae length) and concentration of Tebuconazole in soil. AMF sensitivity varied greatly among each experiment and even within the same experiment among the different parameters.

While *R. clarus* seems to be very sensitive to Tebuconazole in OECD 2.5% artificial soil (e.g. EC10 EHL= 0.000002 mg TBC/kg of soil DW), the same species was less sensitive than *F. mosseae* in the experiment with Bra8 soil when considering total and arbuscular colonization. On the other hand, despite the high sensitivity of total and arbuscular colonization shown by *F. mosseae* to Tebuconazole in Bra8 soil, extra-radicular hyphae length was not significantly affected in any of the test treatments with plant biomass production. Moreover, significant effects found in AMF parameters were generally associated to significant decreases of plant biomass production, which suggest that the reductions in AMF colonization can be related to the lack of plant biomass instead of the presence of Tebuconazole in soil.

The endpoints obtained from these experiments contrast with those published by Campos et al. (2015), where no effects on AMF colonization and spores production of indigenous AMF were observed in a mixture of 2 natural soils (in a ratio of 1:1, w:w) with 3% of granulated and air dried elephant grass (*Pennisetum purpureum*) sprayed with a commercial formulation of a mixture of Tebuconazole and Trifloxystrobin that corresponded to a Tebuconazole concentration of 0.19 mg TBC/kg DW (assuming an homogeneous distribution of the fungicide among the top 5 cm soil layer and a substrate bulk density of 1.5 g/cm^3) in greenhouse experiments using beans as host plant.

The endpoints estimated in the experiments with Tebuconazole on the ERAMYC project are generally lower than most of the endpoints reported in the literature for non-target in-soil organisms (namely invertebrates and plants; Appendix A.23), which suggests that AMF could be more sensitivity to Tebuconazole compared to other groups of non-target soil organisms, some of them considered in current ERA of PPPs.

Tebuconazole showed suitability to be used as a reference substance or in ring test experiments only for with inoculant of *R. clarus* in OECD 2.5% artificial soil. In experiments with *F. mosseae* in both soils or with *R. clarus* in Bra8 soil, data did not show a clear relation between increasing concentrations of Tebuconazole in soil and the AMF colonization and extra-radicular hyphae length.

Table 28: **Summary of the experiments performed in the consortium laboratories with Tebuconazole (TBC) using two AMF species (*Rhizophagus clarus* and *Funneliformis mosseae*) in artificial soil (OECD 2.5%) and a Brazilian natural soil (Bra8). No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction on total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated in valid experiments only (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg TBC/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg TBC/kg DW)	Concentrations with plant biomass (mg TBC/kg DW)	Laboratory	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	Bra8	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50	UDESC	Valid	TC	0.5	5	0.005*	0.03*
						AC	< 0.5	0.5	ND	ND
						EHL	50	> 50	> 50	> 50
F. mosseae	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50	UDESC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
						EHL	-	-	-	-
R. clarus	Bra8	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UDESC	Valid	TC	< 0.5	0.5	74.6*	> 100
						AC	100	> 100	17.0*	> 100
						EHL	100	> 100	2.4*	15.7*
R. clarus	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UDESC	Valid	TC	< 0.5	0.5	0.03*	0.2*
						AC	100	> 100	0.26*	1.73*
						EHL	100	> 100	0.000002*	> 100

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.6.1.4 Experiments with Terbuthylazine (TRZ)

Terbuthylazine is an herbicide widely used in agriculture to control weeds and in commercial and industrial water-cooling systems, and in residential and commercial ornamental ponds, fountains and aquaria as an algicide, microbicide and microbiostat (USEPA, 1995; PPDB, 2024). Physical and chemical properties of Terbuthylazine are described on Table 29.

Table 29: Physical and chemical properties of Terbuthylazine.

Terbuthylazine	
Pesticide type	Herbicide
Substance group	Triazine herbicide, Methoxytriazine herbicide
IUPAC name	2-N-tert-butyl-6-chloro-4-N-ethyl-1,3,5-triazine-2,4-diamine
Empirical formula	$C_9H_{16}ClN_5$
CAS number	5915-41-3
Molecular mass	229.71 g/mol
Melting point (°C)	175
Solubility (water, 20°C)	6.6 mg/L
Dissociation constant (pKa) (25°C)	1.9
Log Kow (20°C)	3.4
Henry's Law constant	2.3 x 10^{-3} Pa/m^3/mol
Degradation point (°C)	224
Degradation in soil (aerobic)	
DT_{50} (lab at 20°C)	72 days
DT_{50} (field)	21.8 days

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

Four experiments were performed with Terbuthylazine, using inoculants of *Rhizophagus clarus* and *Funneliformis mosseae* and using leek as host plant in OECD 2.5% or Bra8 soils. These experiments were carried out in UDESC (Brazil) laboratory (Table 30).

Table 30: Experiments performed in UDESC (Brazil) with Terbuthylazine using inoculants of *Funneliformis mosseae* and *Rhizophagus clarus* on two test soils (artificial soil, OECD 2.5%, and natural Brazilian soil, Bra8).

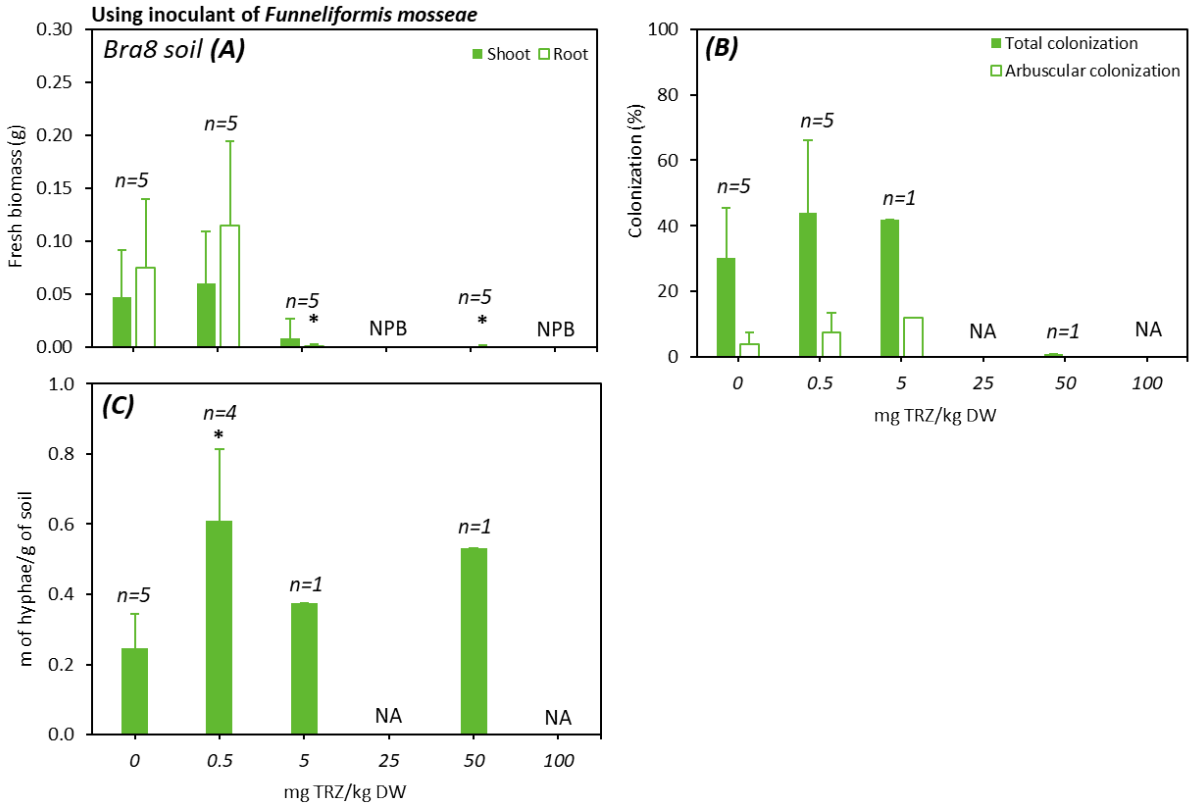
		OECD 2.5%	**Bra8**
Terbuthylazine	*F. mosseae*	UDESC	UDESC

		OECD 2.5%	**Bra8**
	R. clarus	UDESC	UDESC

The experiments were performed according to the procedures described in the SOP Test 2 (Appendix A.18), using a gradient of concentrations composed of the treatments 0, 0.5, 5, 25, 50, 100 mg TRZ/kg of soil. Test concentrations were selected to cover a range of concentrations large enough to provoke effects in AMF symbiotic phase parameters, without taking into consideration realistic application doses of real scenarios.

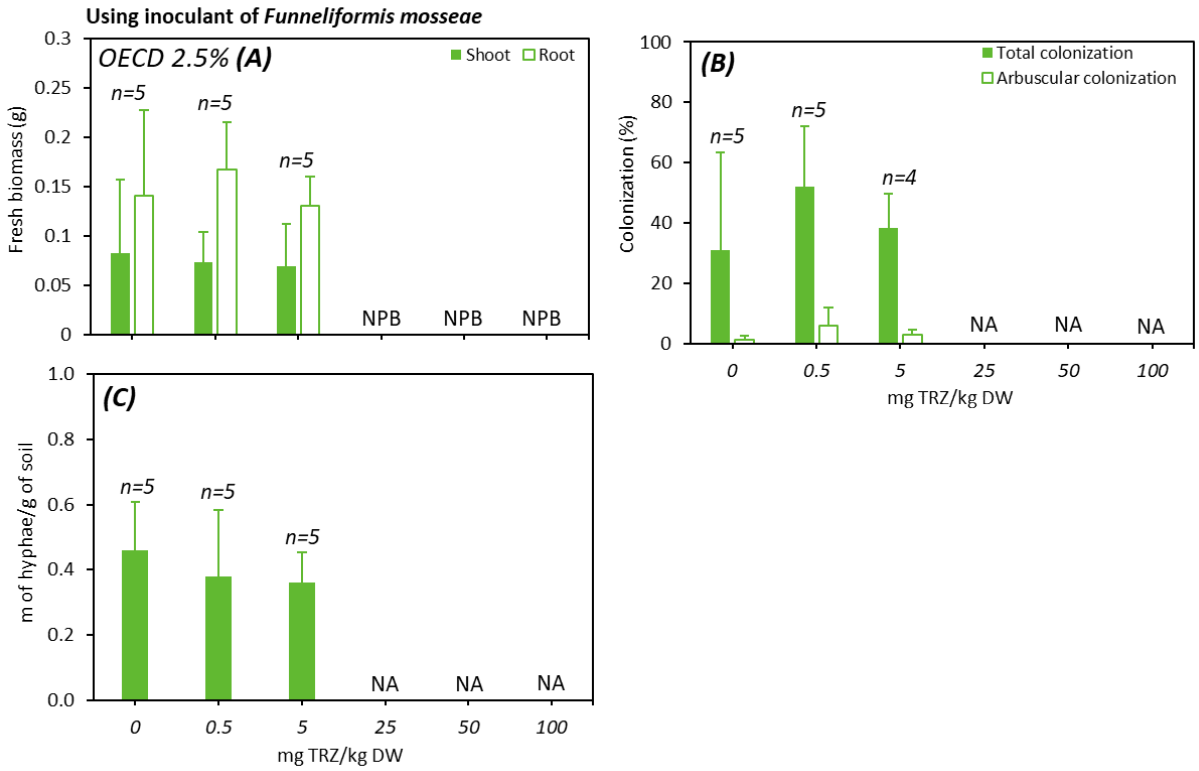
Results obtained in the experiments are presented in graphs of Figures 48 to 51 and raw data are presented in Appendix A.19.

Figure 48: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: Bra8; Test substance: Terbuthylazine; Performing lab: UDESC, Brazil.



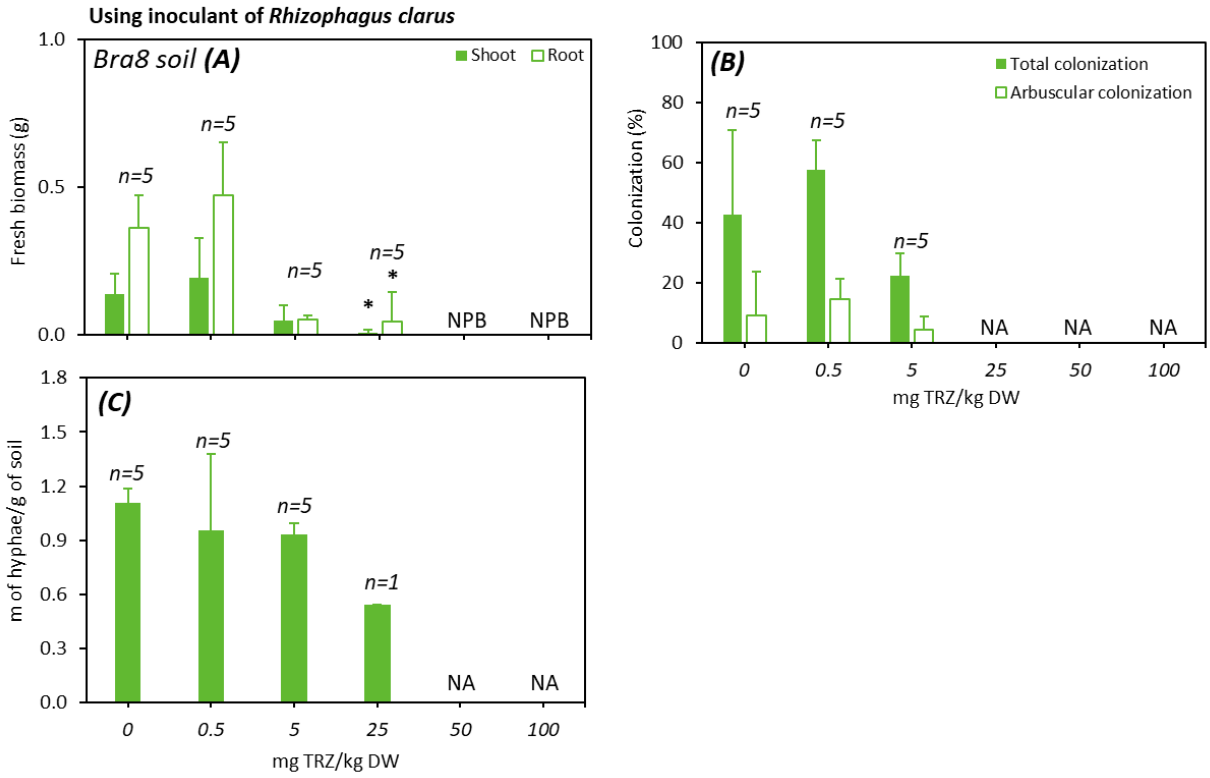
(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 49: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Terbuthylazine; Performing lab: UDESC, Brazil.



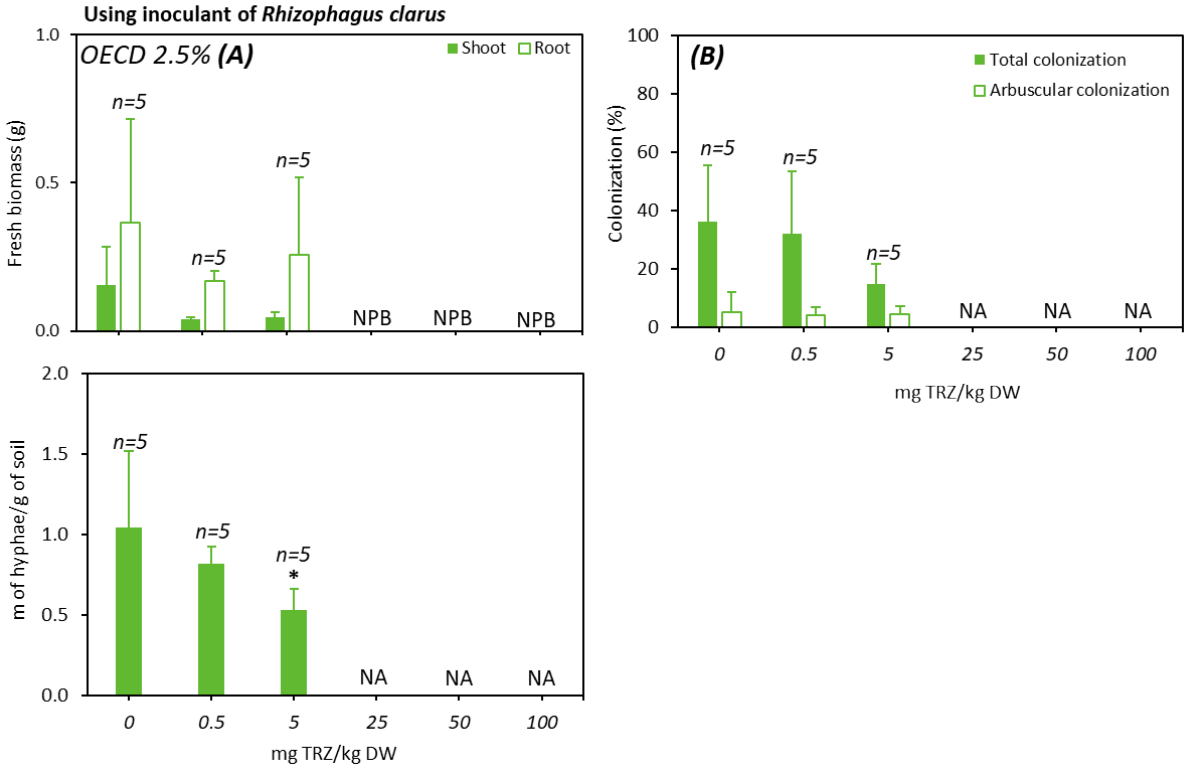
(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 50: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus clarus*; Test soil: Bra8; Test substance: Terbuthylazine; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 51: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus clarus*; Test soil: OECD 2.5%; Test substance: Terbuthylazine; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Chemical analyses were performed to determine the actual contamination of Terbuthylazine in soil collected at the beginning of the experiments, following the same methodology described for the chemical determination of Tebuconazole (Perestrelo et al., 2019; see section 2.6.1.3.) (Table 31).

Terbuthylazine standard by Dr Ehrenstorfer (CAS 5915-41-3; purity 99.28%) was used to assess the quality of the analysis. The percentage of recovery of Terbuthylazine was 98% and the quantification limit was 0.005 mg TRZ/kg.

Table 31: Nominal and initial measured concentrations of Terbuthylazine (in mg TRZ/kg of soil DW), and the respective percentage of nominal concentrations measured in soil samples collected from AMF symbiotic phase tests using inoculants of *F. mosseae* or *R. clarus* and test soils Bra8 and OECD 2.5% artificial soil, carried out in laboratory of UDESC (Brazil).

AMF species	Soil	Lab	Nominal concentration (mg TRZ/kg of soil DW)	Measured concentration (mg TRZ/kg of soil DW)	% nominal concentration
F. mosseae	Bra8	UDESC	0	< 0.005	NA
			0.5	< 0.005	NA
			5	< 0.005	NA
			50	< 0.005	NA
F. mosseae	OECD 2.5%	UDESC	0	0.018	NA
			0.5	0.34	68
			5	3.6	72
R. clarus	Bra8	UDESC	0	< 0.005	NA
			0.5	0.32	64
			5	3.5	70
			25	23.7	94.8
R. clarus	OECD 2.5%	UDESC	0	0.018	NA
			0.5	0.32	64
			5	3.2*	NA
			25	21.4	85.6

NA – not applicable; * - Lost sample –value estimated by multiplying the measured concentration of the previous treatment by the dilution factor between the two treatments (i.e. 10).

2.6.1.4.1 Conclusions of the experiments with Terbuthylazine

The endpoints estimated from the tests performed with Terbuthylazine, as well as their compliance with the validity criterion (i.e. total colonization ≥ 30% in the negative control), are summarized in Table 32.

Results obtained suggest that *R. clarus* is generally more sensitive to the herbicide than *F. mosseae*, independently of the tested soil. This was particularly evident when considering AMF total and arbuscular colonization of roots. Both species were more sensitive to Terbuthylazine when exposed in OECD 2.5% artificial soil than when exposed in Bra8 soil. These results contrast with the data reported by Giovannetti et al. (2006), who carried out a pre-symbiotic phase tests with *Glomus mosseae* in sand with increasing concentrations of a commercial

product of Terbuthylazine. These authors did not find effects on spore germination at concentrations up to 81.2 mg TRZ/l, but did for hyphal growth at concentrations higher or equal to 5.2 mg TRZ/l. On the other hand, considering the endpoints from literature for other soil organisms to Terbuthylazine (either through a commercial product or as a pure active ingredient), the endpoints estimated for AMF to Terbuthylazine in the present project show a tendency of AMF to be, in general, more sensitive than the other non-target soil organisms tested, including NTTPs (Appendix A.23).

Notwithstanding, the endpoints estimated in the present study were generally conditioned by the toxicity of the herbicide to the host plant, that was generally too high, which impaired the plant biomass production in the highest test concentrations. This happened because Terbuthylazine was more (or similarly) toxic to the host plant than to *F. mosseae* and *R. clarus* symbioses in the test soils and therefore, Terbuthylazine cannot be considered suitable to be applied in AMF symbiotic phase experiments as a reference substance.

Table 32: **Summary of the experiments performed by the consortium laboratories with Terbuthylazine (TRZ) using two AMF species (*Rhizophagus clarus* and *Funneliformis mosseae*) in artificial soil (OECD 2.5%) and a natural Brazilian soil (Bra8). No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction on total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg TRZ/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg TRZ/kg DW)	Concentrations with plant biomass (mg TRZ/kg DW)	Laboratory	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	Bra8	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 50	UDESC	Valid	TC	5	> 5	38.7*	> 50
						AC	5	> 5	10.5*	11.6*
						EHL	50	> 50	ND	ND
F. mosseae	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5	UDESC	Valid	TC	5	> 5	> 5	> 5
						AC	5	> 5	> 5	> 5
						EHL	5	> 5	0.03*	0.2*
R. clarus	Bra8	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25	UDESC	Valid	TC	5	> 5	3.7*	5.1*
						AC	5	> 5	4.6*	5.0*
						EHL	25	> 25	3.9*	> 25
R. clarus	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25	UDESC	Valid	TC	5	> 5	0.4*	3.5*
						AC	5	> 5	ND	ND
						EHL	0.5	5	0.09*	5.2*

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.6.1.5 Experiments with Azoxystrobin (AZX)

Azoxystrobin is a post-emergence broad spectrum fungicide widely used in agriculture for the control of fungal diseases affecting crops (Wang et al. 2020). Physical and chemical properties of Azoxystrobin are presented in Table 33.

Table 33: Physical and chemical properties of Azoxystrobin.

Azoxystrobin	
Pesticide type	Fungicide
Substance group	Strobilurin fungicide
IUPAC name	Methyl (E)-2-{2-[6-(2-cyanophenoxy)pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate
Empirical formula	$C_{22}H_{17}N_3O_5$
CAS number	131860-33-8
Molecular mass	403.4 g/mol
Melting point (°C)	116
Solubility (water, 20°C)	6.7 mg/L
Solubility (acetone, 20°C)	86000 mg/L
Dissociation constant (pKa) (25°C)	Not applicable
Log Kow (20°C)	2.5
Henry's Law constant	7.40 x 10^{-9} Pa/m^3/mol
Degradation point (°C)	345
Degradation in soil (aerobic)	
DT_{50} (lab at 20°C)	84.5 days
DT_{50} (field)	180.7 days

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

Eight experiments exposing *R. irregularis* and two exposing *F. mosseae* to Azoxystrobin using leek as host plant in OECD 2.5% artificial soil, #Lufa 2.1, Por3, Lufa 2.2, Est32 and Bra8 natural soils were carried out by the consortium laboratories. For these experiments, a concentration gradient composed of 0, 0.5, 5, 25, 50, 100 mg AZX/kg of soil DW for #Lufa 2.1 and OECD 2.5% soils and 0, 0.5, 1.25, 3.125, 7.8, 19.5 mg AZX/kg soil DW for the rest of the soils were used. The test concentrations were selected with the aim of estimating NOEC, LOEC, EC10 and EC50 values, and therefore, did not aim to cover realistic application doses.

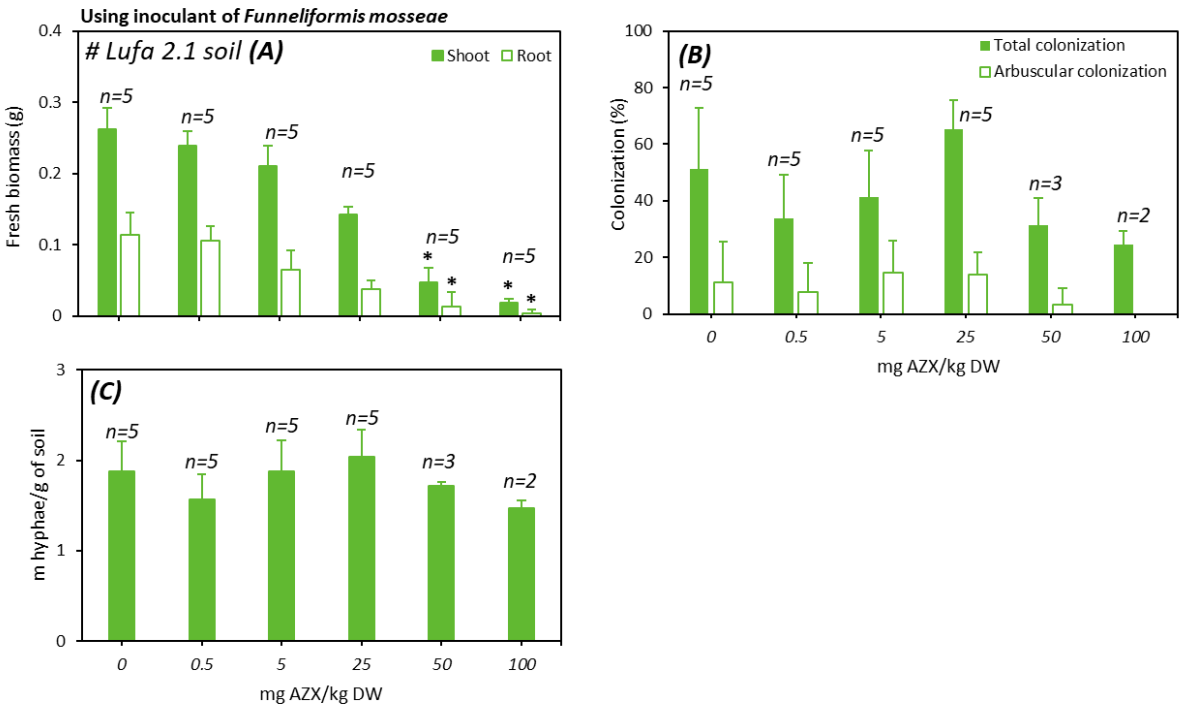
The list of experiments carried out with Azoxystrobin are summarized in Table 34 and were performed according to the procedures described in the SOP Test 2 (Appendix A.18).

Table 34: **Experiments performed by the consortium laboratories (UC, Portugal; UDESC, Brazil; UT, Estonia) with Azoxystrobin using inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* in OECD 2.5%, #Lufa 2.1, Por3, Lufa 2.2, Est32 and Bra8 soils.**

	OECD 2.5%	#Lufa 2.1	Por3	Lufa 2.2	Est32	Bra8
F. mosseae	UC	UC	-	-	-	-
R. irregularis	UC	UC	UC	UC, UDESC, UT	UC	UDESC

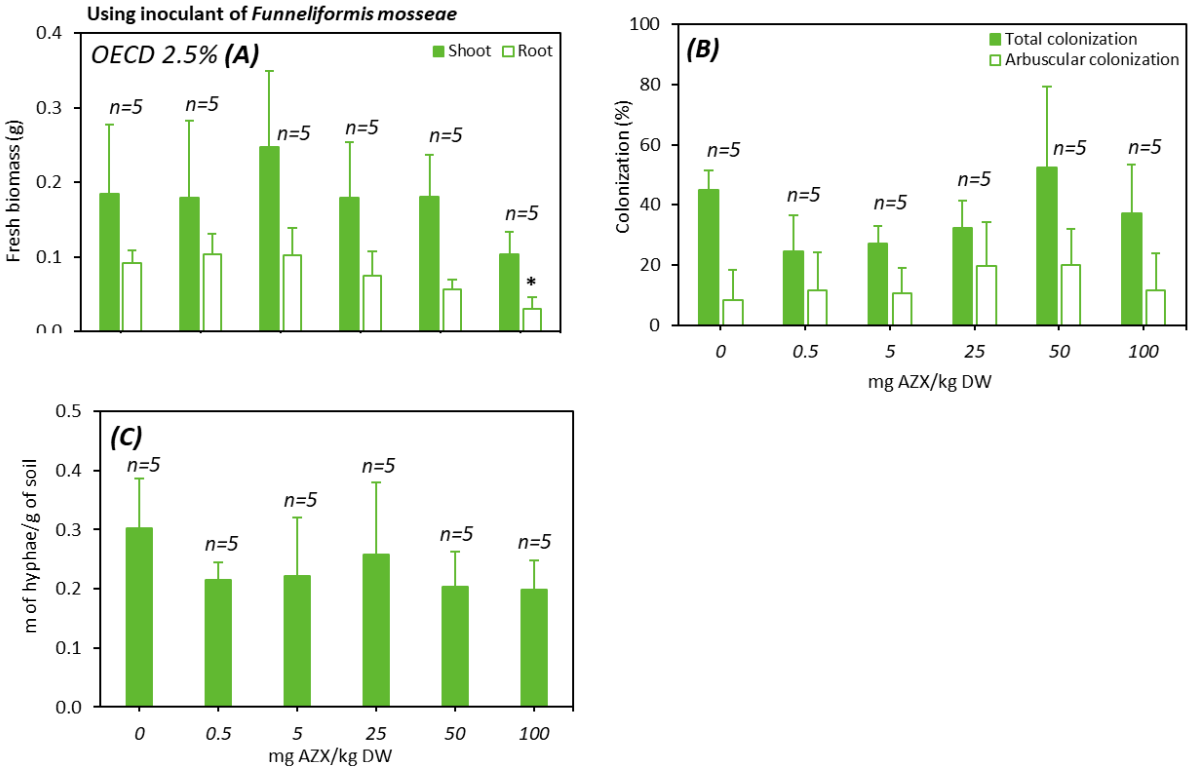
Results obtained in the experiments with Azoxystrobin are presented in Figures 52 to 60 and the corresponding raw data are presented in Appendix A.19.

Figure 52: **Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: #Lufa 2.1; Test substance: Azoxystrobin; Performing lab: UC, Portugal.**



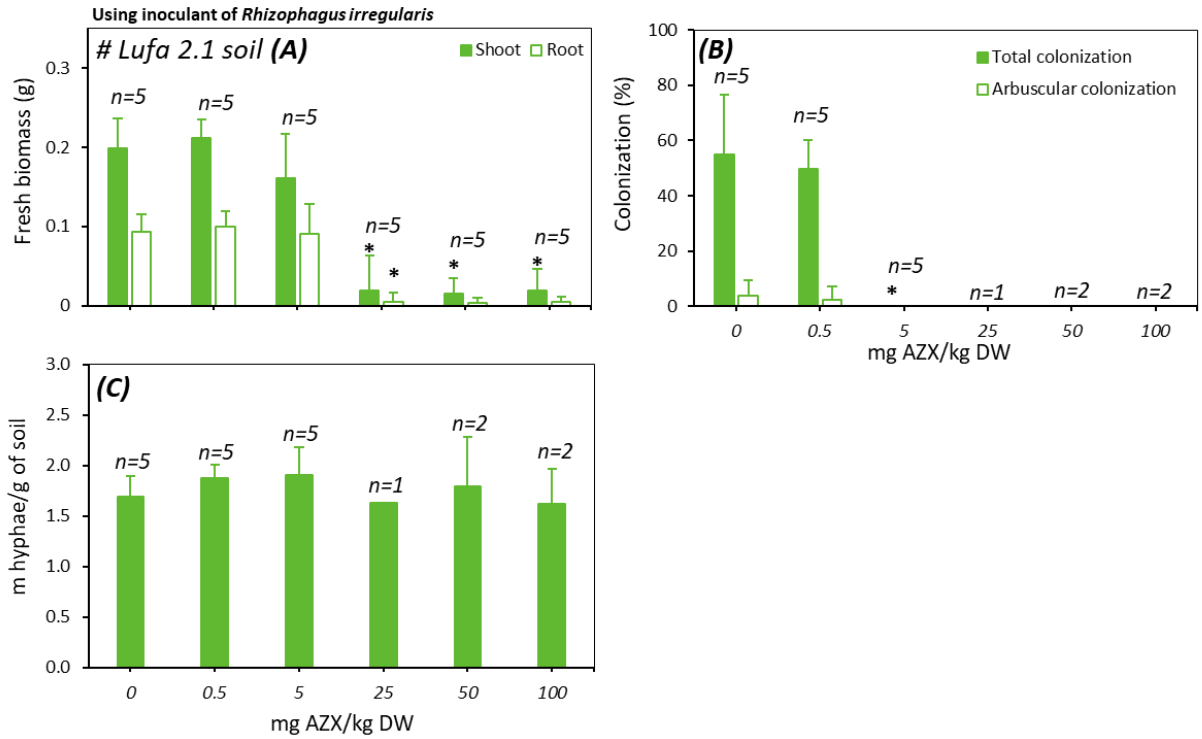
(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 53: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: UC, Portugal.



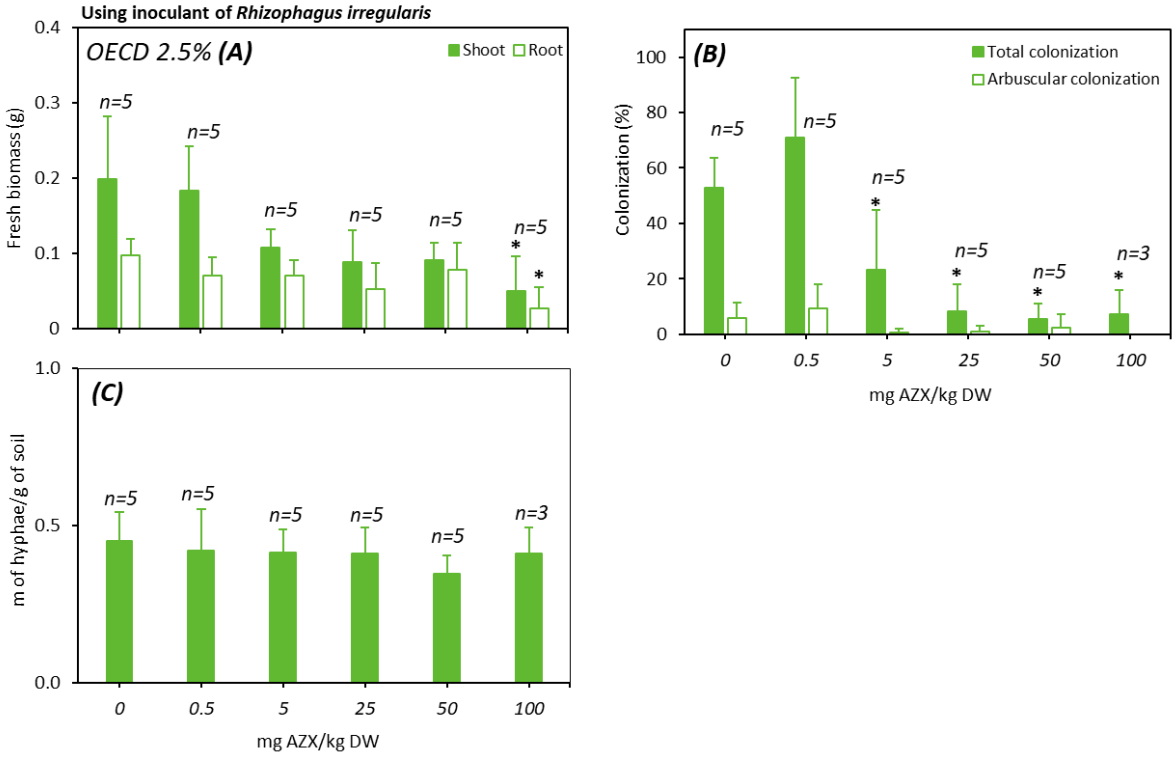
(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 54: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: #Lufa 2.1; Test substance: Azoxystrobin; Performing lab: UC, Portugal.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

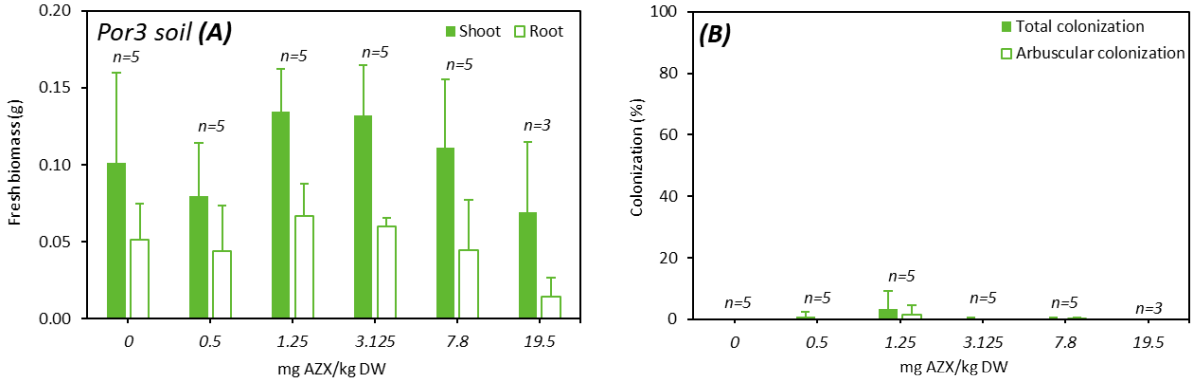
Figure 55: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: UC, Portugal.



(A) Fresh root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.

Source: own illustration, CloverStrategy.

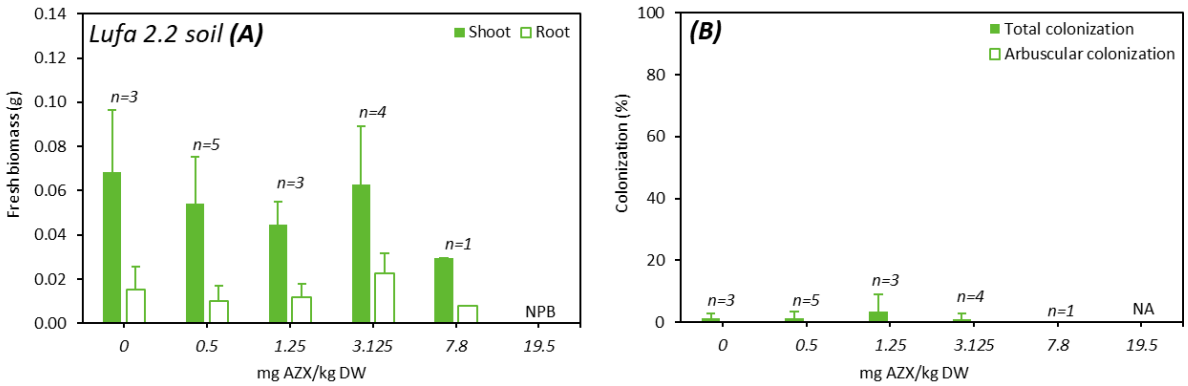
Figure 56: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Por3; Test substance: Azoxystrobin; Performing lab: UC, Portugal.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.

Source: own illustration, CloverStrategy.

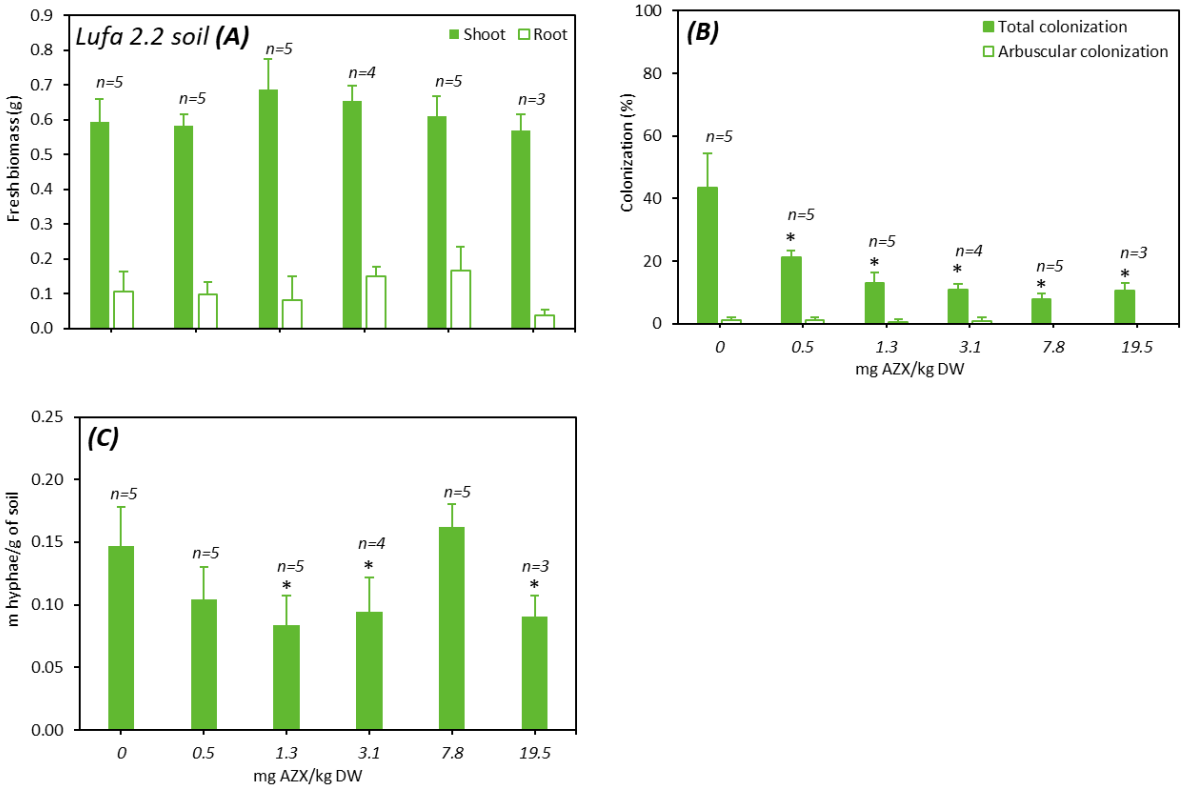
Figure 57: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Lufa 2.2; Test substance: Azoxystrobin; Performing lab: UC, Portugal.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars. NPB – no plant biomass produced; NA – not assessed.

Source: own illustration, CloverStrategy.

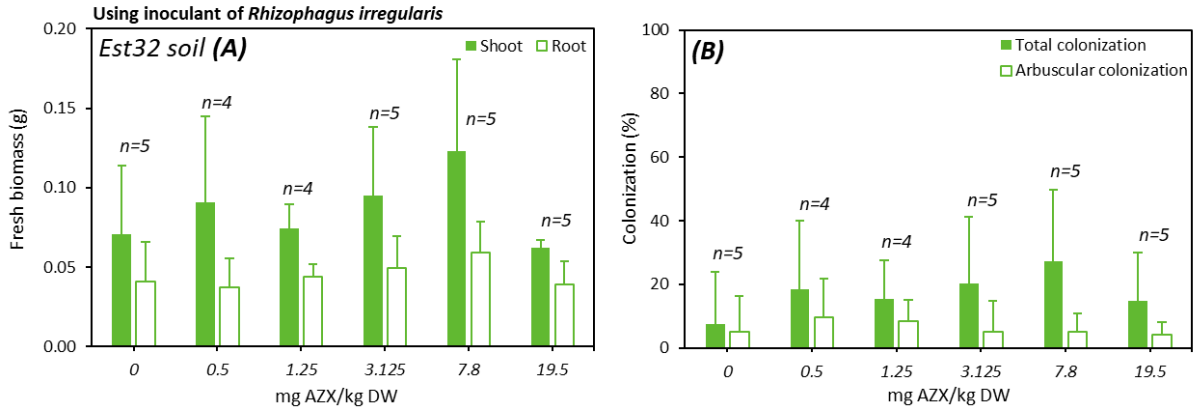
Figure 58: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Lufa 2.2; Test substance: Azoxystrobin; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each

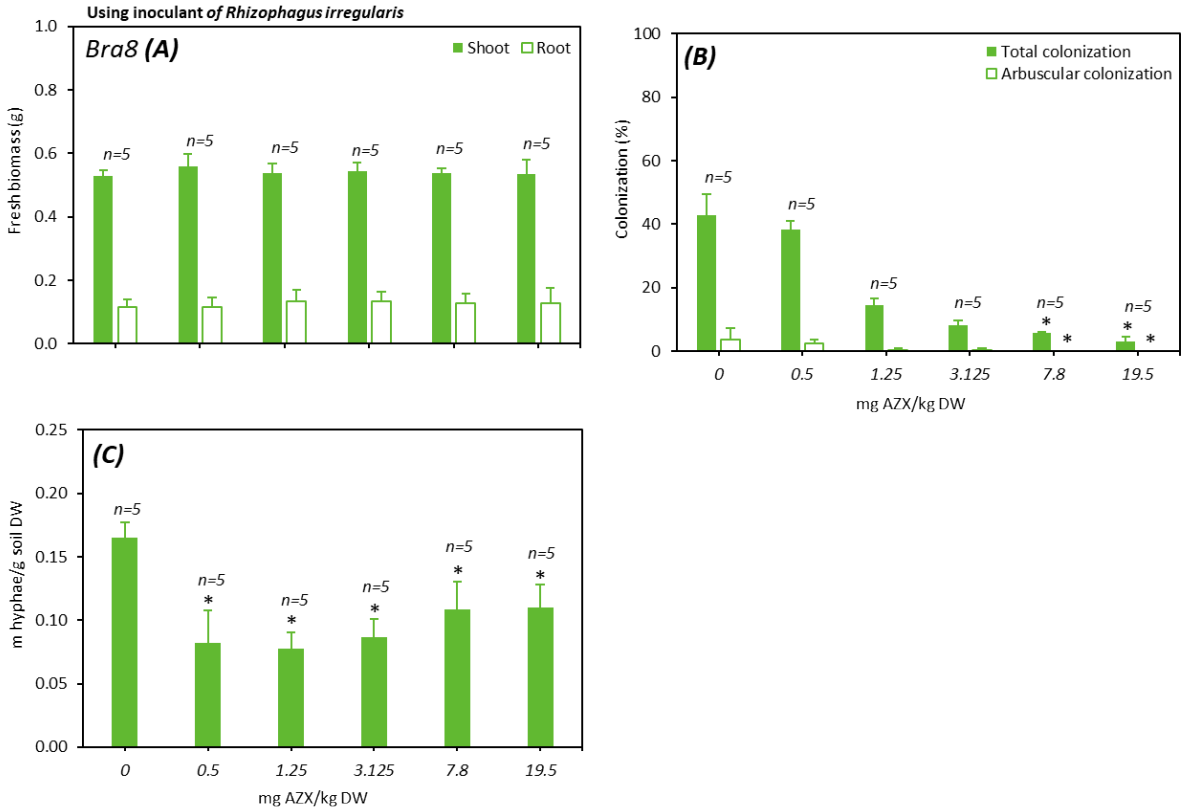
treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 59: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Est32; Test substance: Azoxystrobin; Performing lab: UC, Portugal.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 60: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Bra8; Test substance: Azoxystrobin; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Seeds germination of leek in the experiments with Lufa 2.2 soil was very low in the experiments carried out in UC (Figure 59) and UT (*data not shown*). Because of that, after the 2-week time for seeds germination (as established in the SOP Test 2) replicates were resown. After the second 2-week period for resown seeds germination, in the experiment carried out in UT, there were only 2 replicates with germinated seeds in controls (because of that, the test was discarded and no data was generated), while in the experiment carried out in UC, some of the germinated plants ended up by dying during the exposure/test period, which led to the loss of some replicates. Seeds germination in the UDESC experiment with Lufa 2.2 (Figure 60) was also initially low, but there was no need to resow the leek.

Chemical analyses were performed to determine the actual contamination of Azoxystrobin in soil collected at the beginning of the experiments, following the same methodology described for the chemical determination of the previous test substances (Perestrelo et al., 2019; see section 2.6.1.3.) (Table 35).

Azoxystrobin standard by Sigma-Aldrich (CAS 131860-33-8; purity >98%) was used to assess the quality of the analysis. The percentage of Azoxystrobin recovery was 97% and the quantification limit was 0.005 mg AZX/kg.

Table 35: Nominal and initial measured concentrations of Azoxystrobin (in mg AZX/kg of soil DW), and the respective percentage of nominal concentrations measured in soil samples collected from AMF symbiotic phase tests using inoculants of *F. mosseae* or *R. irregularis* and test soils #Lufa 2.1, Lufa 2.2, Bra8 and OECD 2.5% artificial soil, carried out in UC (Portugal) and UDESC (Brazil).

AMF species	Soil	Lab	Nominal concentration (mg AZX/kg of soil DW)	Measured concentration (mg AZX/kg of soil DW)	% nominal concentration
F. mosseae	#Lufa 2.1	UC	0	< 0.005	NA
			0.5	0.54	108.0
			5	1.4	28.0
			25	3.9	15.6
			50	6.4	12.8
			100	17.2	17.2
F. mosseae	OECD 2.5%	UC	0	< 0.005	NA
			0.5	0.31	62.0
			5	1.6	32.0
			25	3.3	13.2
			50	7.5	15.0
			100	10.8	10.8
R. irregularis	#Lufa 2.1	UC	0	< 0.005	NA
			0.5	0.36	72.0
			5	1.1	22.0
			25	4.4	17.6
			50	5.4	10.8
			100	13.4	13.4
R. irregularis	OECD 2.5%	UC	0	< 0.005	NA
			0.5	0.27	54.0
			5	1.1	22.0

AMF species	Soil	Lab	Nominal concentration (mg AZX/kg of soil DW)	Measured concentration (mg AZX/kg of soil DW)	% nominal concentration
			25	3.8	15.2
			50	4.1	8.2
			100	13.8	13.8
R. irregularis	Bra8	UDESC	0	< 0.005	NA
			0.5	0.34	68.0
			1.25	1.3	104.0
			3.125	3.0	96.0
			7.8	5.8	74.4
			19.5	17.5	89.7
R. irregularis	Lufa 2.2	UDESC	0	< 0.005	NA
			0.5	0.66	132.0
			1.25	1.6	128.0
			3.125	2.2	70.4
			7.8	6.4	82.1
			19.5	15.2	77.9

NA – not applicable.

2.6.1.5.1 Conclusions of the experiments with Azoxystrobin

The endpoints estimated from the data generated in the experiments carried out with Azoxystrobin, as well as their respective compliance with the validity criterion (i.e. total colonization on the control treatment ≥ 30%), are summarized in Table 36.

In experiments with #Lufa 2.1, TC and AC of *R. irregularis* revealed higher sensitivity to Azoxystrobin than *F. mosseae*, however, EHL showed lower sensitivity to *R. irregularis* compared to *F. mosseae*. Moreover, these differences were not observed in experiments with OECD 2.5% artificial soil. Actually, sensitivity of *R. irregularis* to Azoxystrobin varied with the test soil and the AMF parameter measured. While TC of *R. irregularis* was more sensitive in Lufa 2.2, being in the same order of magnitude in the rest of the test soils, AC showed the highest sensitivity in #Lufa 2.1, Bra8 and Lufa 2.2 soils and EHL was generally the least sensitive parameter (of the three measured AMF parameters), except in the experiment with Bra8, where the EHL showed the most sensitive pattern of all experiments with Azoxystrobin.

Results obtained in the present experiments showed higher sensitivity of AMF to Azoxystrobin than the data reported by Campos et al. (2015) who conducted a pot experiment using a mixture of two natural soils and *Pennisetum purpureum* (previously granulated and dried) in a ratio of

50:50:3 as substrate, beans as host plant and total colonization and spores production of indigenous AMF as parameters to evaluate the effect of a commercial product of Azoxystrobin. Authors did not find effects neither on AMF intra-radicular colonization nor in spores production in substrate sprayed with the fungicide in a concentration corresponding to 0.16 mg AZX/kg DW (assuming an homogeneous distribution of the fungicide among the top 5 cm soil layer and a substrate bulk density of 1.5 g/cm^3). On the other hand, a reduction on spore germination and production, total root colonization and production of extra-radicular mycelium of *R. irregularis* in *in-vitro* experiments with solutions with increasing concentrations of pure Azoxystrobin was observed for concentrations higher than 1 mg AZX/l by Buysens et al. (2015). Moreover, the endpoints reported in the literature for non-target soil organisms other than AMF (e.g. EC20 *Eisenia andrei*= 12.2 mg AZX/kg of soil (Leitão et al., 2017), LC50 *Eisenia fetida*= 21.3 mg AZX/kg of soil (Xu et al., 2021) and EC10 *Enchytraeus crypticus*= 17 mg AZX/kg of soil (Gomes et al. 2024)) suggest that the TC and/or AC of the AMF *R. irregularis* is more sensitive to Azoxystrobin then other non-target in-soil organisms (Appendix A.23).

Results obtained in the experiments of the 2nd phase of WP2 evidenced that Azoxystrobin is a suitable chemical to be used as a reference substance for AMF testing. Actually, the toxic effects observed on the establishment of the AMF-plant symbiosis do not seem to be caused by side effects of the contaminant on the host plant.

Table 36: **Summary of the experiments performed by the consortium laboratories with Azoxystrobin using two AMF species (*Rhizophagus irregularis* and *Funneliformis mosseae*) in artificial soil (OECD 2.5%) and natural soils (#Lufa 2.1, Por3, Est32, Lufa 2.2 and Bra8). No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction (and the respective 95% confidence intervals) in total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated in valid experiments only (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg AZX/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg AZX/kg DW)	Concentrations with plant biomass (mg AZX/kg DW)	Lab	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	#Lufa 2.1	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UC	Valid	TC	100	> 100	53.1 (28.1-78.1)	78.4 (20.1-136.8)
						AC	100	> 100	10.7*	70.2*
						EHL	100	> 100	0.3*	2.2*
F. mosseae	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UC	Valid	TC	100	> 100	ND	ND
						AC	100	> 100	0.9*	5.8*
						EHL	100	> 100	ND	ND
R. irregularis	#Lufa 2.1	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UC	Valid	TC	0.5	5	0.2 (0.1-0.4)	1.5 (0.4-2.6)
						AC	100	> 100	0.1*	0.7*
						EHL	100	> 100	99.4*	> 100
R. irregularis	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UC	Valid	TC	0.5	5	0.5 (0.1-0.9)	3.4 (0.8-5.9)

AMF species	Soil	Concentrations (mg AZX/kg DW)	Concentrations with plant biomass (mg AZX/kg DW)	Lab	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
						AC	100	> 100	4.2*	4.6*
						EHL	100	> 100	1.7*	11.4*
R. irregularis	Por3	0; 0.5; 1.25; 3.125; 7.8; 19.5	0; 0.5; 1.25; 3.125; 7.8; 19.5	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
R. irregularis	Lufa 2.2	0; 0.5; 1.25; 3.125; 7.8; 19.5	0; 0.5; 1.25; 3.125; 7.8	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
R. irregularis	Est32	0; 0.5; 1.25; 3.125; 7.8; 19.5	0; 0.5; 1.25; 3.125; 7.8; 19.5	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
R. irregularis	Bra8	0; 0.5; 1.25; 3.125; 7.8; 19.5	0; 0.5; 1.25; 3.125; 7.8; 19.5	UDESC	Valid	TC	3.125	7.8	0.3 (0.1 - 0.5)	1.0 (0.8 - 1.3)
						AC	3.125	7.8	0.1 (0.01 - 0.2)	0.6 (0.1 - 1.1)
						EHL	< 0.5	0.5	0.002*	0.01*
R. irregularis	Lufa 2.2	0; 0.5; 1.25; 3.125; 7.8; 19.5	0; 0.5; 1.25; 3.125; 7.8; 19.5	UDESC	Valid	TC	< 0.5	0.5	0.08 (0.05-0.12)	0.6 (0.3-0.8)
						AC	19.5	> 19.5	0.5*	3.0*
						EHL	0.5	1.25	17.3 (4.5-30.1)	> 19.5

AMF species	Soil	Concentrations (mg AZX/kg DW)	Concentrations with plant biomass (mg AZX/kg DW)	Lab	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
R. irregularis	Lufa 2.2	0; 0.5; 1.25; 3.125; 7.8; 19.5		UT	Invalid	TC	-	-	-	-
						AC	-	-	-	-
						EHL	-	-	-	-

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.6.1.6 Experiments with Fluazinam (FLZ)

Fluazinam is a fungicide widely used in agriculture, mainly to protect crops against Oomycetes. This chemical acts by inhibiting spores' germination, hyphal penetration, growth and sporulation (EFSA, 2008), and it is also known to have some acaricidal activity (PPDB, 2024). Physical and chemical properties of Fluazinam are summarized on Table 37.

Table 37: Physical and chemical properties of Fluazinam.

Fluazinam	
Pesticide type	Fungicide, Acaricide
Substance group	Pyridine fungicide, Aminopyridine fungicide, Chloropyridine fungicide
IUPAC name	3-chloro-N-(3-chloro-5-trifluoromethyl-2-pyridyl)-α,α,α-trifluoro-2,6-dinitro-p-toluidine
Empirical formula	$C_{13}H_4Cl_2F_6N_4O_4$
CAS number	79622-59-6
Molecular mass	465.09 g/mol
Melting point (°C)	117
Solubility (water. 20°C)	0.135 mg/L
Solubility (acetone. 20°C)	853000 mg/L
Dissociation constant (pKa) (25°C)	7.22
Log Kow (20°C)	4.1
Henry's Law constant	5.93 x 10^{-2} Pa/m^3/mol
Degradation point (°C)	150
Degradation in soil (aerobic)	
DT_{50} (lab at 20°C)	124 days
DT_{50} (field)	25.9 days

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

Seven experiments exposing *R. irregularis* and two exposing *F. mosseae* to Fluazinam using leek as host plant in OECD 2.5%, #Lufa 2.1, Por3, Lufa 2.2, Est32 and Bra8 soils were carried out in the consortium laboratories. For these experiments, a concentration gradient composed of 0, 0.5, 5, 25, 50, 100 mg FLZ/kg of soil DW for Est32 and OECD 2.5% soils and 0, 0.5, 1.0, 2.0, 4.0 8.0 mg FLZ/kg soil DW for the rest of the soils were used. The test concentrations were selected with the aim of estimating NOEC, LOEC, EC10 and EC50 values, and therefore, did not aim to cover realistic application doses.

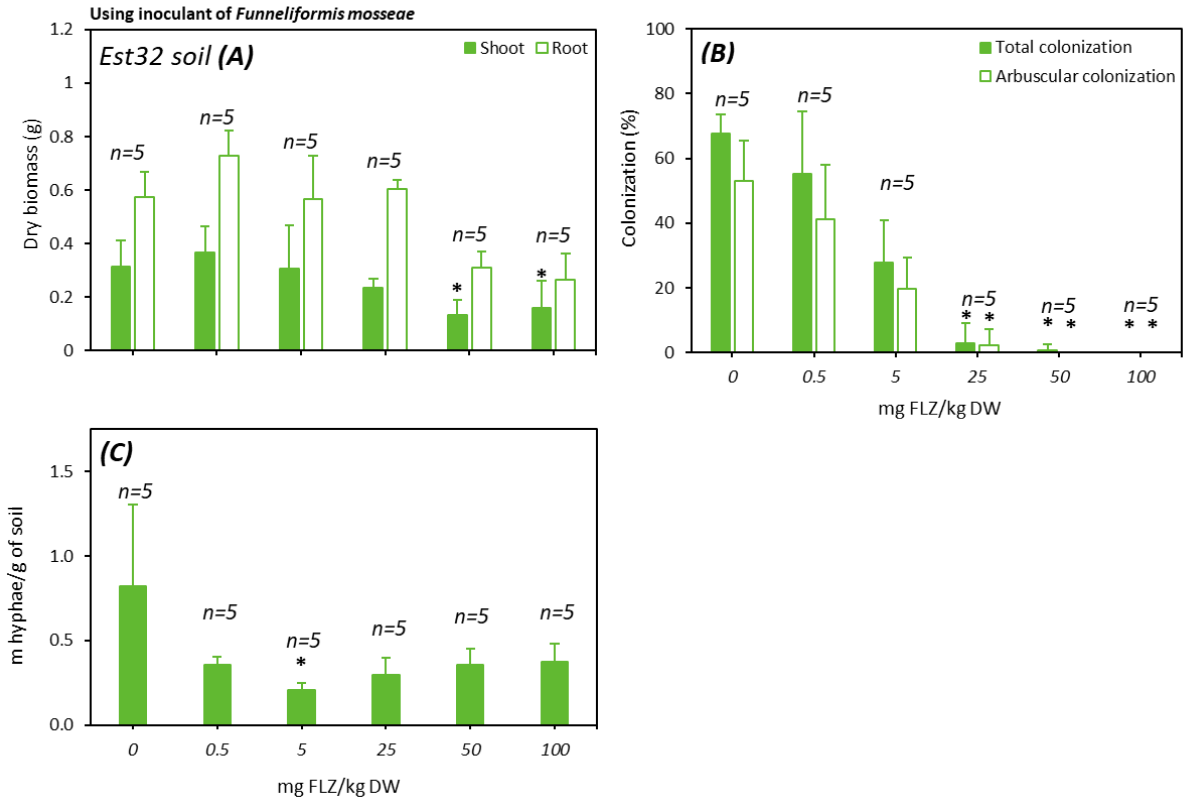
These experiments are summarized in Table 38 and were performed according to the procedures described in the SOP Test 2 (Appendix A.18).

Table 38: Experiments performed by UT (Estonia) with Fluazinam using inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* in OECD 2.5% and Est32 soils.

		OECD 2.5%	Est32	Por3	Lufa 2.2	Bra8
Fluazinam	*F. mosseae*	UT	UT	-	-	-
	R. irregularis	UT	UT	UC	UC, UT, UDESC	UDESC

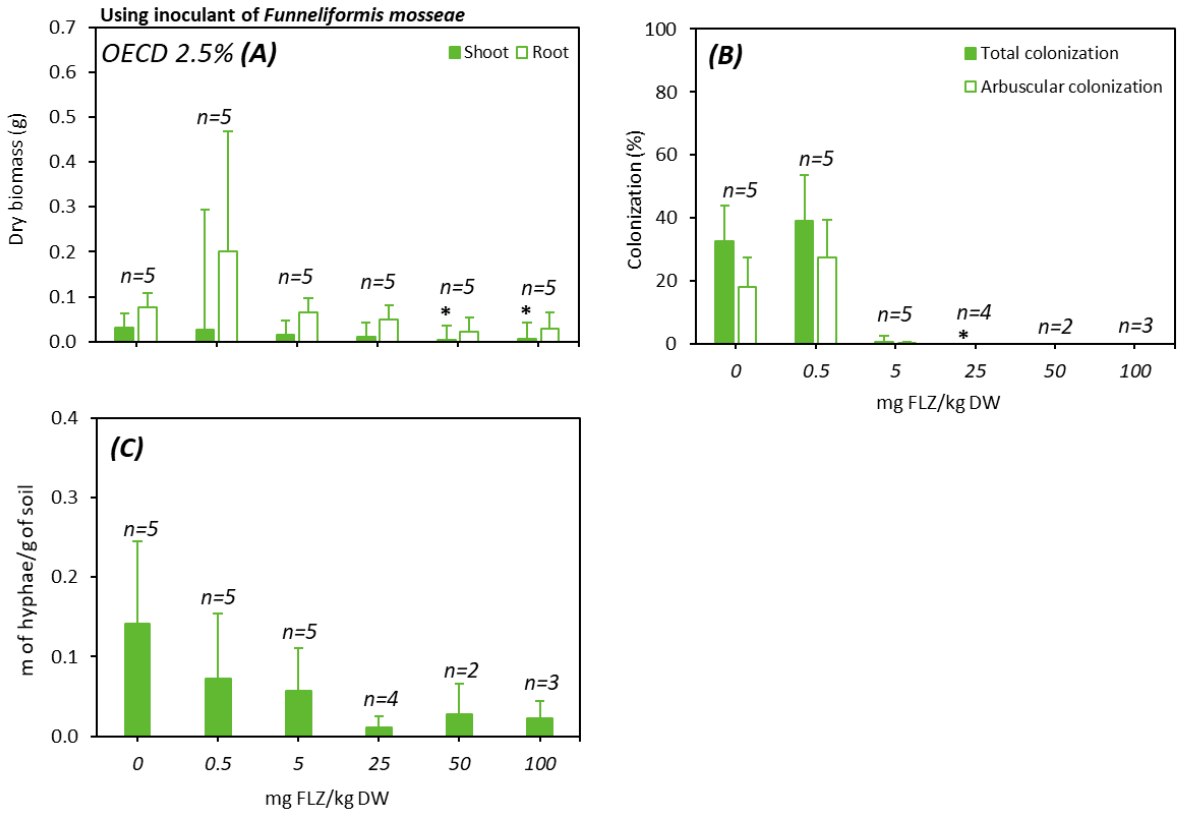
Results obtained on the experiments with Fluazinam are presented in graphs of Figures 61 to 68 and raw data are presented in Appendix A.19.

Figure 61: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: Est32; Test substance: Fluazinam; Performing lab: UT, Estonia.



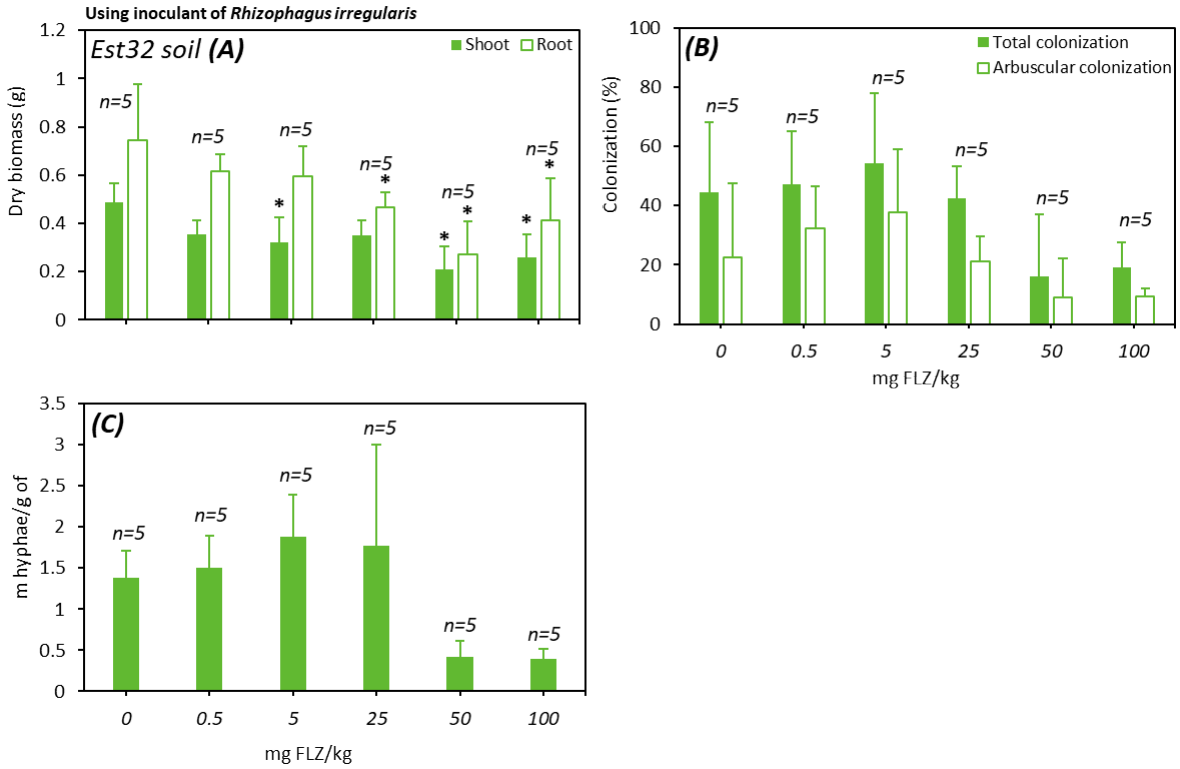
(A) Dry root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e., total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 62: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: UT, Estonia.



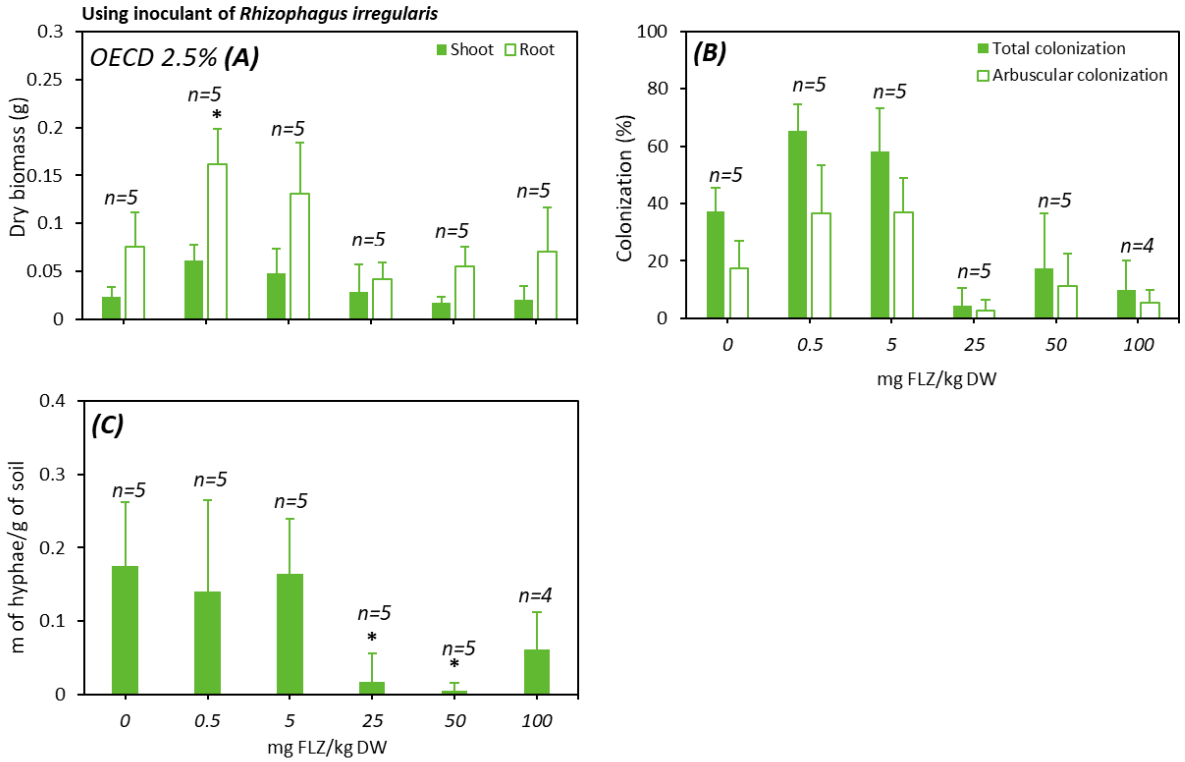
(A) Dry root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e., total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 63: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Est32; Test substance: Fluazinam; Performing lab: UT, Estonia.



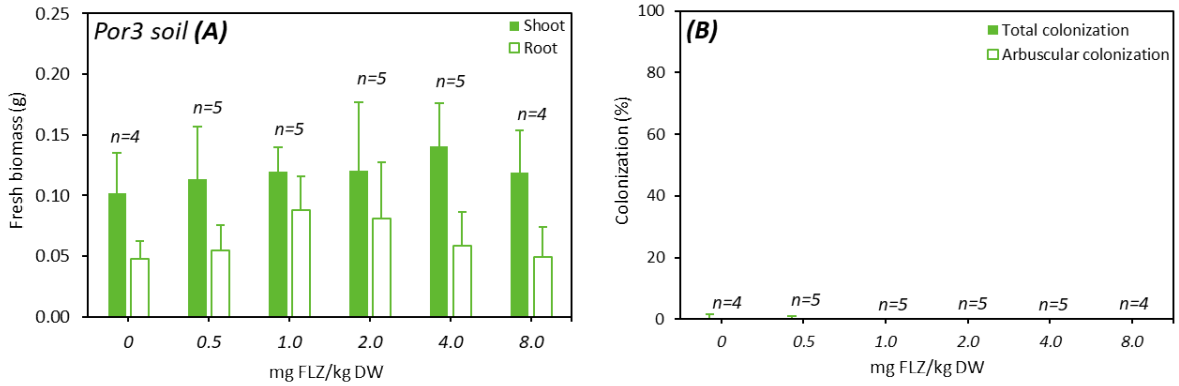
(A) Dry root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e., total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 64: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: UT, Estonia.



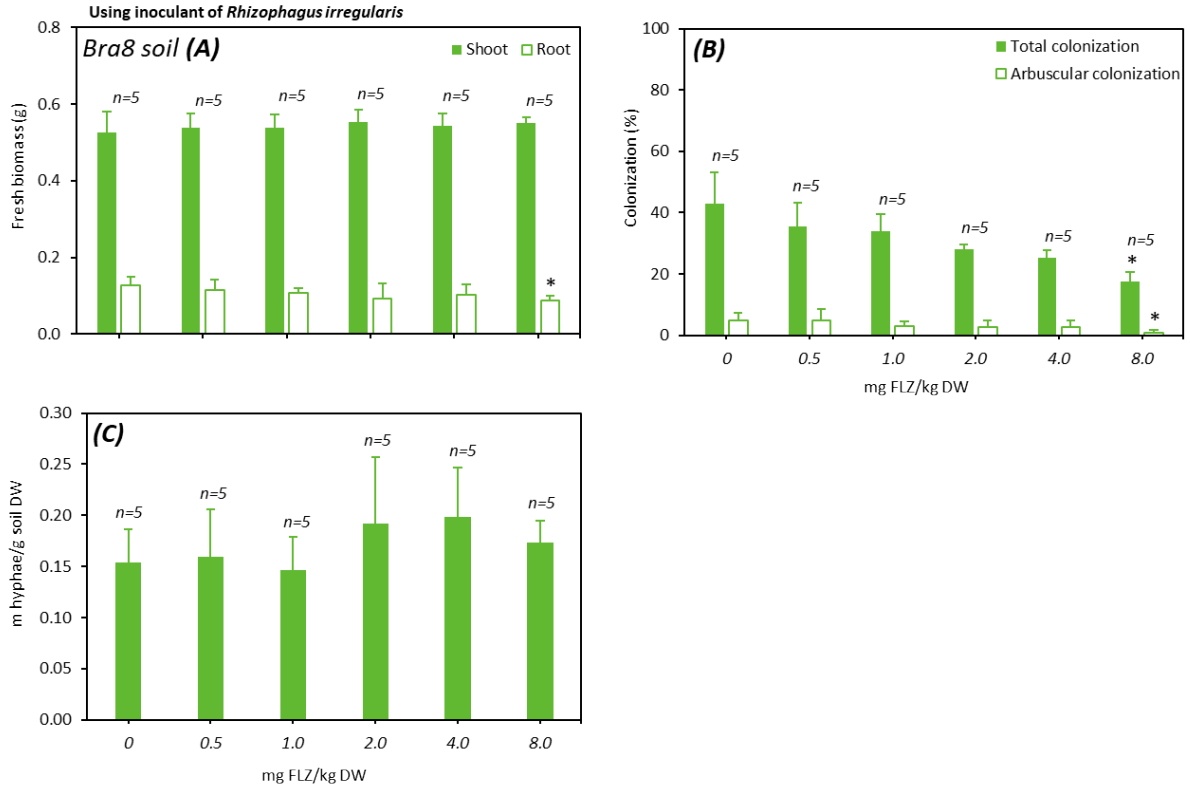
(A) Dry root and shoot biomass (average ± standard deviation), (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e., total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 65: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Por3; Test substance: Fluazinam; Performing lab: UC, Portugal.



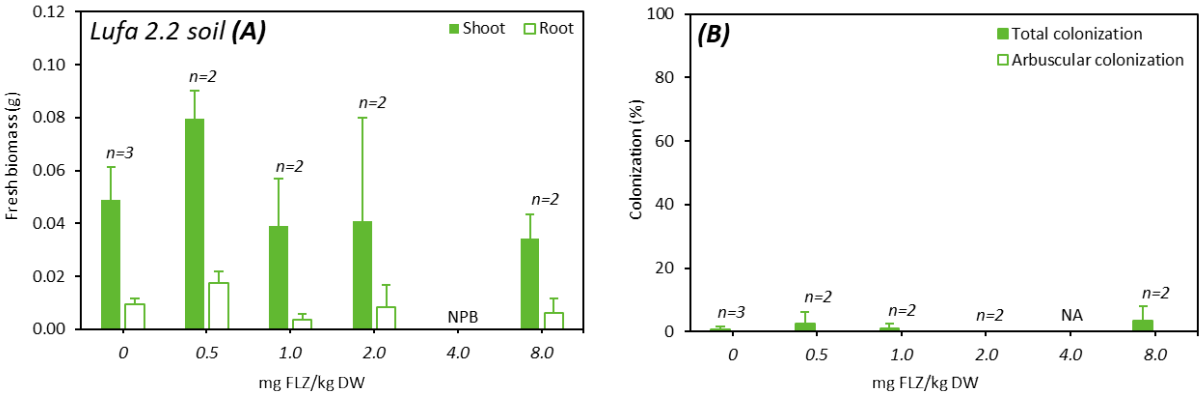
(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 66: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Bra8; Test substance: Fluazinam; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

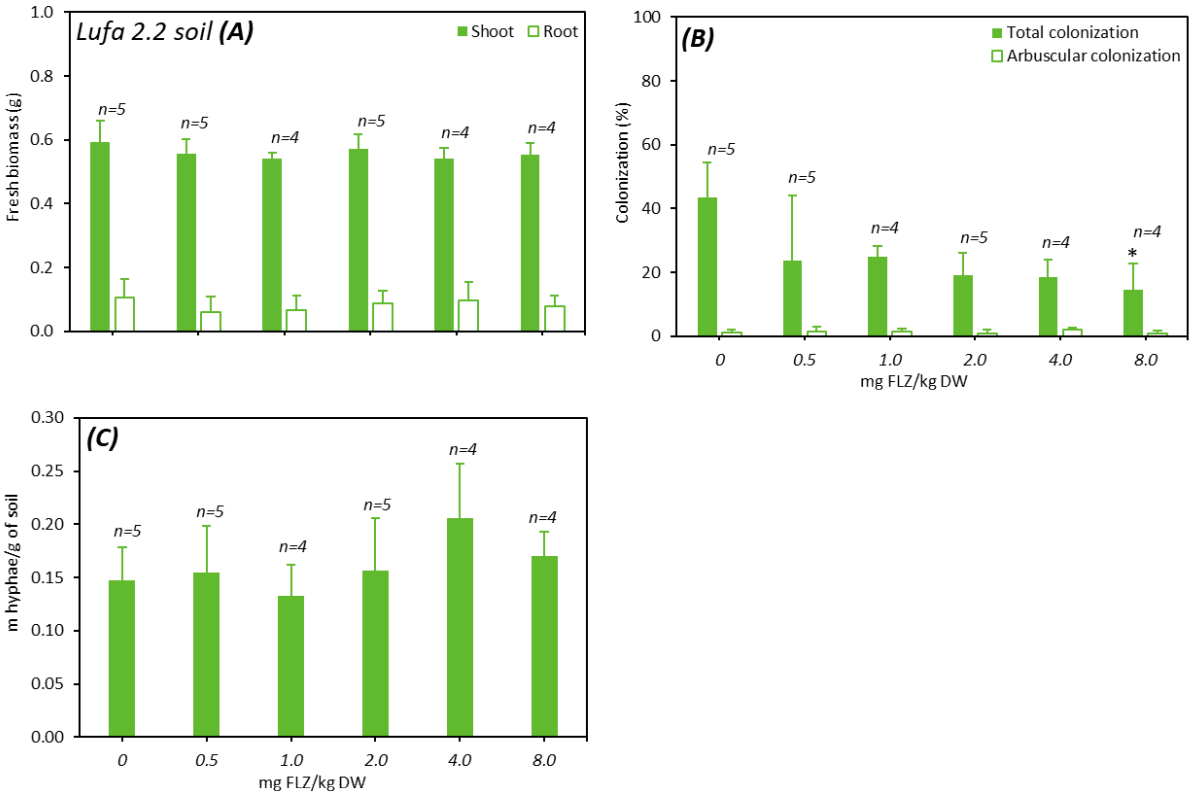
Figure 67: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Lufa 2.2; Test substance: Fluazinam; Performing lab: UC, Portugal.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (i.e. total colonization < 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.

Source: own illustration, CloverStrategy.

Figure 68: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Lufa 2.2; Test substance: Fluazinam; Performing lab: UDESC, Brazil.



(A) Fresh root and shoot biomass (average ± standard deviation) and (B) total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

As for the experiments with Azoxystrobin, seeds germination of leek in the experiments with Lufa 2.2 soil was very low in the experiments carried out in UC (Figure 67) and UT (*data not shown*). Because of that, after the 2-week time for seeds germination (as established in the SOP Test 2) replicates were resown. After the second 2-week period for resown seeds germination, in the experiment carried out in UT, there were only 2 replicates with germinated seeds in controls and because of that, the test was discarded, and no data was generated. In the experiment carried out in UC, some of the germinated plants ended up by dying during the exposure/test period, which led to the loss of some replicates. Seeds germination in the UDESC experiment with Lufa 2.2 (Figure 68) was also initially low, however, it was sufficient to not need to resow the leek.

Chemical analyses were performed to determine the actual contamination of Fluazinam in soil collected at the beginning of the experiments, following the same methodology described for the chemical determination of the previous test substances (Perestrelo et al., 2019; see section 2.6.1.3.) (Table 39).

Fluazinam standard by Dr Ehrenstorfer (CAS 79622-59-6; purity 98.7%), was used to assess the quality of the analysis. The percentage of recovery of Fluazinam was 102% and the quantification limit was 0.005 mg FLZ/kg.

Due to a problem in the freezer where the soil samples were stored, no chemical analyses could be carried out for the experiments with conducted in UT.

Table 39: Nominal and initial measured concentrations of Fluazinam (in mg FLZ/kg of soil DW), and the respective percentage of nominal concentrations measured in soil samples collected from AMF symbiotic phase tests using inoculants of *R. irregularis* and test soils Lufa 2.2 and Bra8, carried out in UDESC (Brazil).

AMF species	Soil	Lab	Nominal concentration (mg FLZ/kg of soil DW)	Initial measured concentration (mg FLZ/kg of soil DW)	% nominal concentration
R. irregularis	Bra8	UDESC	0	< 0.005	NA
			0.5	0.086	17.2
			1	0.76	76.0
			2	2.3	115.0
			4	2.5	62.5
			8	8.5	106.25
R. irregularis	Lufa 2.2	UDESC	0	< 0.005	NA
			0.5	0.30	60.0
			1	0.68	68.0
			2	1.7	85.0
			4	3.8	95.0
			8	5.6	70.0

NA – not applicable.

2.6.1.6.1 Conclusions of the experiments with Fluazinam

Validity of experiments with Fluazinam (considering total colonization on the control treatment ≥ 30% validity criterion) and the respective estimated endpoints for each parameter are summarized in Table 40.

Experiments with Est32 and OECD 2.5% soils that were carried out for both inoculants with *R. irregularis* and *F. mosseae*, evidenced a higher sensitivity of *F. mosseae* to Fluazinam compared to *R. irregularis* for the three AMF parameters TC, AC and EHL. Considering all experiments, the total intra-radicular colonization was the most sensitive AMF parameter, in general.

Despite the lack of toxicity data of Fluazinam from literature to AMF, toxicity data of this chemical to other non-target in-soil organisms have been reported from previous studies

(Appendix A.23). These data suggest that AMF species can be more sensitive to Fluazinam than other non-target in-soil species such as *Eisenia* sp.*, Folsomia candida* and cucumber in artificial soil (EFSA, 2008).

In general, the experiments carried out with Fluazinam showed that the establishment of the AMF-plant symbiosis decreases with the increase of Fluazinam concentration in soil. Therefore, Fluazinam seems to be a suitable substance to be used as reference substance in AMF symbiotic phase tests.

Table 40: **Summary of the experiments performed by the consortium laboratories with Fluazinam using two AMF species (*Rhizophagus irregularis* and *Funneliformis mosseae*) in artificial soil (OECD 2.5%) and natural soils (#Lufa 2.1, Por3, Est32, Lufa 2.2 and Bra8). No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction (and the respective 95% confidence intervals) in total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated in valid experiments only (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg FLZ/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg FLZ/kg DW)	Concentrations with plant biomass (mg FLZ/kg DW)	Lab	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	Est32	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UT	Valid	TC	5	25	0.6 (0.3-0.9)	3.9 (2.1-5.7)
						AC	5	25	0.5 (0.2-0.8)	3.5 (1.5-5.5)
						EHL	0.5	5	ND	ND
F. mosseae	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UT	Valid	TC	5	25	0.3 (0.1-0.5)	1.8 (0.6-3.1)
						AC	100	> 100	0.3 (0.1-0.6)	2.0 (0.4-3.7)
						EHL	100	> 100	0.0001*	0.0007*
R. irregularis	Est32	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UT	Valid	TC	100	> 100	23.6 (2.6-44.6)	53.7 (13.1-94.3)
						AC	100	> 100	20.2*	58.5*
						EHL	100	> 100	35.5*	44.8 (27.1-62.6)

AMF species	Soil	Concentrations (mg FLZ/kg DW)	Concentrations with plant biomass (mg FLZ/kg DW)	Lab	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
R. irregularis	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5; 5; 25; 50; 100	UT	Valid	TC	< 0.5	0.5	9.7 (4.6-14.7)	18.8 (4.8-32.8)
						AC	< 0.5	0.5	13.6 (1.1-26.1)	25.5*
						EHL	5	25	10.9*	16.7*
R. irregularis	Por3	0; 0.5; 1; 2; 4; 8	0; 0.5; 1; 2; 4; 8	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
R. irregularis	Lufa 2.2	0; 0.5; 1; 2; 4; 8	0; 0.5; 1; 2; 8	UC	Invalid	TC	-	-	-	-
						AC	-	-	-	-
R. irregularis	Bra8	0; 0.5; 1; 2; 4; 8	0; 0.5; 1; 2; 4; 8	UDESC	Valid	TC	4.0	8.0	0.3*	5.5 (2.4-8.6)
						AC	4.0	8.0	0.5 (0.03-1.0)	3.2 (0.2-6.3)
						EHL	8.0	> 8.0	0.1*	1.0*
R. irregularis	Lufa 2.2	0; 0.5; 1; 2; 4; 8	0; 0.5; 1; 2; 4; 8	UDESC	Valid	TC	4.0	8.0	0.05*	0.3*
						AC	8.0	> 8.0	7.6 (2.1-13.2)	> 8.0
						EHL	8.0	> 8.0	0.27*	1.78*

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.6.1.7 Experiments with Pendimethalin (PDM)

Pendimethalin is a preemergent herbicide widely used in agriculture to inhibit germination of annual grasses and weeds (Belden et al. 2005). Physical and chemical properties of Pendimethalin are summarised in Table 41.

Table 41: Physical and chemical properties of Pendimethalin.

Pendimethalin	
Pesticide type	Herbicide
Substance group	Dinitroaniline herbicide
IUPAC name	N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine
Empirical formula	$C_{13}H_{19}N_3O_4$
CAS number	40487-42-1
Molecular mass	281.31 g/mol
Melting point (°C)	56
Solubility (water. 20°C)	0.33 mg/L
Dissociation constant (pKa) (25°C)	2.8
Log Kow (20°C)	5.4
Henry's Law constant	1.27 Pa/m³/mol
Degradation point (°C)	246
Degradation in soil (aerobic)	
DT_{50} (lab at 20°C)	182.3 days
DT_{50} (field)	100.6 days

Data retrieved from PubChem (https://pubchem.ncbi.nlm.nih.gov/) and PPDB (https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm).

Four experiments exposing the AMFs *R. clarus* and *F. mosseae* to Pendimethalin in OECD 2.5% artificial soil and Est32 natural soil were carried in laboratory of UT (Estonia) (Table 42).

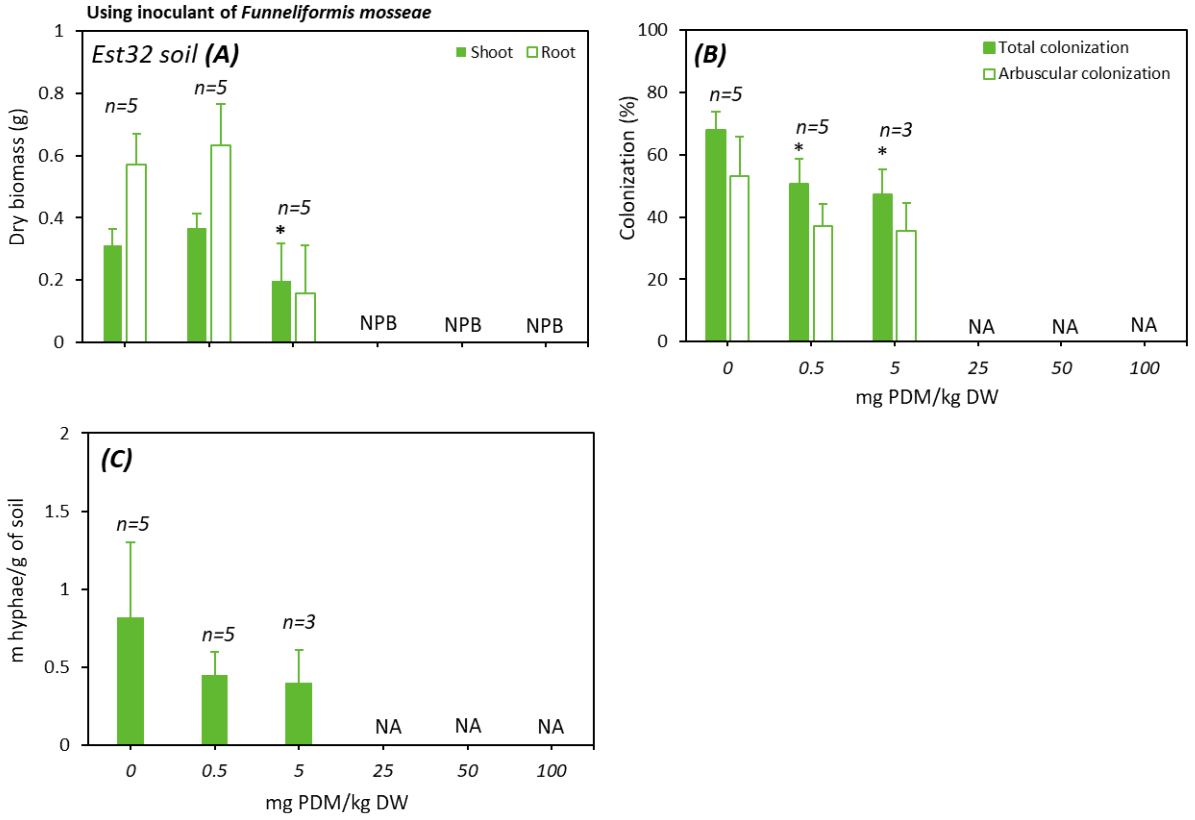
Table 42: Experiments performed by UT (Estonia) with Pendimethalin using inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* in OECD 2.5% and Est32 soils.

		OECD 2.5%	**Est32**
Pendimethalin	*F. mosseae*	UT	UT
	R. irregularis	UT	UT

The experiments were performed according to the procedures described in the SOP Test 2 (Appendix A.18). The concentration gradient defined for the experiments was composed of the following concentrations: 0, 0.5, 5, 25, 50, 100 mg PDM/kg of soil. Test concentrations were selected with the objective to find significant effects in the establishment of the AMF-plant symbiosis. Therefore, the concentration gradient selected for the experiments did not aim to cover realistic application doses of the test substance.

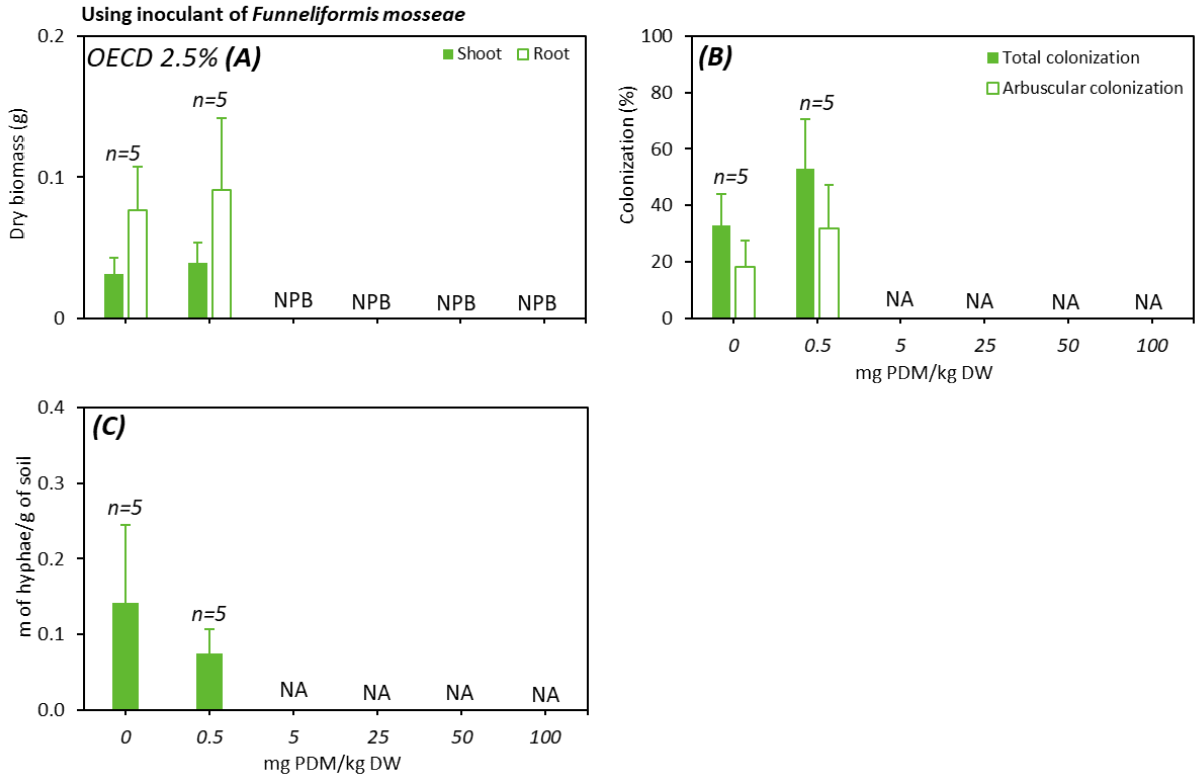
Results obtained in the experiments with Pendimethalin are presented in graphs of Figures 69 to 72 and raw data are presented in Appendix A.19.

Figure 69: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: Est32; Test substance: Pendimethalin; Performing lab: UT, Estonia.



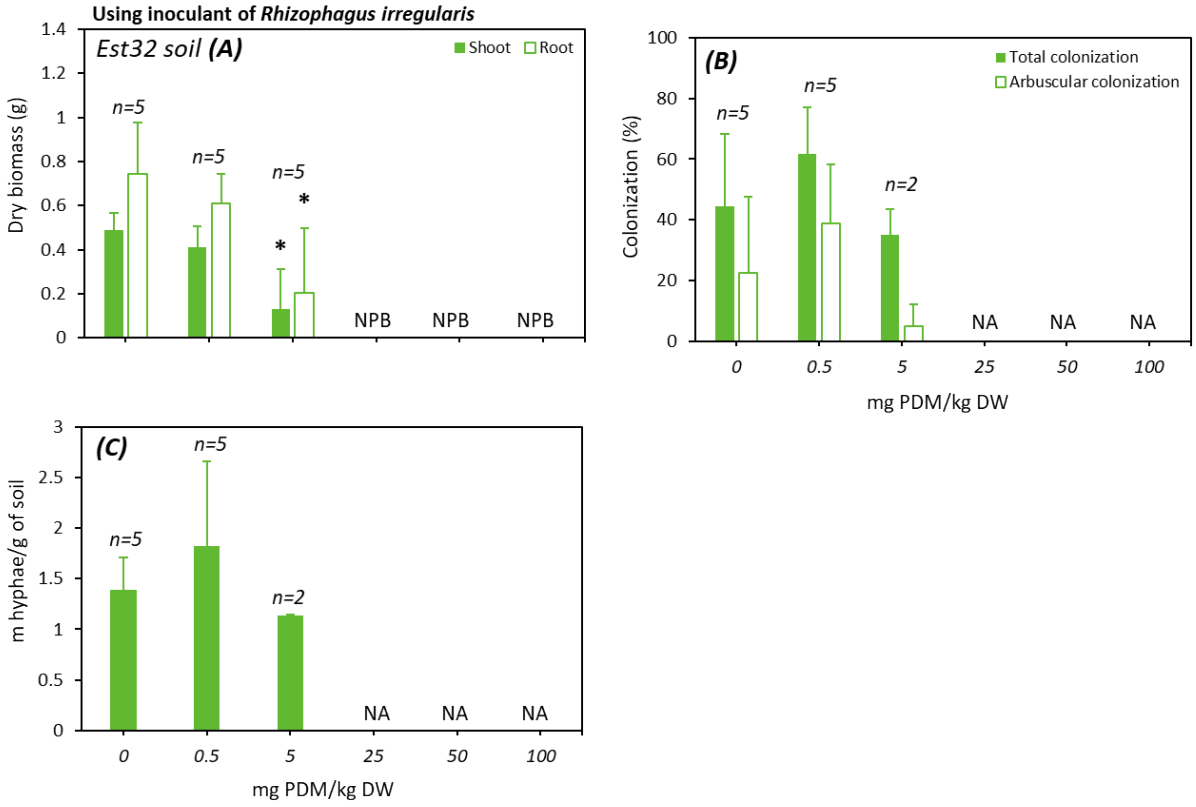
(A) Dry root and shoot biomass (average ± standard deviation). (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 70: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Funneliformis mosseae*; Test soil: OECD 2.5%; Test substance: Pendimethalin; Performing lab: UT, Estonia.



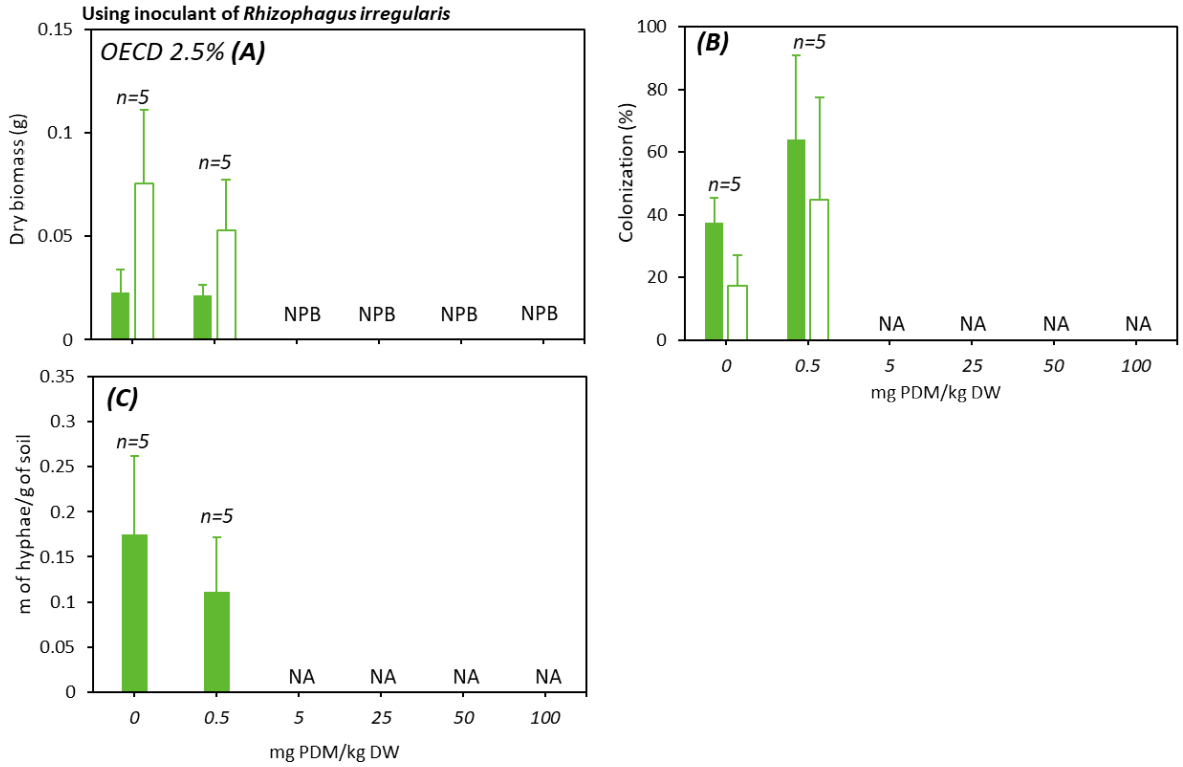
(A) Dry root and shoot biomass (average ± standard deviation). (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 71: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: Est32; Test substance: Pendimethalin; Performing lab: UT, Estonia.



(A) Dry root and shoot biomass (average ± standard deviation). (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - significantly lower compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. NPB – no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Figure 72: Host plant: leek (*Allium ampeloprasum*); Inoculant: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Pendimethalin; Performing lab: UT, Estonia.



(A) Dry root and shoot biomass (average ± standard deviation). (B) total and arbuscular colonization (average ± standard deviation) and (C) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (i.e. total colonization ≥ 30%). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; NPB – means no plant biomass produced; NA – not assessed.
Source: own illustration, CloverStrategy.

Due to a problem in the freezer where the soil samples collected at the beginning of the experiments were stored, no chemical analyses could be carried out for the experiments with Pendimethalin conducted in UT (Estonia).

2.6.1.7.1 Conclusions of the experiments with Pendimethalin

Endpoints estimated from the results obtained in the experiments performed with Pendimethalin, as well as the compliance of each experiment with the validity criterion (i.e., total colonization in the control treatment ≥ 30%) are summarized in Table 43.

EC10 and EC50 values estimated in experiments with Est32 were based on AMF parameters measured in concentrations up to 5 mg PDM/kg, which means that the lack of points in the dose-response curve (only two points plus control in total) makes these values weakly supported. EC10 and EC50 values could not be estimated in experiments with OECD 2.5% artificial soil because there was no plant biomass production at concentrations higher than the lowest tested concentration (i.e. 0.5 mg PDM/kg). In treatments with plant biomass production, the sensitivity in the establishment of the AMF-plant symbiosis, in general, did not significantly decrease compared to the control. The exception was the TC of *F. mosseae* in tests with Est32 soil, where

both 0.5 and 5 kg/kg treatments showed significantly lower TC than the control, although the AMF TC in these treatments was still relatively high (around 50%). Results obtained in most experiments agree with the data reported by Giovannetti et al. (2006) who found no significant effects of Pendimethalin in spores' germination of *Glomus mosseae* (IMA1), despite the decreasing dose-response trend observed in growth of extra-radicular mycelium. During the experiments conducted in the ERAMYC project, both AMF species were sensitive to Pendimethalin, with the AMF *F. mosseae* showing more sensitivity to this chemical than *R. irregularis*.

Despite the reduced number of treatments with plant biomass supporting the estimated endpoints, it is worth comparing the estimated endpoints with the toxic values reported in the literature. The endpoints estimated are generally lower than those from literature (Appendix A.23), excepting the results obtained by Sanyal et al. (2015), who estimated LC50 values ranging from 0.016 to 0.018 mg PDM/kg of soil for the earthworm *Perionyx excavatus* after exposure to Pendimethalin in a natural soil and OECD 10% artificial soil, respectively. Another exception is found for the endpoints estimated in the present project to *R. irregularis* that are in the same order of magnitude of the literature data for the non-target terrestrial plant *Lactuca sativa* (IC50 for 7 days seed growth= 7.74 (5.67-10.57) mg PDM/kg of soil).

Results obtained suggest that Pendimethalin is not suitable to be used as reference substance or as test substance for the ring test of AMF symbiotic phase tests, as relatively high AMF colonization (i.e. TC around or higher than 50%) was always found in treatments with plant biomass production. The trends observed in the experiments with PDM lead to suppose that, even refining the concentration gradients, the total inhibition of plant biomass production would occur at concentrations not high enough to allow obtaining a clear dose-response for the establishment of AMF-plant symbiosis.

Table 43: **Summary of the experiments performed by UT (Estonia) with Pendimethalin using two AMF species (*Rhizophagus irregularis* and *Funneliformis mosseae*) in artificial soil OECD 2.5% and natural soils Est32. No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) reduction (and the respective 95% confidence intervals) in total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated in valid experiments only (effects on plant biomass were not estimated) using nominal concentrations. Results are expressed in mg PDM/kg of soil dry weight. Non-linear models applied and the respective r^2 values are presented in Appendix A.20.**

AMF species	Soil	Concentrations (mg PDM/kg DW)	Concentrations with plant biomass (mg PDM/kg DW)	Lab	State (valid/invalid)	Parameters	NOEC	LOEC	EC10	EC50
F. mosseae	Est32	0; 0.5; 5; 25; 50; 100	0; 0.5; 5	UT	Valid	TC	> 0.5	0.5	0.03*	0.2*
						AC	5	> 5	1.7*	> 5
						EHL	5	> 5	ND	ND
F. mosseae	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5	UT	Valid	TC	0.5	> 0.5	ND	ND
						AC	0.5	> 0.5	ND	ND
						EHL	0.5	> 0.5	ND	ND
R. irregularis	Est32	0; 0.5; 5; 25; 50; 100	0; 0.5; 5	UT	Valid	TC	5	> 5	4.4*	> 5
						AC	5	> 5	1.4*	1.8*
						EHL	5	> 5	4.2*	> 5
R. irregularis	OECD 2.5%	0; 0.5; 5; 25; 50; 100	0; 0.5	UT	Valid	TC	0.5	> 0.5	ND	ND
						AC	0.5	> 0.5	ND	ND
						EHL	0.5	> 0.5	ND	ND

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

2.7 Conclusions and discussion of the results obtained on WP2

2.7.1 Selection of a reference substance for AMF testing

Forty experiments were carried out in the 2nd phase of the WP2 to evaluate the suitability of seven chemicals (Boric acid, Copper sulphate, Tebuconazole, Terbuthylazine, Azoxystrobin, Fluazinam and Pendimethalin) to be used as reference substance in the AMF symbiotic phase tests (Table 44). These experiments were performed in the three consortium laboratories using three AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis* and *Rhizophagus clarus*), one host plant (*Allium ampeloprasum*) and six test soils (OECD 2.5% artificial soil and Bra8, Lufa 2.2, Est32, Por3 and #Lufa 2.1 natural soils). The selection of two test substances to be used as reference substance in the ring tests to be performed on WP3 were based on the results of these experiments.

Table 44: Suitability of seven chemicals to be used as reference substance for the ring test experiments, based on the results of experiments performed in the 2nd phase of WP2 of the ERAMYC project using three AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis* and *Rhizophagus clarus*), one artificial soil and five natural soils (OECD 2.5% artificial soil and Bra8, Lufa 2.2, Est32, Por3 and #Lufa 2.1 natural soils). Tests were performed by the consortium laboratories (UC, UT and UDESC). S – suitable to be used as reference substance; NS – not suitable to be used as reference substance; NS/S – further tests required for suitability confirmation.

Chemical	AMF species	OECD 2.5%	Bra8	Lufa 2.2	Est32	Por3	#Lufa 2.1
Boric acid	*F. mosseae*	NS (UDESC)	NS (UDESC)	-	-	NS (UC)	NS (UC)
	R. clarus	-	-	-	-	-	-
	R. irregularis	NS (UT)	-	-	-	NS (UC)	NS (UC)
Copper sulphate	*F. mosseae*	NS/S (UDESC)	-	-	-	-	-
	R. clarus	-	-	-	-	-	-
	R. irregularis	NS/S (UDESC)	-	-	-	-	-
Terbuthylazine	*F. mosseae*	NS (UDESC)	NS (UDESC)	-	-	-	-
	R. clarus	NS	NS	-	-	-	-

Chemical	AMF species	OECD 2.5%	Bra8	Lufa 2.2	Est32	Por3	#Lufa 2.1
		(UDESC)	(UDESC)				
	R. irregularis	-	-	-	-	-	-
Tebuconazole	*F. mosseae*	NS (UDESC)	NS (UDESC)	-	-	-	-
	R. clarus	S (UDESC)	NS (UDESC)	-	-	-	-
	R. irregularis	-	-	-	-	-	-
Fluazinam	*F. mosseae*	NS (UT)	-	-	S (UT)	-	-
	R. clarus	-	-	-	-	-	-
	R. irregularis	S (UT)	S (UDESC)	S (UDESC)	S (UT)	NS (UC)	-
Azoxystrobin	*F. mosseae*	NS (UC)	-	-	-	-	NS (UC)
	R. clarus	-	-	-	-	-	-
	R. irregularis	S (UC)	S (UDESC)	S (UDESC)	NS (UC)	NS (UC)	NS (UC)
Pendimethalin	*F. mosseae*	NS (UT)	-	-	NS (UT)	-	-
	R. clarus	-	-	-	-	-	-
	R. irregularis	NS (UT)	-	-	NS (UT)	-	-

Boric acid is not suitable to be used as reference substance to the AMF symbiotic phase tests as the decrease of AMF colonization observed in the experiments (with the two AMF species) seems related to the toxic effects of the substance in leek (i.e., decreased biomass) for the four tested soils (OECD 2.5%, Por3, #Lufa 2.1 and Bra8). Similarly, the toxicity of Pendimethalin and Terbuthylazine was also higher in the host plant (leek) than in the establishment of the AMF-plant symbiosis with *F. mosseae* and *R. irregularis*, in OECD 2.5%, Bra8 and Est32 soils. Therefore, Pendimethalin and Terbuthylazine were not considered for the selection of a reference substance.

Copper sulphate was inconclusive regarding its suitability to be used as reference substance, due to the unclear relationship between the concentrations of Copper sulphate and the effects on plant biomass production and root AMF colonization of *F. mosseae*. Toxic effects on the host plant appeared to be higher than the effects on AMF colonization on experiments with *R. irregularis*, but this could be due to the concentration gradient used, possibly too large between test concentrations. Therefore, further AMF symbiotic phase tests using refined concentration gradients are advised to better clarify this relationship.

Thus, the main candidates to be used as reference substance were the fungicides Tebuconazole, Azoxystrobin and Fluazinam.

Tebuconazole seemed a suitable test substance but only when using *R. clarus* inoculant in OECD 2.5% soil, as the experiments with *R. clarus* in Bra8 soil and the experiments with *F. mosseae* did not evidence a coherent dose-response relationship between the Tebuconazole increasing concentrations and the AMF root colonization.

Azoxystrobin showed toxic effects in experiments with *R. irregularis* inoculant, regardless the test soil used. Clear dose-response effects (especially regarding total colonization) on the AMF parameters were observed on experiments with this species to contaminated OECD 2.5%, Bra8 and Lufa 2.2 soils. Furthermore, the effects observed seem to be more connected to the toxicity of Azoxystrobin on AMF root colonization than to effects in the host plant biomass production.

Fluazinam also showed higher toxicity to AMF symbiotic parameters (e.g. root colonization) than to leek growth. This was particularly evident in experiments with *F. mosseae* inoculant in Est32 and in the experiments with *R. irregularis* inoculant in OECD 2.5%, Est32, Bra8 and Lufa 2.2 soils.

Finally, the substances Azoxystrobin and Fluazinam were selected as test substances for WP3 since these were the substances that showed clearer effects in the experiments conducted in WP2 (for most AMF species and tested soils).

2.7.2 Selection of an AMF species for WP3 testing

The three AMF species used on the experiments conducted during the 2nd phase of WP2 (*Rhizophagus irregularis*, *Rhizophagus clarus* and *Funneliformis mosseae)* led to a high number of valid experiments, showing that these species are suitable for the performance of AMF symbiotic phase tests.

The relative sensitivity of the three AMF species was evaluated for each test substance by comparing the endpoints obtained with each species on experiments using the same test soil (Table 45).

Table 45: Relative sensitivity of three AMF species to the different test substances. Only clusters of experiments conducted with different AMF species and using the same test soil were considered.

Test substance	Test soil	Upward sensitivity of AMF species
Boric acid	OECD 2.5%	*F. mosseae* > *R. irregularis*
$CuSO_4$	OECD 2.5%	*R. irregularis* ⇆ *F. mosseae*
Tebuconazole	Bra8	*F. mosseae* > *R. clarus*
Terbuthylazine	Bra8	*R. clarus* > *F. mosseae*
Terbuthylazine	OECD 2.5%	*F. mosseae* ⇆ *R. clarus*
Azoxystrobin	#Lufa 2.1	*R. irregularis* ⇆ *F. mosseae*
Azoxystrobin	OECD 2.5%	*R. irregularis* ⇆ *F. mosseae*
Fluazinam	Est32	*F. mosseae* > *R. irregularis*
Fluazinam	OECD 2.5%	*R. irregularis* ⇆ *F. mosseae*
Pendimethalin	Est32	*R. irregularis* ⇆ *F. mosseae*
Pendimethalin	OECD 2.5%	*R. irregularis* = *F. mosseae*

⇆ - sensitivity higher or lower depending on the parameter (whether TC, AC or EHL is considered) and/or the estimated endpoint (whether NOEC, LOEC, EC10 or EC50 is considered)

Given the similar sensitivity of *R. irregularis* and *F. mosseae* for most of the test substances, the consortium selected *R. irregularis* as the species to be used on the experiments from WP3. The decision was supported by the good performance of this species on the experiments from WP2 (especially in the experiments conducted with Azoxystrobin and Fluazinam, the test substances selected for the ring test) and by the availability of sufficient *R. irregularis* inoculant from a culture bank (Agroscope, Switzerland). This ensured the performance of WP3 experiments, without the need for inoculant propagation by the consortium, which would be very time-consuming and unfeasible within the project timeline.

2.7.3 Influence of soil properties on AMF colonization and on the sensitivity of AMF to the presence of chemicals in soil

2.7.3.1 Statistical analysis

Predictive Generalised Linear Models (GLMs) were conducted to assess the influence of soil properties on AMF colonization and on the sensitivity of AMF to chemicals in soil.

For assessing the influence of soil properties on the establishment of the AMF-plant symbiosis, an independent GLM was applied for each test parameter (TC, AC and EHL), considering the respective average measurements in negative controls of experiments from both phases of WP2 with *F. mosseae* and *R. irregularis* (as these were the AMF species used by all project partners) and leek (as this was the host plant used in most of the 1st phase experiments and in all of the 2nd phase experiments of WP2) (i.e. a total of 32 valid tests) as response variable and soil pH, organic matter content, cation exchange capacity, sand, silt and clay contents, aluminium exchangeable concentration and phosphorus, iron and manganese extractable concentrations as continuous explanatory variables. Additionally, the influence of AMF species, and laboratories were evaluated, considering these factors as nominal explanatory variables (variables and data considered in the GLMs are presented in Table 46 and Appendix A.21, respectively).

For assessing the influence of soil properties on the sensitivity of AMF colonization to the presence of chemicals, a GLM independent for each endpoint (estimated EC10 and EC50 values based on nominal test concentrations) and test parameter (i.e. TC, AC and EHL) was applied using the respective estimated endpoints of valid tests of the 2nd phase of WP2 using *R. irregularis* and Azoxystrobin and Fluazinam (as this AMF species and these substances were tested by all project partners) as response variables (i.e. data from a total of 8 valid tests), soil pH, organic matter content, cation exchange capacity, sand, silt and clay contents, aluminium exchangeable concentration and phosphorus, iron and manganese extractable concentrations as continuous explanatory variables, and test substance and laboratory as nominal explanatory variables (Table 49). Data considered in GLMs to evaluate the influence of soil properties in AMF sensitivity to chemicals are presented in Appendix A.22.

Previous to the GLM analysis, the presence of outliers was graphically checked through Boxplot and Dotplot methods. Data exploration also included elimination of collinear variables (i.e. variables with variance inflation factors ≥ 5) and/or highly correlated variables (i.e. Pairplots of variables with $r \geq 0.8$) and looking for possible interactions between variables by visual evidence on Coplot graphs. Binomial distribution GLMs were adopted, using the logit link function when the response variable was a percentage (i.e. when using TC and AC measurements as response variable, values were divided by 100 to scale them between 0 and 1) while Gamma distribution GLMs were adopted, using inverse link function when using continuous data (i.e. when EHL measurements or TC, AC or EHL endpoints). Best models were selected based on the lowest overdispersion and Arkaic information criterion (AIC) values. GLMs were applied in Brodgar 2.7.5 (www.brodgar.com).

2.7.3.2 AMF colonization *vs* Soil properties

Data exploration did not evidence any outliers (outliers were graphically checked as explained above) and no interactions were found between explanatory variables. Variables considered in the analysis are summarized in Table 46.

Table 46: Summary of the variables considered on the GLM analysis regarding AMF colonization vs Soil properties.

AMF colonization vs Soil properties		
Response variables	**Continous explanatory variables**	**Nominal explanatory variables**
Average TC, AC and EHL on experiments with *F. mosseae* and *R. irregularis* with host plant leek	Soil pH Organic matter content Cation exchange capacity Sand, silt and clay contents Aluminium exchangeable concentration Phosphorous extractable concentration Iron extractable concentration Manganese extractable concentration	AMF species Laboratory

There were strong correlations (i.e. correlations with $r \geq 0.80$) between continuous explanatory variables. Organic matter content, sand, silt and clay contents, and aluminium exchangeable concentrations and manganese extractable concentrations were highly correlated, while iron extractable concentration was correlated with cation exchange capacity. On the other hand, TC and AC response variables were generally weakly correlated with the explanatory variables, except EHL for soil pH and P extractable concentrations that showed correlations with r of 0.79 and 0.81, respectively (Table 47). Collinearity of variables was also considered for their selection for the models. In GLMs using TC or AC measurements as response variables, the explanatory variables, either continuous or nominal, were not significant (i.e. $p > 0.05$) independently of the combination of the selected variables. In addition to having no significant explanatory variables, GLMs using TC measurements always explained less than 5% of the observed variation while GLMs using AC measurements only explained 15 to 35% of the observed variation. Therefore, TC was the parameter least influenced by the soil properties, AMF species and laboratory, followed by AC. On the other hand, GLMs using EHL measurements as response variable, showed statistical significance when using soil pH, iron extractable concentration, laboratory, cation exchange capacity and phosphorous extractable concentration. From the GLMs with significant explanatory variables, the GLM using only phosphorous presented the highest percentage of explained variation (55.48%), while the GLM with iron was the model that less explained the observed variation (15.80%). GLM using laboratory as explanatory variable was significant only for UC laboratory, explaining 25.27% of the observed variation (Table 48 and Figure 73).

Therefore, data statistical analysis evidenced that TC and AC measurements of *F. mosseae* and *R. irregularis* in unspiked soils are not influenced by soil properties or laboratory. EHL, however, showed to be influenced by soil properties such as iron and phosphorous extractable concentrations, soil pH and cation exchange capacity. Note that soil properties such as organic matter content, sand, silt and clay contents, aluminium exchangeable concentration and manganese extractable concentrations did not show significant influence in EHL measurements. The “Laboratory” variable also had significant influence in the EHL outcomes, as measurements performed in experiments carried out in the UC were significantly different from those measured in UDESC laboratory (Figure 73).

Table 47: **Coefficients of correlations (*r*) between ten continuous explanatory variables (pH – soil KCl pH, OM – % of organic matter content, CEC – cation exchange capacity, Sand - % of sand content, Silt - % of silt content, Clay - % of clay content, Al – exchangeable aluminium concentration, P – phosphorus extractable concentration, Fe – iron extractable concentration, Mn – manganese extractable concentration) and AMF total root colonization (TC), AMF arbuscular colonization (AC) or AMF extra-radicular hyphae length (EHL) measured in symbiotic phase tests (using different unspiked soils) of WP2 of ERAMYC project as response variable. Bold values mean coefficients of correlation ≥ 0.80.**

	pH	OM	CEC	Sand	Silt	Clay	Al	P	Fe	Mn
TC	0.03	0.13	0.18	-0.08	0.13	0.06	0.05	0.15	0.20	0.16
AC	0.03	0.08	0.39	0.06	-0.01	-0.07	-0.18	0.28	0.38	0.10
EHL	0.79	-0.20	0.15	0.26	0.10	-0.40	-0.20	**0.81**	0.43	0.05
	pH	-0.55	-0.02	0.62	-0.23	-0.75	-0.52	0.77	0.23	-0.30
		OM	0.58	**-0.95**	**0.92**	**0.91**	**0.88**	-0.00	0.52	**0.96**
			CEC	-0.31	0.49	0.21	0.18	0.60	**0.95**	0,62
				Sand	**-0.90**	**-0.98**	**-0.97**	0.21	-0.26	**-0.90**
					Silt	0.79	**0.90**	0.22	0.55	**0.98**
						Clay	**0.94**	-0.39	0.12	**0.80**
							Al	-0.21	0.17	**0.85**
								P	0.79	0.24
									Fe	0.64

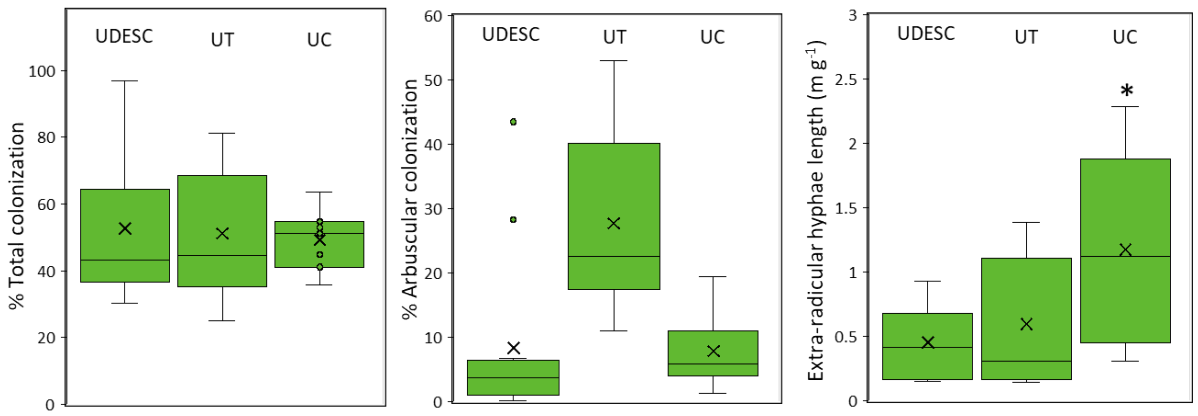
Table 48: **Percentage of explained observed variance (Exp. var.) of regression models (the ones that best explain the observations), response variable and explanatory variables and their respective regression coefficients (Reg. coef.) and *p*-value. EHL - AMF extra-radicular hyphae length; pH - soil pH; CEC - cation exchange capacity; Fe - extractable iron concentration; P – extractable phosphorous concentration; Lab - laboratory (Lab2 – University of Tartu, Lab3 – University of Coimbra).**

Response variable	Explanatory variable	Reg. coef.	*p*-value	Exp. var.
EHL	[1]Fe	-0.0046	0.0385*	15.80%
EHL	[1]pH [1]Fe	-0.5933 -0.0052	0.00004* 0.00622*	55.15%

Response variable	Explanatory variable	Reg. coef.	*p*-value	Exp. var.
EHL	[2](Lab)2	-0.1958	0.76119	25.27%
	[2](Lab)3	-1.3318	0.01802*	
EHL	[1]pH	-0.5664	0.00009*	41.59%
EHL	[1]pH	-0.7310	0.00004*	52.60%
	[1]CEC	-0.1337	0.0133*	
EHL	[1]P	-0.0966	0.00002*	55.48%

[1] – Continuous explanatory variable; [2] – Class of nominal explanatory variable; * - statistically significant value ($p \leq 0.05$)

Figure 73: **Boxplots representing the percentage of total (left graph), arbuscular (center graph) mycorrhiza fungi root colonization and the extra-radicular hyphae length (right graph) measured in AMF symbiotic phase tests using leek as host plant and inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis*, carried out in laboratories of the State University of Stanta Catarina, Brazil (UDESC), University of Tartu, Estonia (UT) and University of Coimbra, Portugal (UC) in the WP2 of the ERAMYC project.**



* - Statistically significant different ($p \leq 0.05$) compared to UDESC (evidenced in a GLM described in Table 48).
Source: own illustration, CloverStrategy.

2.7.3.3 AMF sensitivity *vs* Soil properties

No outliers were found in the used datasets of TC, AC or EHL endpoints (outliers were graphically checked as explained above) for data exploration. Variables considered in the analysis are summarized in Table 49.

Table 49: Summary of the variables considered on the GLM analysis regarding AMF sensitivity vs Soil properties.

AMF sensitivity vs Soil properties		
Response variables	**Continous explanatory variables**	**Nominal explanatory variables**
EC10 and EC50 of TC, AC and EHL from experiments using *R. irregularis* with Azoxystrobin and Fluazinam	Soil pH Organic matter content Cation exchange capacity Sand, silt and clay contents Aluminium exchangeable concentration Phosphorous extractable concentration Iron extractable concentration Manganese extractable concentration	Test substance Laboratory

The selection of the explanatory variables to be included in the models was conditioned by the fact that there were strongly correlated continuous explanatory variables (i.e. correlations with $r \geq 0.80$). These correlations showed that organic matter content, sand, silt and clay contents and aluminium exchangeable concentrations and manganese extractable concentrations were highly autocorrelated, while cation exchange capacity was correlated with iron extractable concentration and soil pH was correlated to phosphorous extractable concentrations (Table 50).

No statistically significant explanatory variables were found in GLMs using the estimated endpoins for the three parameters considered (i.e. EC10 or EC50 of TC, AC and EHL) as response variable, except for a GLM with "Laboratory" as explanatory variable for EC10 values based on TC measurements, where this variable showed statistical significance for data from experiments carried out in UT laboratory (Figure 74), and explained 90.81% of the observed variance (Table 51). Since the data generated in UT laboratory is composed of only two tests (n=2), additional tests would be needed to confirm the influence of laboratory in the AMF sensitivity outcomes based on TC measurements.

Although GLMs generally did not evidence statistically significant explanatory variables, they explained a higher percentage of the observed variance when considering the "Laboratory" and "Test substance" variables for all parameters. Among them, the GLMs for TC explained the highest percentages, followed by the GLMs for AC, while the GLMs for EHL exhibited the lowest percentages of explained variance. The absence of statistical significance for the "Test substance" variable in the GLMs suggests that a dataset composed of endpoints estimated for a larger number of substances is needed to enhance the robustness of this analysis.

Table 50: Coefficients of correlations (*r*) between ten continuous explanatory variables (pH – soil KCl pH, OM – % of organic matter content, CEC – cation exchange capacity, Sand - % of sand content, Silt - % of silt content, Clay - % of clay content, Al – exchangeable aluminium concentration, P – phosphorus extractable concentration, Fe – iron extractable concentration, Mn – manganese extractable concentration) and EC10 and EC50 values of AMF total root colonization (TC_EC10 and TC_EC50, respectively), AMF arbuscular colonization (AC_EC10 and AC_EC50, respectively) and AMF extra-radicular hyphae length (EHL_EC10 and EHL_EC50, respectively)

estimated in symbiotic phase tests of WP2 of ERAMYC project as response variable. Bold values mean coefficients of correlation ≥ 0.80.

	pH	OM	CEC	Sand	Silt	Clay	Al	P	Fe	Mn
TC_EC10	0.08	-0.00	0.31	0.11	-0.10	-0.10	-0.30	0.37	0.43	0.06
TC_EC50	0.06	0.05	0.33	0.05	-0.04	-0.06	-0.25	0.39	0.47	0.13
AC_EC10	0.02	-0.18	0.25	0.28	-0.34	-0.24	-0.45	0.20	0.25	-0.18
AC_EC50	0.06	-0.03	0.36	0.15	-0.16	-0.15	-0.34	0.36	0.44	0.01
EHL_EC10	**0.95**	-0.48	-0.21	0.42	-0.14	-0.51	-0.38	**0.81**	0.14	-0.19
EHL_EC50	**0.95**	-0.52	-0.21	0.47	-0.20	-0.56	-0.45	**0.81**	0.13	-0.24
	pH	-0.68	-0.14	0.66	-0.39	-0.74	-0.60	**0.80**	0.12	-0.45
		OM	0.30	**-0.95**	**0.92**	**0.93**	**0.92**	-0.25	0.36	**0.94**
			CEC	0.00	0.12	-0.05	-0.01	0.38	**0.89**	0.16
				Sand	**-0.93**	**-0.99**	**-0.98**	0.37	-0.10	**-0.94**
					Silt	**0.88**	**0.94**	-0.04	0.32	**0.98**
						Clay	**0.95**	-0.49	0.01	**0.90**
							Al	-0.36	0.07	**0.91**
								P	0.67	-0.05
									Fe	0.36

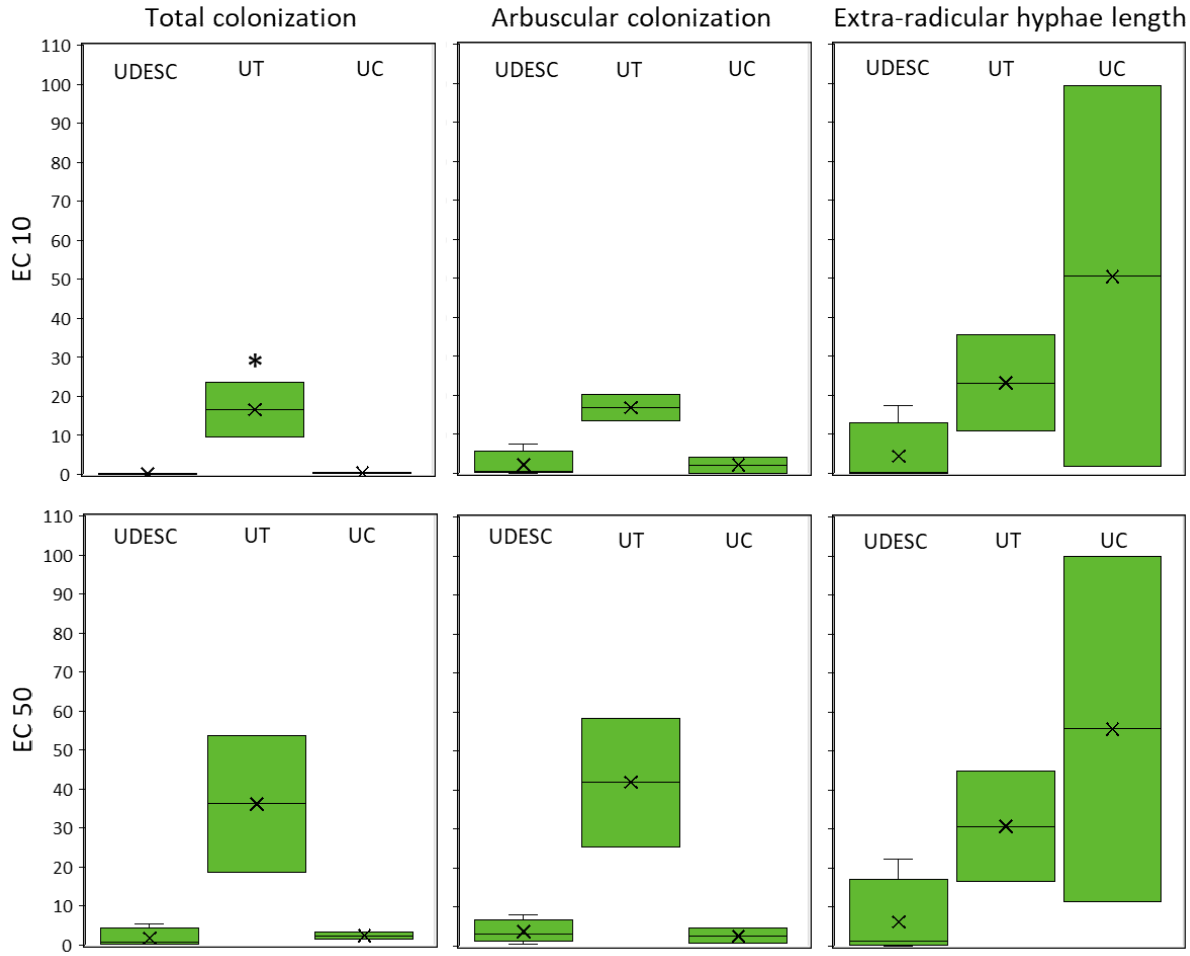
Table 51: **Percentage of explained observed variance (Exp. var.) of regression models (the ones that best explain the observations), response variable and explanatory variables and their respective regression coefficients (Reg. coef.) and *p*-value. TC - AMF total root colonization; Lab - laboratory (Lab2 – University of Tartu, Lab3 – University of Coimbra).**

Response variable	Explanatory variable	Reg. coef.	*p*-value	Exp. var.
TC	[1](Lab)2	-5.419	0.0353*	90.81%
	[1](Lab)3	-2.622	0.3156	

[1] – Class of nominal explanatory variable; * - Significant value ($p \leq 0.05$)

Figure 74: **Boxplots representing the EC10 (first line of graphs) and EC50 values (second line of graphs) estimated based of total (left graphs) and arbuscular (center graphs)**

mycorrhiza fungi root colonization and extra-radicular hyphae length (right graphs) measured in AMF symbiotic phase tests using leek as host plant and inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and using different soils spiked with increasing concentrations of Azoxystrobin and Fluazinam, carried out in laboratories of the State University of Stanta Catarina, Brazil (UDESC), University of Tartu, Estonia (UT) and University of Coimbra, Portugal (UC) in the WP2 of the ERAMYC project.



* - Statistically significant different ($p \leq 0.05$) compared to UDESC (evidenced in the GLM described in Table 51).
Source: own illustration, CloverStrategy.

2.7.4 Relative sensitivity of AMF parameters

From the 28 valid symbiotic phase tests carried out in the 2nd phase of WP2, the obtained data allowed to estimate endpoints (i.e. EC10 and EC50 values) for the 3 test parameters in 18 tests (Table 52). From these 18 tests, EHL was the most sensitive parameter in 9 tests (including data from 5 test substances), TC in 5 tests (including 3 test substances) and AC in 4 tests (including 4 test substances). Endpoints based on TC could be estimated in 26 tests, while AC endpoints could be estimated in 23 tests and EHL endpoints in 22 tests. Therefore, although the EHL was the most sensitive parameter in the majority of tests, it was also the parameter that least often allowed the estimation of endpoints. Moreover, from the 5 test substances that allowed the estimation of endpoints for all three AMF parameters, EHL was the most sensitive to Boric acid

and Tebuconazole in all tests carried out with these substances. Notwithstanding, in tests with Terbuthylazine both EHL and TC were the most sensitive parameters and in tests with Fluazinam and Azoxystrobin, the highest sensitivity was found in the three parameters depending on the variables AMF species and test soil. Therefore, the relative sensitivity of the test parameters to the presence of test chemicals in soil seems to depend on the test substance properties, which agrees with the GLMs described above that highlighted the strong influence of the test substance in the sensitivity of AMF measurements.

Table 52: Relative sensitivity of the different AMF test parameters (total colonization [TC], arbuscular colonization [AC] and extra-radicular hyphae length [EHL]) based on the estimated endpoints (EC10 and EC50s ordered from the lowest to the highest values; i.e. from the most to the least sensitive) obtained in laboratory AMF symbiotic phase tests with different test substances, using inoculants with propagules of different AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis* or *Rhizophagus clarus*) and different test soils (OECD 2.5% artificial soil or Bra8, #Lufa 2.1, Lufa 2.2, Est32 natural soils) carried out in different laboratories (UDESC, UT or UC).

Test substance	AMF species	Soil	Lab	Upward sensitivity of endpoints
Boric acid	*F. mosseae*	Bra8	UDESC	AC (EC10) > TC (EC10) > TC (EC50) = AC (EC50)
Boric acid	*F. mosseae*	OECD 2.5%	UDESC	EHL (EC10) > TC (EC10) > EHL (EC50) > AC (EC10) > AC (EC50) > TC (EC50)
Boric acid	*R. irregularis*	OECD 2.5%	UT	EHL (EC10) > AC (EC10) > AC (EC50) > TC (EC10) > TC (EC50) > EHL (EC50)
Boric acid	*F. mosseae*	#Lufa 2.1	UC	TC > EHL
Copper sulphate	*F. mosseae*	OECD 2.5%	UDESC	AC > TC > EHL
Copper sulphate	*R. irregularis*	OECD 2.5%	UDESC	TC > EHL
Tebuconazole	*F. mosseae*	Bra8	UDESC	TC > EHL
Tebuconazole	*R. clarus*	Bra8	UDESC	EHL (EC10) > EHL (EC50) > AC (EC10) > TC (EC10) > TC (EC50) = AC (EC50)
Tebuconazole	*R. clarus*	OECD 2.5%	UDESC	EHL (EC10) > TC (EC10) > TC (EC50) > AC (EC10) > AC (EC50) > EHL (EC50)

Test substance	AMF species	Soil	Lab	Upward sensitivity of endpoints
Terbuthylazine	*F. mosseae*	Bra8	UDESC	AC > TC
Terbuthylazine	*F. mosseae*	OECD 2.5%	UDESC	EHL > TC = AC
Terbuthylazine	*R. clarus*	Bra8	UDESC	TC (EC10) > EHL (EC10) > AC (EC10) > AC (EC50) > TC (EC50) > EHL (EC50)
Terbuthylazine	*R. clarus*	OECD 2.5%	UDESC	EHL (EC10) > TC (EC10) > TC (EC50) > EHL (EC50)
Azoxystrobin	*F. mosseae*	#Lufa 2.1	UC	EHL > TC > AC
Azoxystrobin	*F. mosseae*	OECD 2.5%	UC	AC
Azoxystrobin	*R. irregularis*	#Lufa 2.1	UC	AC > TC > EHL
Azoxystrobin	*R. irregularis*	OECD 2.5%	UC	TC (EC10) > EHL (EC10) > TC (EC50) > AC (EC10) > AC (EC50) > EHL (EC50)
Azoxystrobin	*R. irregularis*	Bra8	UDESC	EHL > AC > TC
Azoxystrobin	*R. irregularis*	Lufa 2.2	UDESC	TC > AC > EHL
Fluazinam	*F. mosseae*	Est32	UT	AC > TC
Fluazinam	*F. mosseae*	OECD 2.5%	UT	EHL > TC > AC
Fluazinam	*R. irregularis*	Est32	UT	AC (EC10) > TC (EC10) > EHL (EC10) > EHL (EC50) > TC (EC50) > AC (EC50)
Fluazinam	*R. irregularis*	OECD 2.5%	UT	TC (EC10) > EHL (EC10) > AC (EC10) > EHL (EC50) > TC (EC50) > AC (EC50)
Fluazinam	*R. irregularis*	Bra8	UDESC	EHL (EC10) > TC (EC10) > AC (EC10) > EHL (EC50) > AC (EC50) > TC (EC50)

Test substance	AMF species	Soil	Lab	Upward sensitivity of endpoints
Fluazinam	*R. irregularis*	Lufa 2.2	UDESC	TC > EHL > AC
Pendimethalin	*F. mosseae*	Est32	UT	TC > AC
Pendimethalin	*F. mosseae*	OECD 2.5%	UT	ND
Pendimethalin	*R. irregularis*	Est32	UT	AC (EC10) > AC (EC50) > EHL (EC10) >TC (EC10) > TC (EC50) = EHL (EC50)
Pendimethalin	*R. irregularis*	OECD 2.5%	UT	ND

ND – Endpoints not determined.

2.7.5 Relative sensitivity of AMF to different test soils

The relative sensitivity of AMF parameters on the different soils tested was compared within each tested substance, considering clusters of experiments for which at least two different soils were tested using the same AMF species. A total of 21 experiments were considered in this analysis (Table 53).

Table 53: Relative sensitivity of the different AMF test parameters (total colonization [TC], arbuscular colonization [AC] and extra-radicular hyphae length [EHL]) in the different test soils (OECD 2.5% artificial soil or Bra8, #Lufa 2.1, Lufa 2.2, Est32 natural soils), based on the estimated nominal endpoints (EC10 and EC50s ordered from the lowest to the highest values; i.e. from the most to the least sensitive) obtained in laboratory AMF symbiotic phase tests with different test substances, using inoculants with propagules of different AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis* or *Rhizophagus clarus*). A summary of the endpoints is listed in Table 56.

Test substance	AMF species	Parameter	Upward sensitivity of test parameters to the tested soils
Boric acid	*F. mosseae*	TC	$EC10_{L2.1\#} > EC10_B > EC50_{L2.1\#} > EC10_O > EC50_O > EC50_B$
		AC	$EC10_B > EC10_O > EC50_O > EC50_B$
		EHL	$EC10_O > EC50_O > EC10_{L2.1\#} = EC50_{L2.1\#}$
Tebuconazole	*R. clarus*	TC	$EC10_O > EC50_O > EC10_B > EC50_B$

Test substance	AMF species	Parameter	Upward sensitivity of test parameters to the tested soils
		AC	$EC10_O > EC50_O > EC10_B > EC50_B$
		EHL	$EC10_O > EC10_B > EC50_B > EC50_O$
Terbuthylazine	*F. mosseae*	TC	$EC10_B = EC10_O > EC50_B = EC50_B$
		AC	$EC10_B = EC10_O > EC50_B = EC50_B$
		EHL	$EC10_O > EC50_O$
Terbuthylazine	*R. clarus*	TC	$EC10_O > EC50_O > EC10_B > EC50_B$
		AC	$EC10_B > EC50_B$
		EHL	$EC10_O > EC10_B > EC50_O > EC50_B$
Azoxystrobin	*F. mosseae*	TC	$EC10_{L2.1\#} > EC50_{L2.1\#}$
		AC	$EC10_O > EC50_O > EC10_{L2.1\#} > EC50_{L2.1\#}$
		EHL	$EC10_{L2.1\#} > EC50_{L2.1\#}$
Azoxystrobin	*R. irregularis*	TC	$EC10_{L2.2} > EC10_{L2.1\#} > EC10_B > EC10_O > EC50_{L2.2} > EC50_B > EC50_{L2.1\#} > EC50_O$
		AC	$EC10_{L2.1\#} = EC10_B > EC10_{L2.2} > EC50_B > EC50_{L2.1\#} > EC50_{L2.2} > EC10_O > EC50_O$
		EHL	$EC10_B > EC50_B > EC10_O > EC50_O > EC10_{L2.2} > EC50_{L2.2} = EC10_{L2.1\#} > EC50_{L2.1\#}$
Fluazinam	*F. mosseae*	TC	$EC10_O > EC10_E > EC50_O > EC50_E$
		AC	$EC10_O > EC10_E > EC50_O > EC50_E$
		EHL	$EC10_O > EC50_O$
Fluazinam	*R. irregularis*	TC	$EC10_{L2.2} > EC50_{L2.2} = EC10_B > EC50_B > EC10_O > EC50_O > EC10_E > EC50_E$
		AC	$EC10_B > EC50_B > EC10_{L2.2} > EC50_{L2.2} = EC10_O > EC10_E > EC50_O > EC50_E$
		EHL	$EC10_B > EC10_{L2.2} > EC50_B > EC50_{L2.2} > EC10_O > EC50_O > EC10_E > EC50_E$

O – OECD 2.5% artificial soil; B – Bra8 soil; L2.1# - #Lufa 2.1 soil; L2.2 – Lufa 2.2 soil; E – Est32 soil. Endpoints higher than the highest tested concentration were considered equal to the next highest estimated endpoint.

The test soil that is most often ranked in the first place of sensitivity (i.e., AMF parameters are more sensitive in that soil) is OECD 2.5% artificial soil (it appears 11 times in first place, Bra8 appears 5 times, #Lufa 2.1 appears 3 times, Lufa 2.2 appears 2 times, Est32 never appears as the most sensitive and in 3 occasions there are two soils with the same highest relative sensitivity). However, conclusions regarding the relative sensitivity of the AMF parameters to the different

tested soils should be drawn with caution, as they may be biased by the number of experiments conducted with each test soil. Actually, the sensitivity of AMF parameters to the different tested soils seems directly proportional to the number of times that each soil is used in the different experiments (e.g. OECD 2.5% was the most used soil, followed by Bra8 soil).

2.7.6 Data of WP2 in the perspective of risk assessment

In terms of in-soil organisms, current ERA schemes of PPPs require effect data on earthworms *Eisenia andrei/fetida*, collembolans *Folsomia candida*, predatory mites *Hypoaspis aculeifer*, non-target terrestrial plants (NTTPs) and on nitrogen and carbon transformation by in-soil microorganisms. Whereby effect data for NTTP are used to describe effects outside the field, only, effect data for earthworms, collembolans, mites and microorganisms are used to estimate effects in-field. Despite the effort to cover a large spectrum of sensitivities by requiring effect data on different non-target soil organism groups, other soil groups not represented by these non-target species could be under protected. For instance, there is a growing concern on improving the current ERA for the protection of some “disregarded” organisms. This is particularly true for microorganisms, such as beneficial fungi like AMF (EFSA, 2017), that represent a group of soil organisms important for soil functioning. Actually, the effects on soil microorganisms are generally evaluated via functional responses on carbon and nitrogen turnover and relatively few efforts have been made to standardize methods for testing effects on beneficial fungi. From this point of view, the effect data generated in WP2 of the ERAMYC project are highly relevant, as they allow comparing the effect data reported in the literature to in-soil non-target organisms (namely to the in-soil non-target species currently considered in ERA schemes for the in-field area) and, by that way, to understand if AMF are being adequately protected. Aiming to facilitate the comparison between effect data from literature and effect data from ERAMYC project, Table 54 presents the lowest endpoints reported in the literature for the non-target soil organisms considered on current ERA schemes and the endpoints estimated for AMF in the ERAMYC project for the seven substances evaluated in WP2. Note that only the endpoints generated from experiments with OECD artificial soil were considered, as this is the soil required to generate the effect data in actual ERA, and only endpoints estimated from total colonization measurements were considered as this parameter was, in generally, the most robust and least variable from the three AMF parameters used in ERAMYC tests (see sections above).

Table 54: Lowest endpoints estimated for the test substances of ERAMYC WP2 (Boric acid, Copper sulphate, Tebuconazole, Terbuthylazine, Azoxystrobin, Fluazinam and Pendimethalin) retrieved from literature and from the ERAMYC outcomes.

Test substance	**Lowest endpoint reported from literature**[a]	**Lowest endpoint estimated on ERAMYC WP2**[b] – symbiotic phase	**Number of folds between lowest endpoint from literature and the lowest endpoint from ERAMYC**
Boric acid	*Eisenia fetida* (NOEC) = 316 mg BA/kg of soil (Becker et al., 2011)	*Funneliformis mosseae* (EC10) = 94.6 (58.9-130.4) mg BA/kg of soil	3.3
Copper sulphate	*Folsomia candida* (NOEC, chronic) = 1.480 mg Cu/kg of soil (obtained from study with copper nitrate; EFSA, 2013)	*Rhizophagus irregularis* (EC10) = 2.4 (0.01-4.9) mg $CuSO_4$/kg of soil = 0.96 mg Cu/kg of soil	1.5
Tebuconazole	*Eisenia fetida* (NOEC, chronic) = 10 mg TBC/kg of soil (EFSA, 2014)	*Rhizophagus clarus* (EC10) = 0.03 mg TBC/kg of soil	**333.3**
Terbuthylazine	*Eisenia fetida* (NOEC, chronic) = 0.563 mg TRZ/kg of soil (EFSA, 2010)	*Rhizophagus clarus* (EC10) = 0.4 mg TRZ/kg of soil	1.4
Azoxystrobin	*Eisenia fetida* (NOEC, chronic) = 4 mg AZX/kg of soil (EFSA, 2010)	*Rhizophagus irregularis* (EC10) = 0.5 (0.1-0.9) mg AZX/kg of soil	**8**
Fluazinam	*Eisenia andrei* (NOEC, chronic) < 0.175 mg FLZ/kg of soil (EFSA, 2008)	*Funneliformis mosseae* (EC10) = 0.3 (0.1-0.5) mg FLZ/kg of soil	0.6
Pendimethalin	Soil microorganisms (EC25, nitrogen transformation) = 2.66 mg PDM/kg of soil (EFSA, 2016)	*R. irregularis* and *F. mosseae* (NOEC) = 0.5 mg PDM/kg of soil	**5.32**

[a] - Only experiments conducted with artificial soil and standard species were considered, see Appendix A.23; [b] – Only endpoints for total colonization from experiments conducted on artificial soil were considered. Values in bold are higher than the assessment factor (AF) considered on current risk assessment schemes (AF=5).

The comparison between the endpoints from the literature with those estimated for AMF total colonization shows that, from the seven substances evaluated, three (i.e., Tebuconazole, Azoxystrobin and Pendimethalin) are clearly more toxic to AMF comparing to the non-target soil species as the number of folds between the literature endpoints and the estimated endpoints is higher than the assessment factor of 5 considered in current risk assessment schemes. This suggests that AMF are not being sufficiently protected by current ERA and, therefore, the inclusion of this group of microorganisms in the ERA schemes is urgent.

2.7.7 Relevance of chemical analysis for endpoints estimation

In the initial measured concentrations, the percentage of recovery of test substances was lower than 80% of the nominal concentrations for 4 out of the 6 test substances for which chemical analyses were conducted in soil samples (Table 55).

Table 55: Mean percentages (± standard deviation) of nominal concentration measured initially on soil samples from the experiments performed on WP2.

Test substance	Mean (± SD) % of nominal concentration, initially measured
Boric acid *(n=7)*	91.2 ± 41.7
Copper sulphate *(n=10)*	222.0 ± 49.1
Tebuconazole *(n=13)*	**69.0 ± 57.7**
Terbuthylazine *(n=7)*	**74.1 ± 11.7**
Azoxystrobin *(n=30)*	**49.5 ± 39.3**
Fluazinam *(n=10)*	**75.5 ± 27.6**
Pendimethalin *(n=0)*	NA

Values in bold: mean % of nominal concentration < 80%; NA – not estimated due to lack of soil samples for chemical analysis; *n* – number of soil samples evaluated for the determination of each test substance.

The estimated EC10 and EC50 values for TC, AC and EHL were based on nominal and initial measured concentrations for the test substances (Table 56). It was not possible to estimate ECx values based on initial measured concentrations in the experiment using inoculant of *F. mosseae* in Bra8 soil contaminated with Terbuthylazine because the initial measured concentration of

TRZ in all test treatments was lower than the quantification limit (i.e. < 0.005 mg TRZ/kg of soil; Table 31).

Chemical analysis did not considerably change the outcomes of the majority of the tests since the endpoints estimated based on the nominal concentrations allowed to estimate endpoints in the same order of magnitude of those estimated based on initial measured concentrations (Table 56).

From the 96 pairs of ECx nominal/measured estimated, 12 pairs showed endpoints based on nominal concentrations more than 5 times higher than the endpoints based on the respective initial measured concentrations. This was observed in endpoints estimated from experiments with Azoxystrobin, Tebuconazole and Terbuthylazine (Table 56) and suggests that, considering the EC10 and/or EC50 value estimated from nominal concentrations, may not be sufficiently protective for AMF.

Table 56: Estimated EC10 and EC50 values based on the data generated in the AMF symbiotic phase tests carried out with several test substances and using inoculants of different AMF species in WP2 of the ERAMYC project considering nominal (EC10/EC50 nom) and initial measured concentrations (EC10/EC50 meas) of the test substances. Ratios between nominal and inital measures (R) (EC10 or EC50) > 5 are highlighted in bold.

Test substance	AMF species	Test soil	Lab	Parameters	EC10 nom	EC10 meas	R EC10	EC50 nom	EC50 meas	R EC50
Boric acid	*F. mosseae*	Bra8	UDESC	TC	41.0*	35.7*	1.1	> 250	> 96.2	2.6
				AC	19.9*	20.9*	1.0	> 250	> 96.2	2.6
				EHL	ND	69.0*	ND	ND	> 96.2	ND
Boric acid	*F. mosseae*	OECD 2.5%	UDESC	TC	94.6 (58.9-130.4)	91.4 (47.0-135.8)	1.0	143.6 (78.9-208.4)	138.6 (72.6-204.5)	1.0
				AC	98.5 (59.1-138.0)	101.2*	1.0	130.2 (43.6-216.8)	116.8*	1.1
				EHL	83.9*	66.0 (36.2-95.8)	1.3	97.1 (36.5-157.7)	101.1 (26.4-175.8)	1.0
Boric acid	*F. mosseae*	#Lufa 2.1	UC	TC	36.8 (6.0-67.6)	70.7 (57.9-83.4)	0.5	69.0 (33.6-104.4)	80.3 (74.4-86.3)	0.9
				AC	ND	ND	ND	ND	ND	ND
				EHL	> 100	84.0 (77.1-90.9)	1.2	> 100	87.7 (66.6-108.8)	1.1
Copper sulphate	*F. mosseae*	OECD 2.5%	UDESC	TC	20.9 (8.8-33.0)	15.2*	1.4	137.6 (58.0-217.1)	243.8*	0.6
				AC	0.00004*	7.9*	0.00001	2.66*	51.9*	0.1
				EHL	207.2	557.7	0.4	> 300	> 824.8	0.4

Test substance	AMF species	Test soil	Lab	Parameters	EC10 nom	EC10 meas	R EC10	EC50 nom	EC50 meas	R EC50
					(38.8-375.5)	(112.8-1042.6)				
Copper sulphate	*R. irregularis*	OECD 2.5%	UDESC	TC	2.4 (0.01-4.9)	4.1 (0.1-8.1)	0.6	16.1 (0.05-32.0)	26.8 (0.4-53.2)	0.6
				AC	ND	ND	ND	ND	ND	ND
				EHL	38.4*	75.8 (12.4-139.2)	0.5	90.8 (56.7-125.0)	151.3 (105.6-197.1)	0.6
Tebuconazole	*F. mosseae*	Bra8	UDESC	TC	0.005*	0.01 (0.001-0.03)	0.5	0.03*	0.07 (0.01-0.1)	0.4
				AC	ND	> 0.072*	ND	ND	> 0.072*	ND
				EHL	> 50	0.001*	**50000**	> 50	0.004*	**12500**
Tebuconazole	*R. clarus*	Bra8	UDESC	TC	74.6*	48.4*	1.5	> 100	> 94.8	1.1
				AC	17.0*	ND	ND	> 100	ND	ND
				EHL	2.4*	1.9*	1.3	15.7*	12.2*	1.3
Tebuconazole	*R. clarus*	OECD 2.5%	UDESC	TC	0.03*	0.06*	0.5	0.2*	0.4*	0.5
				AC	0.26*	0.3*	0.9	1.73*	1.7*	1.0
				EHL	0.000002*	0.04*	0.0001	> 100	0.3*	**333**
Terbuthylazine	*F. mosseae*	Bra8	UDESC	TC	38.7*	NA	ND	> 50	NA	ND
				AC	10.5*	NA	ND	11.6*	NA	ND
				EHL	ND	NA	ND	ND	NA	ND

Test substance	AMF species	Test soil	Lab	Parameters	EC10 nom	EC10 meas	R EC10	EC50 nom	EC50 meas	R EC50
Terbuthylazine	*F. mosseae*	OECD 2.5%	UDESC	TC	> 5	> 3.6	1.4	> 5	> 3.6	1.4
				AC	> 5	0.01*	**500**	> 5	0.03*	**167**
				EHL	0.03*	0.02*	1.5	0.2*	0.14*	1.4
Terbuthylazine	*R. clarus*	Bra8	UDESC	TC	3.7*	1.9*	1.9	5.1*	3.3 (0.9-5.7)	1.5
				AC	4.6*	3.2*	1.4	5.0*	3.5*	1.4
				EHL	3.9*	1.9*	2.1	> 25	> 23.7	1.1
Terbuthylazine	*R. clarus*	OECD 2.5%	UDESC	TC	0.4*	0.2*	2.0	3.5*	1.1*	3.2
				AC	ND	ND	ND	ND	ND	ND
				EHL	0.09*	0.02*	4.5	5.2*	2.1*	2.5
Azoxystrobin	*F. mosseae*	#Lufa 2.1	UC	TC	53.1 (28.1-78.1)	8.6 (0.9-16.2)	**6.2**	78.4 (20.1-136.8)	14.0*	**5.6**
				AC	10.7*	2.8*	3.8	70.2*	18.7*	3.8
				EHL	0.3*	6.7 (1.1-12.3)	0.04	2.2*	43.9 (7.0-80.8)	0.1
Azoxystrobin	*F. mosseae*	OECD 2.5%	UC	TC	ND	1.5*	ND	ND	9.9*	ND
				AC	0.9*	ND	ND	5.8*	ND	ND
				EHL	ND	0.0004*	ND	ND	0.003*	ND
Azoxystrobin	*R. irregularis*	#Lufa 2.1	UC	TC	0.2 (0.1-0.4)	0.07 (0.04-0.1)	2.9	1.5 (0.4-2.6)	0.5 (0.2-0.7)	3.0

Test substance	AMF species	Test soil	Lab	Parameters	EC10 nom	EC10 meas	R EC10	EC50 nom	EC50 meas	R EC50
				AC	0.1*	0.06*	1.7	0.7*	0.4*	1.8
				EHL	99.4*	5.5*	**18.1**	> 100	36.4*	2.7
Azoxystrobin	*R. irregularis*	OECD 2.5%	UC	TC	0.5 (0.1-0.9)	0.5 (0.2-0.9)	1.0	3.4 (0.8-5.9)	1.0 (0.4-1.6)	3.4
				AC	4.2*	0.1*	**42.0**	4.6*	0.6*	**7.7**
				EHL	1.7*	0.1*	**17.0**	11.4*	0.7*	**16.3**
Azoxystrobin	*R. irregularis*	Bra8	UDESC	TC	0.3 (0.1 - 0.5)	0.2 (0.1-0.3)	1.5	1.0 (0.8 - 1.3)	1.0 (0.8-1.2)	1.0
				AC	0.1 (0.01 - 0.2)	0.1*	1.0	0.6 (0.1 - 1.1)	0.5 (0.02-1.0)	1.2
				EHL	0.002*	0.002*	1.0	0.01*	0.01*	1.0
Azoxystrobin	*R. irregularis*	Lufa 2.2	UDESC	TC	0.08 (0.05-0.12)	0.1 (0.07-0.2)	0.8	0.6 (0.3-0.8)	0.8 (0.5-1.0)	0.8
				AC	0.5*	0.4*	1.3	3.0*	2.3*	1.3
				EHL	17.3 (4.5-30.1)	13.4 (5.2-21.6)	1.3	> 19.5	> 15.2	1.3
Fluazinam	*R. irregularis*	Bra8	UDESC	TC	0.3*	0.2*	1.5	5.5 (2.4-8.6)	5.9 (1.9-10.0)	0.9
				AC	0.5 (0.03-1.0)	0.1*	5.0	3.2 (0.2-6.3)	2.1*	1.5
				EHL	0.1*	0.1*	1.0	1.0*	0.7*	1.4

Test substance	AMF species	Test soil	Lab	Parameters	EC10 nom	EC10 meas	R EC10	EC50 nom	EC50 meas	R EC50
Fluazinam	*R. irregularis*	Lufa 2.2	UDESC	TC	0.05*	0.03*	1.7	0.3*	0.2*	1.5
				AC	7.6 (2.1-13.2)	4.0*	1.9	> 8.0	> 5.6*	1.4
				EHL	0.27*	0.3*	0.9	1.78*	1.9*	0.9

ND – Not determined as the data did not allow estimation of the endpoint.

The estimated endpoint based on extra-radicular hyphae length (EHL) measured in the experiment conducted with *F. mosseae* in Bra8 soil contaminated with Tebuconazole were much lower when considering measured concentrations compared to nominal concentrations (50 000 and 12 500 times, for EC10 and EC50, respectively). These differences are related to the percentage of recovery of the test substance found in treatments of this experiment that were particularly low (1.8% on average for all treatment, Table 27).

Azoxystrobin was the test substance for which there were more ECx nominal/measured pairs with a difference higher than 5 (i.e. in 7 pairs out of the 30 pairs estimated), meaning that most of the endpoints calculated on measured concentrations for Azoxystrobin were lower when compared to the endpoints based on initial measured concentrations.

This could be related, not only to the low percentages of recovery generally associated to Azoxystrobin experiments (49.5% on average, Table 55), but also to the fact that Azoxystrobin was the test substance for which more experiments were carried out.

In conclusion, ERA based on endpoints derived from nominal concentrations may not be sufficiently protective for AMF. Data suggest that chemical analyses can provide valuable information to complement and validate the adequacy of the procedures applied in the tests, helping to generate more precise endpoints and allowing to improve the accuracy and effectiveness of risk assessment.

3 Section 3 – WP3: Performance of the ring test

3.1 Preparation of the ring test

A ring test was performed, under coordination of CloverStrategy, with the objective of evaluating the feasibility and reproducibility of the methods developed for AMF symbiotic phase testing and to test the adapted and refined methodological steps and test conditions of the AMF pre-symbiotic test.

Besides the three laboratories composing the ERAMYC consortium (i.e., UDESC, UT and UC), six other laboratories expressed interest in participating in the ring test for AMF symbiotic phase testing, making a total of 9 laboratories. Of these, a total of six laboratories (including the consortium laboratories) were also available to participate on the pre-symbiotic phase ring test.

The list of laboratories and their participation on the different tests is presented in Table 57.

Table 57: List of laboratories participating in the ring test for AMF pre-symbiotic and symbiotic phase tests.

Institution/company	Country	Contact person	Participation on tests
Univ. Coimbra (Consortium member)	Portugal	Susana Echeverria/Patrícia Ferreira	Symbiotic phase Pre-symbiotic phase
Univ. Tartu (Consortium member)	Estonia	Mari Moora/Tanel Vahter	Symbiotic phase Pre-symbiotic phase
UDESC (Consorium member)	Brazil	Osmar Klauberg/Luís Iuñes	Symbiotic phase Pre-symbiotic phase
Eurofins Agroscience Services Ecotox GmbH	Germany	Eva Aderjan	Symbiotic phase
Eurofins Trialcamp SLU	Spain	Mikel Queralt/Sara Varela	Symbiotic phase
BioChem agra GmbH	Germany	Lennart Schulz	Symbiotic phase
Bayer AG	Germany	Ulrich Menke	Symbiotic phase Pre-symbiotic phase

Institution/company	Country	Contact person	Participation on tests
Fraunhofer Institute for Molecular Biology and Applied Ecology IME	Germany	Karsten Schlich	Symbiotic phase Pre-symbiotic phase
BASF AM and EU	Germany	Leticia Carniel/Matthias Bergtold	Symbiotic phase Pre-symbiotic phase

The project consortium established the first contact with the participating institutions/laboratories by sending them a Survey (Appendix A.24) to clarify if they had a list of key equipment and materials available to use in the experiments and also to clarify the capabilities and expertise/experience of each laboratory for the performance of the ring test experiments. It was verified that, in general, the laboratories met the necessary conditions to carry out the ring test experiments. Then, a draft of a Non-Disclosure Agreement (NDA) was sent to the laboratories; after all the laboratories adapted and accepted the NDA, the SOPs describing the methodologies to follow during the ring test experiments were shared with the participants to give them the opportunity to analyse the methods and the descriptions contained therein.

3.2 Symbiotic phase ring test

In July 2023, a first online meeting with all laboratories of the ring test was held, and the methods and the details of the SOPs were presented and discussed with the ring test participants. No methodological changes were required, but the descriptions of the method were improved and clarified taking into account the doubts raised. The objective was to produce the most user-friendly SOPs possible and to facilitate the understanding and execution of the methods described by less experienced laboratories. The updated SOPs were subsequently distributed to the laboratories. The strategy for the ring test was also defined: priority was given to the symbiotic phase experiments due to the higher standardization effort for these experiments compared to the pre-symbiotic experiments, which are based on already established documents.

In October 2023, after gathering all data from the 2nd phase of WP2, and after a discussion within the project consortium members and UBA, the following elements were selected for the symbiotic phase ring test (Table 58).

Table 58: Elements selected for the performance of the symbiotic phase ring test experiments.

Component selected	Justification
Inoculant of *Rhizophagus irregularis* SAF 22	► Good performance on the generality of the experiments of WP2, especially with Azoxystrobin and Fluazinam ► Enough inoculant for the performance of the ring test could be supplied by a culture bank (Agroscope, Switzerland)
Host plant *Allium ampeloprasum*	► High percentages of root colonization were observed in the experiments conducted in WP2 ► Root structure is poorly branched, and roots are easy to process (e.g. collection, selection and staining)
OECD 2.5% artificial soil	► Composed of the same elements used in reference soil for ecotoxicological testing ► Can be easily reproduced by different laboratories
Test substances Azoxystrobin and Fluazinam	► Showed clearer effects in the experiments conducted in WP2 for different soils and AMF inoculants

The concentration gradients for the AMF symbiotic phase ring test were selected according to the toxicity found in the experiments of WP2 and did not consider recommended doses used in real application scenarios. The test treatments defined were:

- Azoxystrobin: 0; 0.5; 1.25; 3.125; 7.8; 19.5 mg AZX/kg soil DW.
- Fluazinam: 0; 0.5; 1; 2; 4; 8 mg FLZ/kg soil DW.
- Each experiment also included a solvent control (with acetone, CTsol) and a sterilization control (CTster).

A sampling scheme for determination of the initial measured concentration of Azoxystrobin or Fluazinam in soil was followed based on soil samplings at the beginning and end of the ring test experiments. More specifically, the sampling scheme comprised the following collections:

- 5 treatments x 2 dates (beginning and end of the test) x 3 samples of 50 g x 2 test substances x 1 test soil x 9 laboratories x 1 extractant.
- One sample of the control treatment was also collected per each laboratory at the beginning of each test.

Chemical analyses for the determination of initial measured concentrations of Azoxystrobin and Fluazinam were conducted by Tentamus LAB S.L.U. (Spain), following the same methodologies used for the chemical determination on sample from the experiments conducted in 2nd phase of WP2.

In December 2023, a Material Transfer Agreement (MTA) was signed by all the ring test participants to enable the distribution of inoculant that was provided by Agroscope (contact person: Marcel van der Heijden). The MTA had the aim of preventing the inoculant exploitation for own profit by the laboratories, that should use it uniquely for the ring test purposes.

Laboratory materials required for the symbiotic phase ring test experiments were purchased by CloverStrategy and distributed throughout the participants (the test substances, the inoculant, the leek seeds and the nutrient solution belonged to the same batch). Updated versions of the SOPs for the performance of symbiotic phase tests (Appendix A.14 to A.16 and Appendix A.18), recording Excel spreadsheets (Appendix A.25) and two supporting videos

- https://youtu.be/5ee9mGHYonU?si=cB4BCkCYOhj96RQP
- https://youtu.be/B5o4ut_3KPk?si=W7gCdEAa_vRi-jrp

were shared with the participant laboratories.

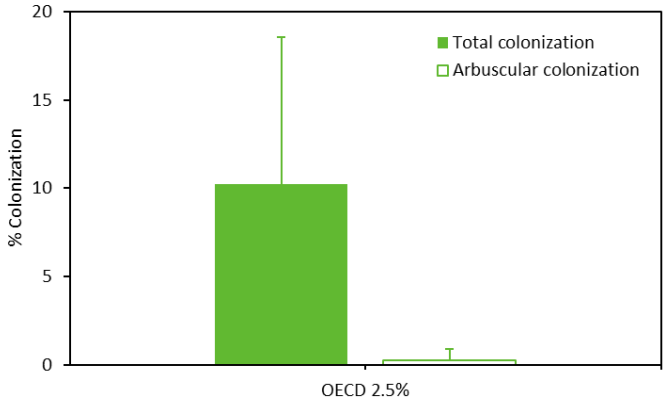
In January 2024, an online training session with the ring test participants was organized by the consortium, to clarify any existing doubts regarding the methodologies described in the supporting materials and to address critical aspects of the methodology and decision criteria required in the measurement of the endpoint parameters.

3.2.1 Assessment of the inoculum potential for the ring test

A potential inoculum test using the same inoculant of *R. irregularis* to be used on the ring test and OECD 2.5% artificial soil was carried out by UC (Portugal), according to SOP 2.1 (Appendix A.13).

The total colonization was lower than 30%, which was established as minimum for the validation of a definitive test (Figure 75).

Figure 75: Total and arbuscular root colonization (average ± standard deviation) of leek (*Allium ampeloprasum*) in OECD 2.5% artificial soil with inoculant of *Rhizophagus irregularis* after 28 days of test period. Tests performed in UC, Portugal.



Source: own illustration, CloverStrategy.

The consortium agreed that the low colonization observed in the experiment with artificial soil can be sufficient to validate the experiments of the ring test, as those tests take 56 days (twice as long compared to the 28 days of the potential inoculum test), which should be enough to increase the percentage of total colonization to, at least, 30%.

3.2.2 Results of the symbiotic phase experiments of the ring test

The experiments for the symbiotic phase ring test started in February 2024. The endpoints assessed included shoot fresh and dry biomass, root fresh biomass and, in replicates where there was plant biomass production, total and arbuscular colonization and extra-radicular hyphae length (note that the measurement of hyphae length was not mandatory for invalid experiments). Doubts regarding the performance of the experiments and the assessment of the endpoint parameters were clarified by the consortium by email, online meetings and during the online training in July 2024.

The validity of the ring test experiments was evaluated considering the following criteria:

- Total intra-radicular colonization of 0% in replicates of the sterilization control (CTster);
- Total intra-radicular colonization higher than 30% on average in replicates of the negative control (0 mg test substance/kg of soil DW);
- Coefficient of variation in the total colonization lower than 50% in the negative control;
- Minimum length of roots of 10 cm, observed in the negative control replicates.

Intra-radicular colonization and extra-radicular hyphae length were analysed using R Studio (version 2023.09.1), through packages drc (v. 3.0-1), car (v. 3.1-2), dunn.test (v. 1.3.6), DescTools (v. 0.99.52) and PMCMRplus (v. 1.9.10). Sterilization control (CTster) was not considered on the statistical analysis of intra-radicular colonization as its purpose was to verify the quality of the soil sterilization and to validate the test. Data on plant biomass are reported in Appendixes A.26 and A.27 (plant biomass data were not statistically analysed).

Normality and homogeneity of data of AMF parameters were assessed through Shapiro and Bartlett tests, respectively. When these assumptions were confirmed, a one-way ANOVA was

performed to assess differences among the treatments for each measured parameter (considering treatments as independent variable and colonization (total or arbuscular) or extra-radicular hyphae length as dependent variable).

When the normality and/or homogeneity assumptions were not confirmed, data were transformed to logarithm (i.e. log (x+1)) or squared root (i.e. $\sqrt{x}$). If the transformed data complied with the assumptions, a one-way ANOVA was performed, following the methodology described above. When the transformed data did not comply with the ANOVA assumptions, a non-parametric Kruskal-Wallis' test was performed, using non-transformed data, to assess the differences among treatments. Kruskal-Wallis' test was also applied when the number of replicates in the treatments was lower than two.

When significant differences were found, rather in one-way ANOVA or Kruskal-Wallis' test, differences between each treatment and the respective control were assessed through a post-hoc Dunnett test or a post-hoc Dunn test (with Bonferroni correction), respectively. The No-Observed Effect Concentration (NOEC) and the Lowest-Observed Effect Concentration (LOEC) were estimated for both approaches. All the statistical analyses were performed considering a significance level of 5%.

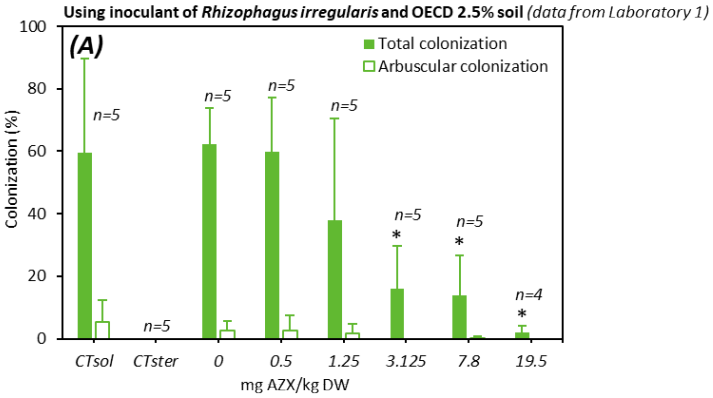
When possible, and only for valid experiments, Effect Concentration values for 10% and 50% reduction (EC10 and EC50, respectively) of total and arbuscular colonization and of extra-radicular hyphae length when compared to control were estimated trough non-linear regression models (using the measured parameters as dependent variable and nominal or measured concentrations as independent variable). Models applied in non-linear regressions were logistic, exponential or Hormesis and its selection was based on the highest coefficient of regression (r^2) and best fit to the data. The 95% confidence intervals were also estimated when data allowed. These estimations were performed on R Studio (version 2023.09.1), using packages drc (v. 3.0-1), tidyverse (v. 2.0.0), readxl (v. 1.4.3) and PMCMRplus (v. 1.9.10).

Results of the symbiotic phase experiments obtained in the ring test are presented below. A code was assigned to each participating laboratory, and results were analysed individually for each laboratory.

3.2.2.1 AMF symbiotic phase experiments of the ring test with Azoxystrobin

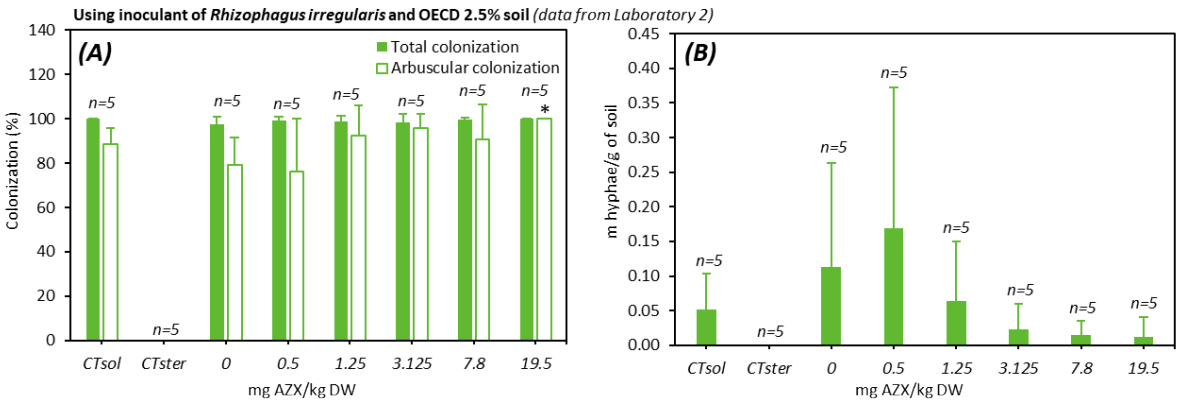
The results obtained by the nine laboratories that conducted the symbiotic phase tests with Azoxystrobin are presented in Figures 76 to 84 and raw data are presented on Appendix A.26.

Figure 76: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 1.



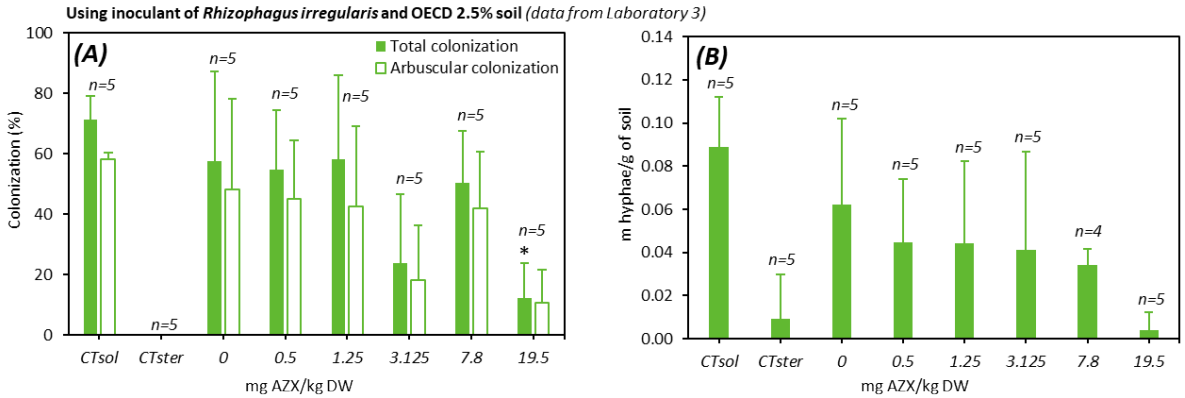
(A) Total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is valid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$. Extra-radicular hyphae length was not assessed within the project's timeframe.
Source: own illustration, CloverStrategy.

Figure 77: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 2.



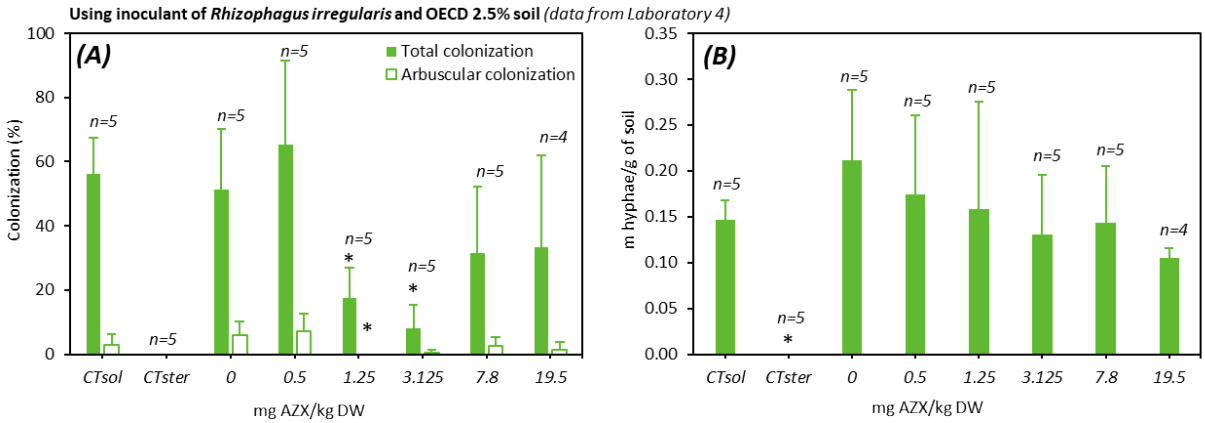
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 78: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 3.



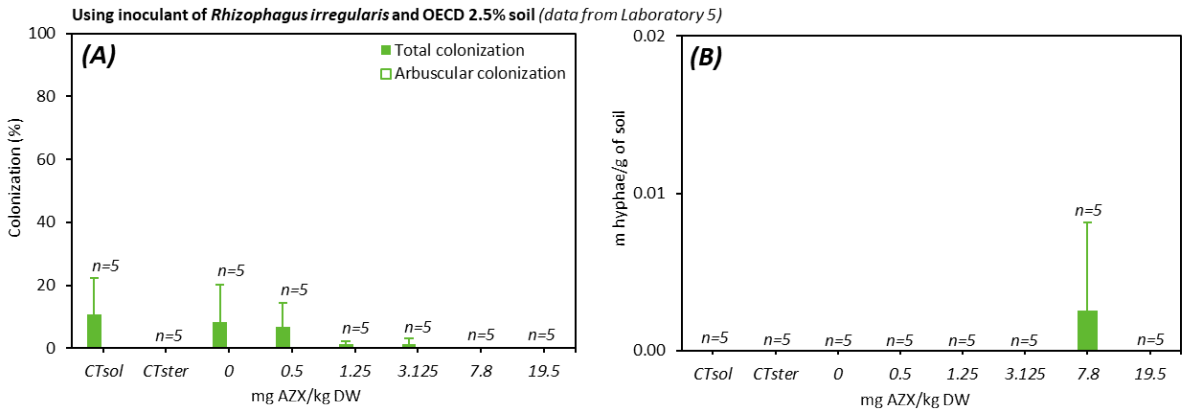
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is invalid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 79: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 4.



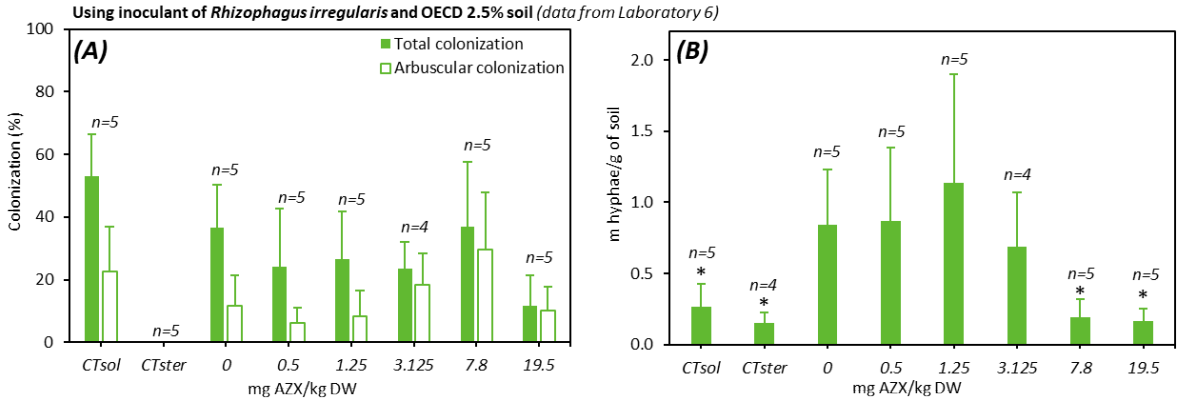
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 80: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 5.



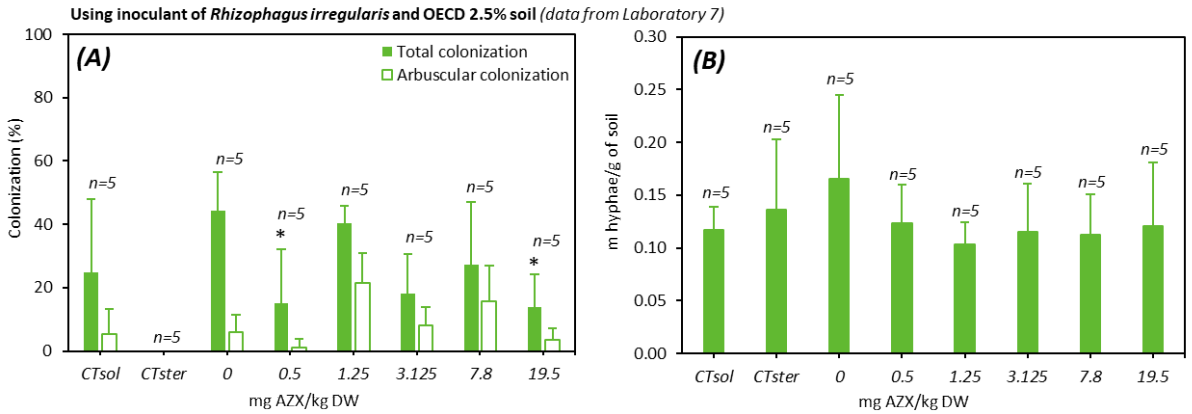
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is invalid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 81: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 6.



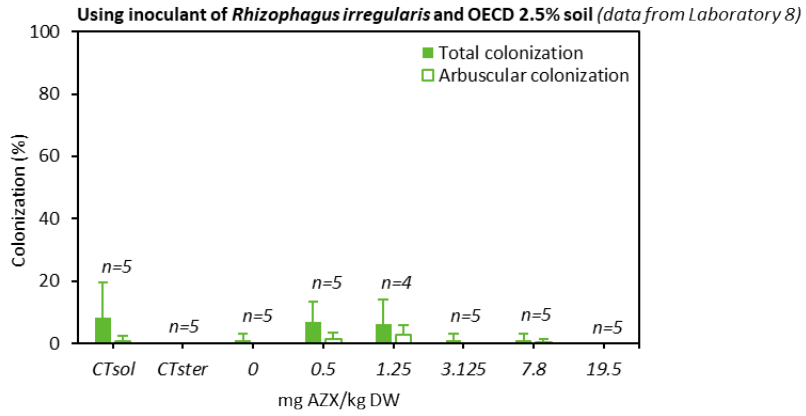
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 82: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 7.



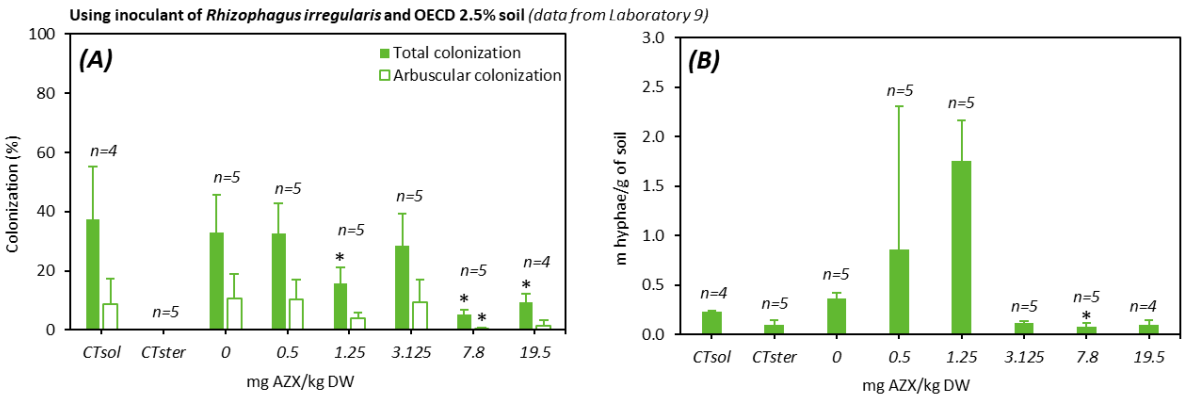
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 83: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 8.



(A) Total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 84: **Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 9.**



(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 59). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

3.2.2.1.1 Summary of results obtained in the AMF symbiotic phase experiments of the ring test using Azoxystrobin

Of the nine experiments conducted exposing *R. irregularis* to Azoxystrobin in OECD 2.5% soil, six were valid and three were not valid. Only two of the experiments were invalid due to the low root colonization observed (i.e. total colonization < 30%) but all three experiments presented coefficients of variation (CV) > 50%. The compliance of each experiment with the validity criteria is summarized in Table 59.

Table 59: **Summary of the compliance with the validity criteria defined in SOP Test 2 of the symbiotic phase experiments of the ring test carried out exposing *R. irregularis* to Azoxystrobin in OECD 2.5% artificial soil. TC – total root colonization in negative control (C0); CTster – sterilization control; CV – coefficient of variation in C0.**

Lab ID	CTster (0% TC)	TC (≥ 30%)	CV (< 50%)	Min. 10 cm root length	Test validity
Laboratory 1	Yes	62.2%	18.8%	Yes	Valid
Laboratory 2	Yes	97.4%	3.8%	Yes	Valid
Laboratory 3	Yes	57.4%	**51.7%**	Yes	**Invalid**
Laboratory 4	Yes	51.2%	36.9%	Yes	Valid
Laboratory 5	Yes	**8.4%**	**141.6%**	Yes	**Invalid**
Laboratory 6	Yes	36.6%	37.2%	Yes	Valid
Laboratory 7	Yes	44.2%	27.6%	Yes	Valid
Laboratory 8	Yes	**1.2%**	**149.2%**	Yes	**Invalid**
Laboratory 9	Yes	33%	38.8%	Yes	Valid

Chemical analyses to determine the actual contamination of Azoxystrobin in soil samples collected at the beginning and end of the experiments were performed following the modified QuEChERS methodology (Perestrelo et al., 2019). Results are presented in Appendix A.31.

The endpoints estimated for the symbiotic phase ring test experiments with Azoxystrobin are presented on Table 60.

Table 60: **Summary of the experiments performed in the symbiotic phase ring test using inoculant of *Rhizophagus irregularis* with Azoxystrobin in OECD 2.5% artificial soil. Nominal concentrations used in the experiments were 0; 0.5; 1.25; 3.125; 7.8; 19.5 mg AZX/kg of soil DW. No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) of reduction on total colonization (TC), arbuscular colonization (AC) and extra-radicular hyphae length (EHL) were estimated in valid experiments only. ECx values were estimated based on nominal and initial measured concentrations. Results are expressed in mg AZX/kg of soil dry weight and presented with the respective 95% confidence interval, when data allowed. Non-linear models applied and the respective r^2 values are presented in Appendix A.32.**

Laboratory	State (valid/invalid)	Parameters	Nominal concentrations				Initial measured concentrations			
			NOEC	LOEC	EC10	EC50	NOEC	LOEC	EC10	EC50
Lab 1	Valid	TC	1.25	3.125	0.4*	1.7 (0.7-2.8)	1.4	4.7	0.4*	2.2 (0.5-3.9)
		AC	19.5	> 19.5	0.9*	1.4*	25.8	> 25.8	1.2*	1.5*
		EHL	Not measured							
Lab 2	Valid	TC	19.5	> 19.5	ND	ND	22.3	> 22.3	ND	ND
		AC	7.8	19.5	0.14*	0.92*	9.4	22.3	0.1*	0.8*
		EHL	19.5	> 19.5	0.9*	1.5*	22.3	> 22.3	0.8*	1.7*
Lab 3	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 4	Valid	TC	0.5	1.25	0.02*	5.4*	0.5	1.0	0.01*	8.3*
		AC	0.5	1.25	0.9*	1.0*	0.5	1.0	0.08*	0.5*
		EHL	19.5	> 19.5	0.1*	0.8*	27.4	> 27.4	0.1*	0.7*

Laboratory	State (valid/invalid)	Parameters	Nominal concentrations				Initial measured concentrations			
			NOEC	LOEC	EC10	EC50	NOEC	LOEC	EC10	EC50
Lab 5	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 6	Valid	TC	19.5	> 19.5	3.5*	> 19.5	20.6	> 20.6	ND	ND
		AC	19.5	> 19.5	0.4*	1.1*	20.6	> 20.6	0.3*	0.9*
		EHL	3.125	7.8	3.1 (0.9-5.4)	5.1 (1.1-9.1)	2.6	9.3	2.6*	5.1*
Lab 7	Valid	TC	< 0.5	0.5	0.7*	4.6*	< 0.3	0.3	0.5*	3.0*
		AC	19.5	> 19.5	ND	ND	3.2	> 3.2	ND	ND
		EHL	19.5	> 19.5	0.0001*	0.0007*	3.2	> 3.2	0.0005*	0.003*
Lab 8	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 9	Valid	TC	0.5	1.25	0.3*	4.0*	0.1	0.7	0.1*	2.7*
		AC	3.125	7.8	0.5*	3.3*	1.7	6.3	0.3*	2.2*
		EHL	3.125	7.8	2.8 (0.03-5.6)	3.0 (1.2-4.8)	1.7	6.3	1.3 (0.03-2.5)	1.5*

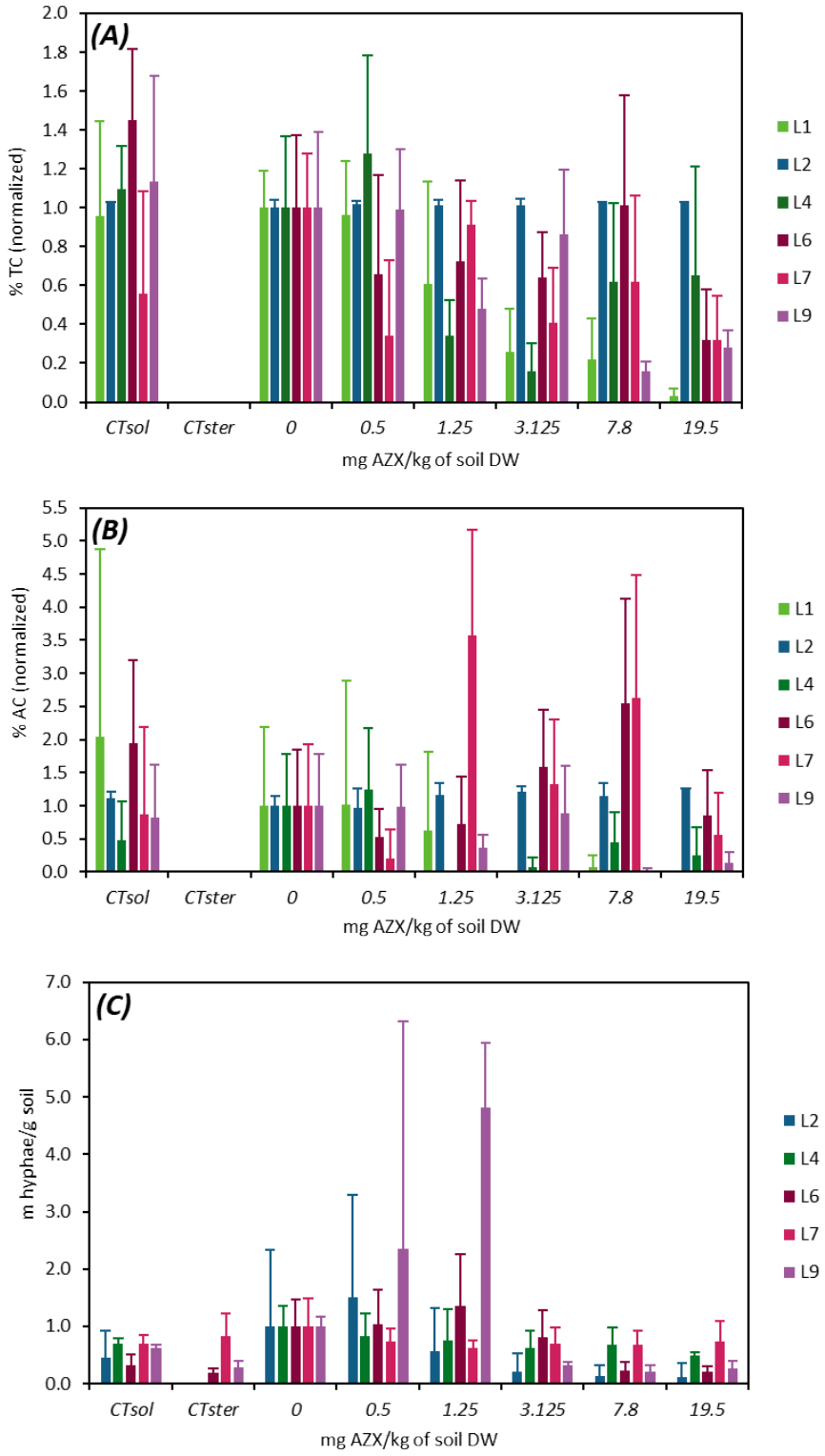
* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

The percentage of total and arbuscular colonization and the extra-radicular hyphae length obtained in the experiments above were normalized by dividing the percentage of total/arbuscular colonization or the extra-radicular hyphae length obtained in each replicate by the mean response observed in the respective negative control (0 mg AZX/kg of soil DW). The normalization of the data makes easier the comparability of the data among laboratories as the response obtained in each experiment (and therefore, by each laboratory) must be analysed in comparison with its own control response. The normalized data for each parameter are presented on Figure 85.

There seems to be a trend towards reduced percentages of total and arbuscular colonization (Figure 85, graphs A and B, respectively) and extra-radicular hyphae length (Figure 85, graph C) along the increase of the concentration gradient, despite the high variability observed in the three measured parameters.

The most variable parameters were arbuscular colonization and extra-radicular hyphae length, and the least variable was the total colonization.

Figure 85: Data obtained in the valid symbiotic phase experiments of the ring test exposing the AMF *R. irregularis* to Azoxystrobin in OECD 2.5% artificial soil (data were normalized to the response in the respective control treatment).

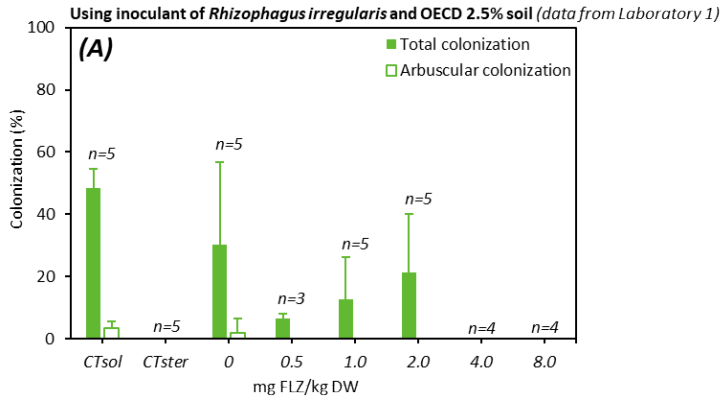


(A) Total colonization (TC, average ± standard deviation), (B) arbuscular colonization (AC, average ± standard deviation) and (C) extra-radicular hyphae length (EHL, average ± standard deviation) after 56 days of exposure.
Source: own illustration, CloverStrategy.

3.2.2.2 AMF symbiotic phase experiments of the ring test with Fluazinam

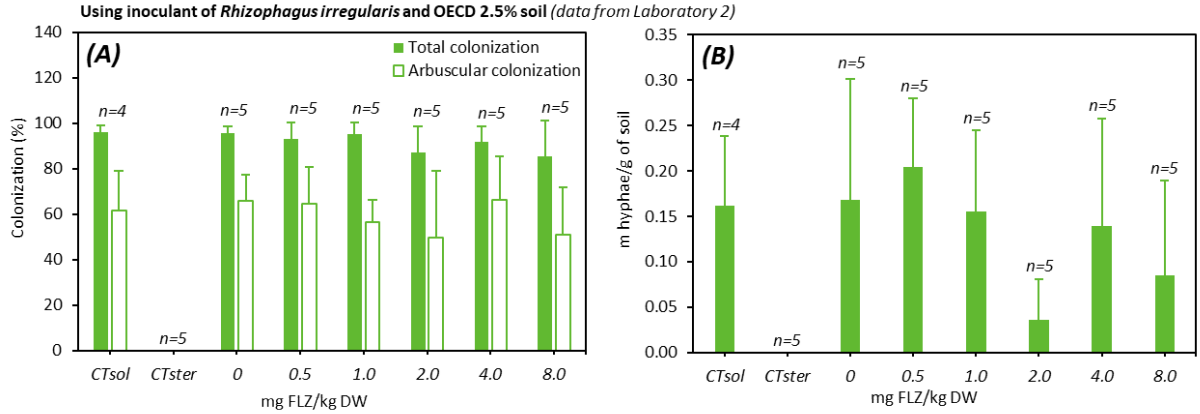
The results obtained by the nine laboratories in the symbiotic phase tests with Fluazinam are presented in Figures 86 to 94. Raw data are presented on Appendix A.27.

Figure 86: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 1.



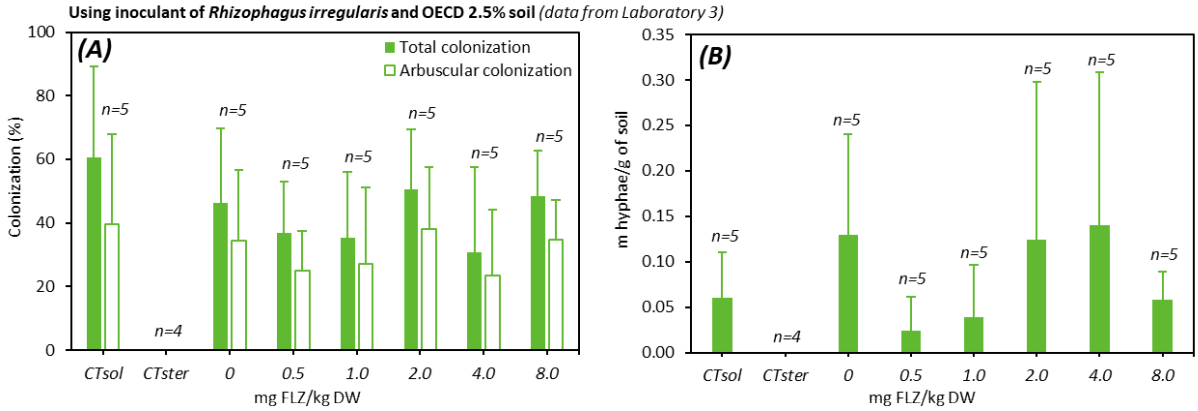
(A) Total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 87: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 2.



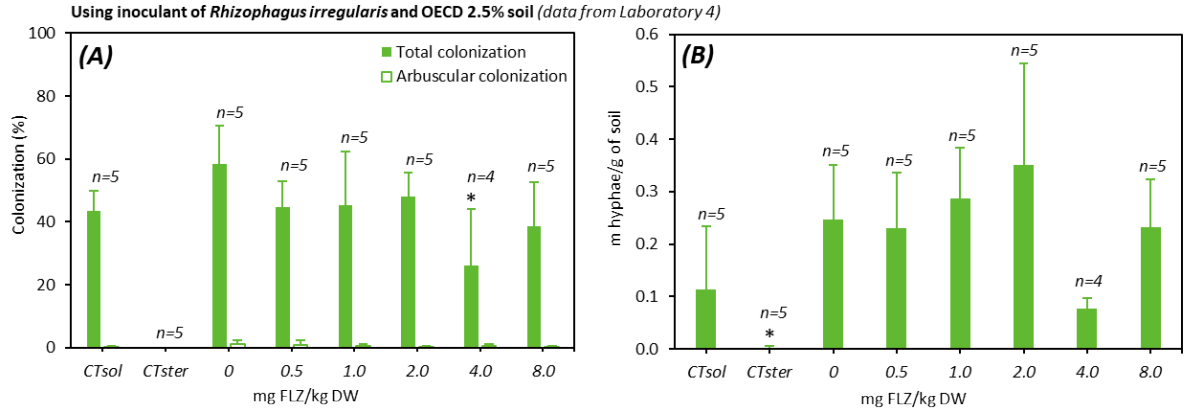
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 88: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 3.



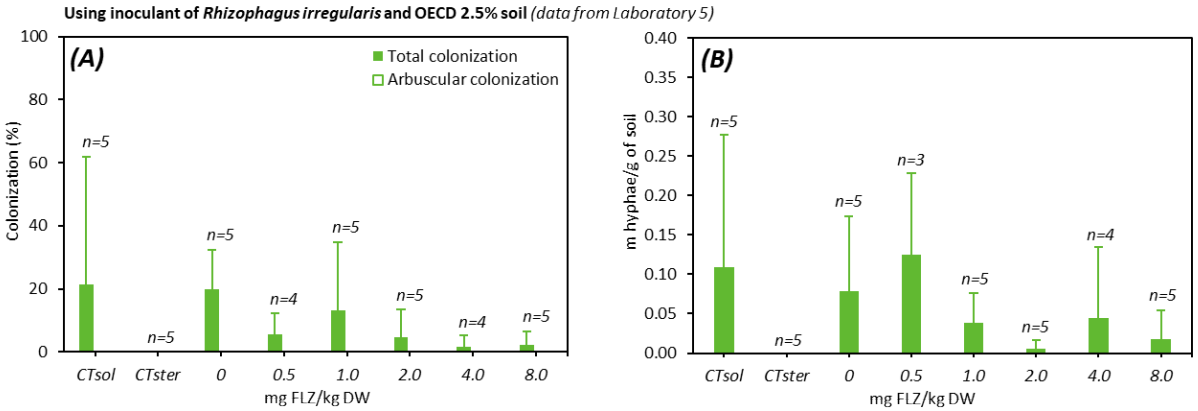
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is invalid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 89: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 4.



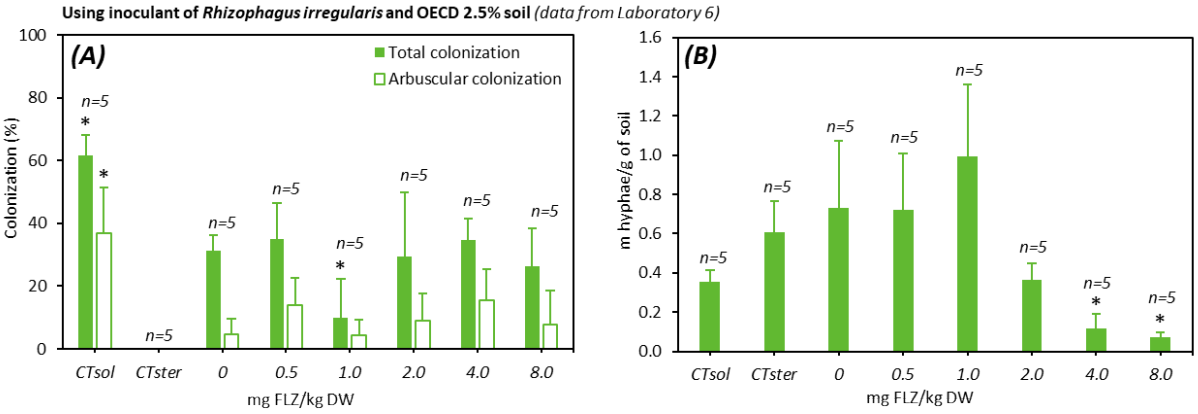
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 90: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 5.



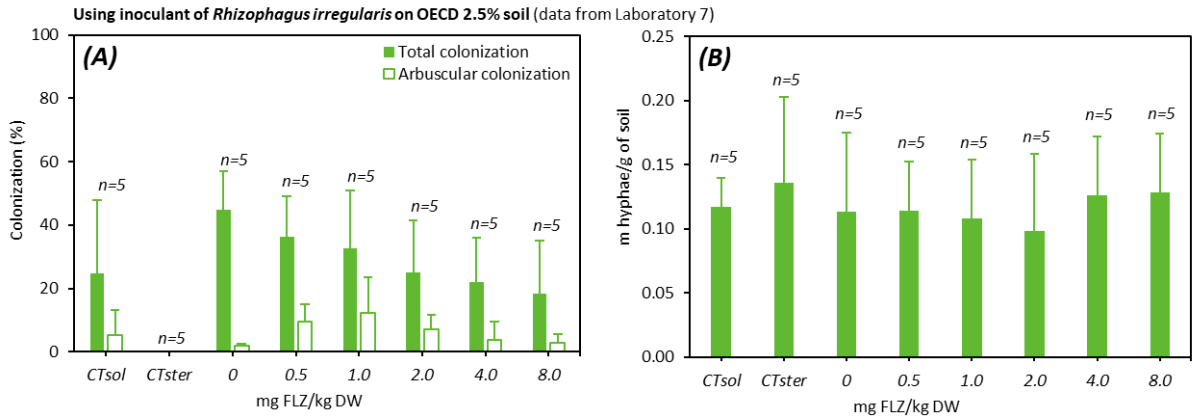
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is not valid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 91: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 6.



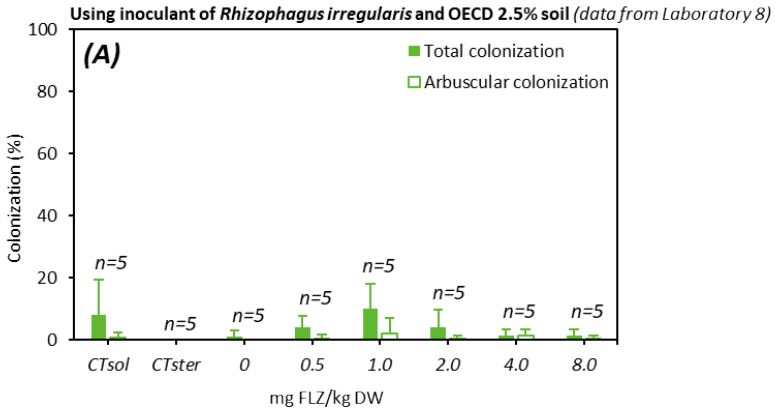
(A) Total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is valid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 92: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 7.



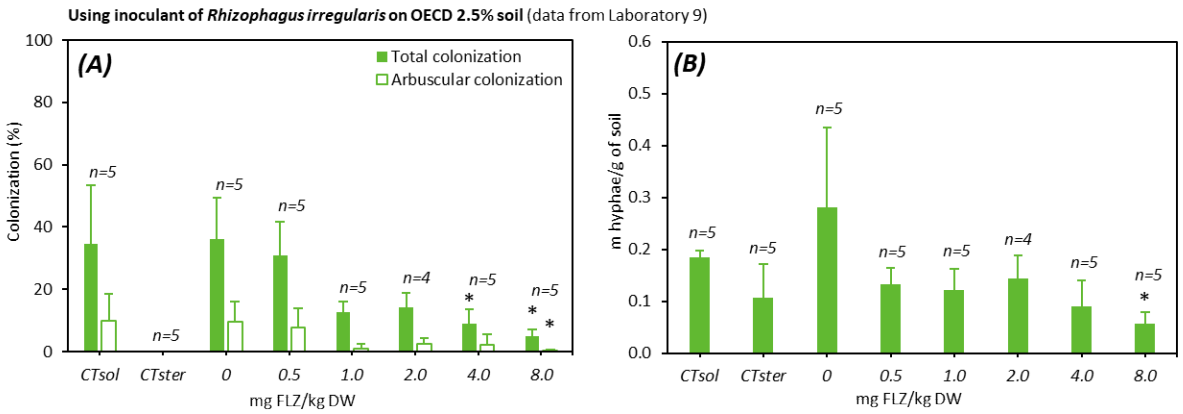
(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 93: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 8.



(A) Total and arbuscular colonization (average ± standard deviation) after 56 days of exposure. Test is invalid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 94: Host plant: leek (*Allium ampeloprasum*); Inoculum: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 9.



(A) Total and arbuscular colonization (average ± standard deviation) and (B) extra-radicular hyphae length (average ± standard deviation) after 56 days of exposure. Test is valid (Table 61). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

3.2.2.2.1 Summary of the results obtained in the AMF symbiotic phase experiments of the ring test using Fluazinam

From the nine experiments conducted during the symbiotic phase ring test exposing *R. irregularis* to Fluazinam in OECD 2.5% artificial soil, five were valid and four were invalid. Only two experiments did not attain sufficient total colonization in the control treatment (i.e. TC > 30%) but all the four invalid experiments presented coefficients of variation (CV) higher than 50%. The compliance of each experiment with the validity criteria is summarized in Table 61.

Table 61: Summary of the compliance with the validity criteria defined in SOP Test 2 of the symbiotic phase experiments of the ring test carried out exposing *R. irregularis* to Fluazinam in OECD 2.5% artificial soil. TC – total colonization in negative control (C0); CTster – sterilization control; CV – coefficient of variation in C0.

Lab ID	CTster (0% TC)	TC (≥ 30%)	CV (< 50%)	Min. 10 cm root length	Test validity
Laboratory 1	Yes	30.4%	**87.1%**	Yes	**Invalid**
Laboratory 2	Yes	95.6%	3.0%	Yes	Valid
Laboratory 3	Yes	46.4%	**50.1%**	Yes	**Invalid**
Laboratory 4	Yes	58.4%	20.6%	Yes	Valid
Laboratory 5	Yes	**19.8%**	**63.9%**	Yes	**Invalid**
Laboratory 6	Yes	31.4%	15.9%	Yes	Valid
Laboratory 7	Yes	44.8%	26.9%	Yes	Valid
Laboratory 8	Yes	**1.2%**	**149.1%**	Yes	**Invalid**
Laboratory 9	Yes	36.2%	36.2%	Yes	Valid

Chemical analyses to determine the actual contamination of Fluazinam in soil samples collected at the beginning and end of the experiments were performed following the modified QuEChERS methodology (Perestrelo et al., 2019). Results are presented in Appendix A.31.

Endpoints estimated for the symbiotic experiments with Fluazinam are presented in Table 62.

Table 62: **Summary of the experiments performed in the symbiotic phase ring test using inoculant of *Rhizophagus irregularis* with Fluazinam in OECD 2.5% artificial soil. Nominal concentrations used in the experiments were: 0; 0.5; 1; 2; 4; 8 mg FLZ/kg of soil DW. No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Effect Concentrations for 10% and 50% (EC10 and EC50) on reduction in total root colonization (TC), arbuscular colonization (AC) and extraradicular hyphae length (EHL) were estimated in valid experiments only. ECx values were estimated based on nominal and initial measured concentrations. Results are expressed in mg FLZ/kg of soil dry weight and presented with the respective 95% confidence interval. when possible. Non-linear models applied and the respective r^2 values are presented in Appendix A.32.**

Laboratory	State (valid/invalid)	Parameters	Nominal concentrations				Initial measured concentrations			
			NOEC	LOEC	EC10	EC50	NOEC	LOEC	EC10	EC50
Lab 1	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 2	Valid	TC	8	> 8	> 8	> 8	9.7	> 9.7	9.1*	> 9.7
		AC	8	> 8	1.3*	> 8	9.7	> 9.7	1.4*	> 9.7
		EHL	8	> 8	0.9*	4.8*	9.7	> 9.7	1.0*	5.1*
Lab 3	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 4	Valid	TC	2	4	0.05*	> 8	1.7	3.6	0.01*	> 11.1
		AC	8	> 8	0.1*	1.1*	11.1	> 11.1	0.1*	1.0*
		EHL	8	> 8	0.5*	3.1*	11.1	> 11.1	0.3*	1.9*

Laboratory	State (valid/invalid)	Parameters	Nominal concentrations				Initial measured concentrations			
			NOEC	LOEC	EC10	EC50	NOEC	LOEC	EC10	EC50
Lab 5	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 6	Valid	TC	0.5	1	ND	ND	0.4	0.8	ND	ND
		AC	8	> 8	ND	ND	5.6	> 5.6	ND	ND
		EHL	2	4	1.8 (0.6-3.1)	2.0 (1.7-2.3)	1.3	2.6	1.2 (0.8-1.6)	1.3 (1.2-1.4)
Lab 7	Valid	TC	8	> 8	0.1*	3.9*	0.6	> 0.6	0.008*	0.05*
		AC	8	> 8	1.4*	> 8	0.6	> 0.6	0.0000001*	> 0.59
		EHL	8	> 8	0.3*	1.8*	0.6	> 0.6	0.6*	3.9*
Lab 8	Invalid	TC	-	-	-	-	-	-	-	-
		AC	-	-	-	-	-	-	-	-
		EHL	-	-	-	-	-	-	-	-
Lab 9	Valid	TC	2	4	0.1 (0.03-0.2)	0.8 (0.2-1.3)	1.8	3.2	0.06 (0.01-0.1)	0.4 (0.09-0.7)
		AC	4	8	0.4 (0.1-0.8)	0.7 (0.3-1.0)	3.2	8.1	0.3 (0.1 - 0.5)	0.4 (0.2-0.6)
		EHL	4	8	0.04*	0.3*	3.2	8.1	0.02*	0.1*

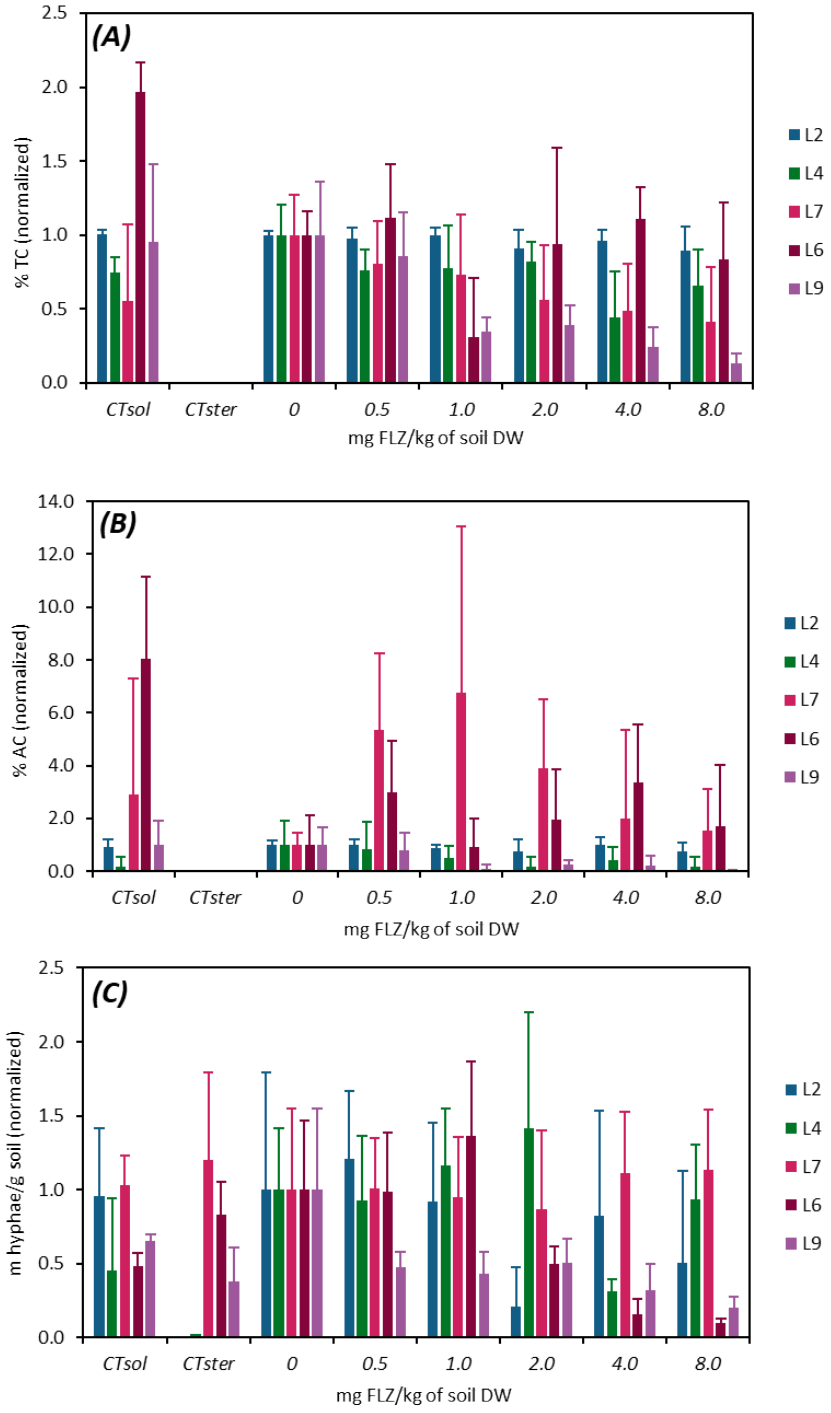
* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of EC10 and EC50.

The percentage of total and arbuscular colonization and the extra-radicular hyphae length obtained in the experiments with Fluazinam were normalized by dividing the percentage of total/arbuscular colonization or the extra-radicular hyphae length obtained in each replicate by the mean response observed on the respective negative control (0 mg FLZ/kg of soil DW). The normalized data are presented in Figure 95.

Apparently, there is a slight tendency to reduce percentages of total and arbuscular colonization and extra-radicular hyphae length along the increase of the Fluazinam concentration, despite the high variability observed on the three measured parameters.

Similarly, to that observed for Azoxystrobin, the most variable parameters were arbuscular colonization and extra-radicular hyphae length, and the least variable was the total colonization.

Figure 95: Data obtained in the valid symbiotic phase experiments of the ring test exposing the AMF *R. irregularis* to Fluazinam in OECD 2.5% artificial soil (data were normalized to the response in the respective control treatment).



(A) Total colonization (TC, average ± standard deviation), (B) arbuscular colonization (AC, average ± standard deviation) and (C) extra-radicular hyphae length (EHL, average ± standard deviation) after 56 days of exposure.
Source: own illustration, CloverStrategy.

3.2.3 Conclusions on the symbiotic phase testing

The symbiotic phase ring test was conducted to evaluate the validity of the method developed for the assessment of effects of chemicals on the symbiotic phase of the AMF life cycle.

From the 18 symbiotic phase experiments performed (including both test substances), 11 complied with the validity criteria. This corresponds to a validation of 61.1% of the experiments performed, supporting the capability and transferability of the method under scrutiny (ISO, 2021). Furthermore, from the 9 laboratories participating in the ring test, 66% validated at least one of the experiments performed (either with Azoxystrobin or Fluazinam).

Guidelines OECD 34 (2005), ISO 5594 (2021), ISO 5725-2 (1994) and Environment Canada EPS 1/RM/46 (2005) were used as guidance to explore the data obtained in the symbiotic phase ring test of the present report.

First, the reproducibility variance of the method was applied for data of each test chemical, according to the following equation (taken from ISO 5594 (2021) and ISO 5725-2 (1994)):

$$S_R^2 = S_L^2 + S_r^2$$

Where S_R^2 is the reproducibility variance, S_L^2 is the variance between laboratories, and S_r^2 is the arithmetic mean of the variance within-laboratory, which is the repeatability.

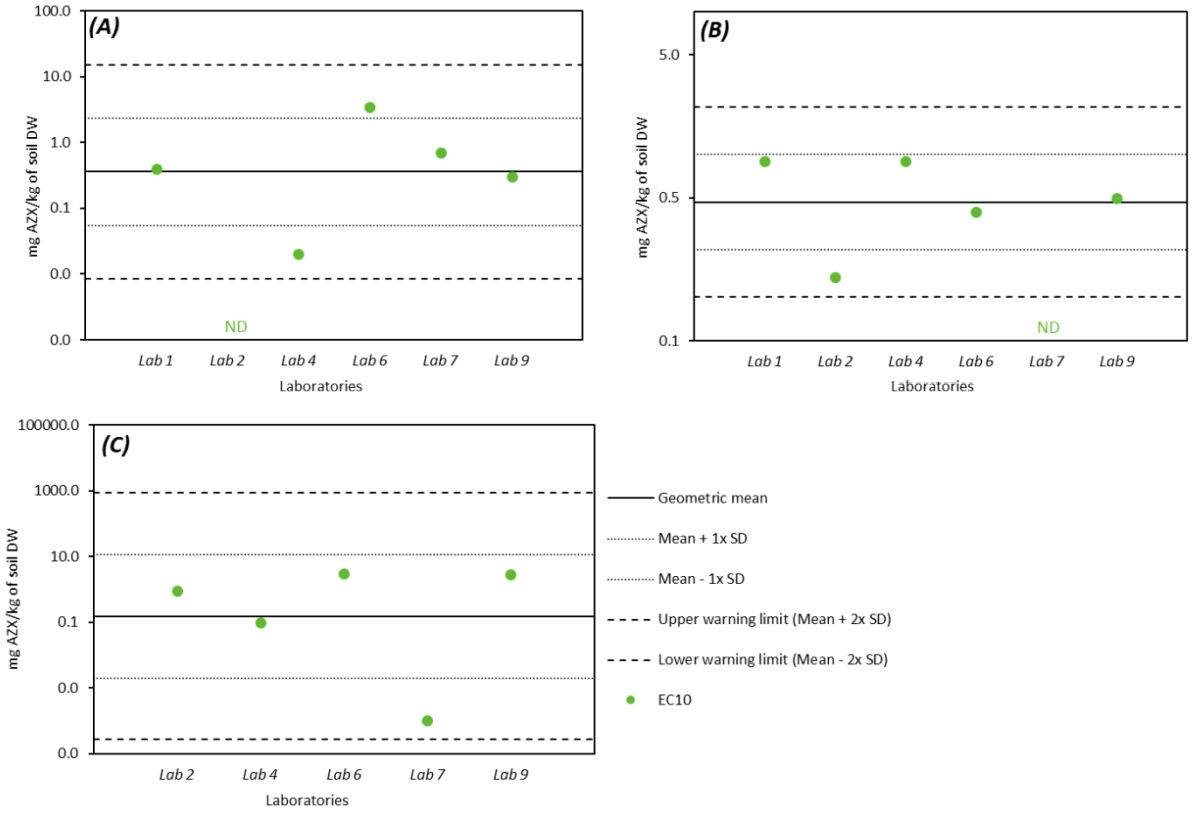
Repeatability estimation using the intervals of confidence of the estimated ECx (as advised in ISO 5594 (2021)) in the ring test could not be achieved as data produced in the ring test did not allow the estimation of the confidence intervals for some of the endpoints. Because of that, the reproducibility of the method could not be confirmed for both test substances.

As an alternative, the statistical evaluation of the precision of the ring test based on the ECx estimations obtained in the experiments was performed by adapting the approach described in EC (2005) for the estimation of warning limits and plotting of warning charts.

Individual warning charts were plotted for EC10 and EC50 estimated for each measured parameter (total colonization, arbuscular colonization and extra-radicular hyphae length) and for each test substance (Figure 96 to 99). Only experiments complying with the validity criteria were considered for the estimation of the warning limits with the exception of the tests for which the ECx could not be estimated or for which the estimated ECx were higher than the highest concentration tested.

Figure 96: Results (EC10 as mg of Azoxystrobin/kg of soil DW) for (A) total colonization, (B) arbuscular colonization and (C) extra-radicular hyphae length from the experiments of symbiotic phase ring test using inoculant of *R. irregularis* and OECD 2.5% artificial soil. EC10 values higher than the highest concentration tested

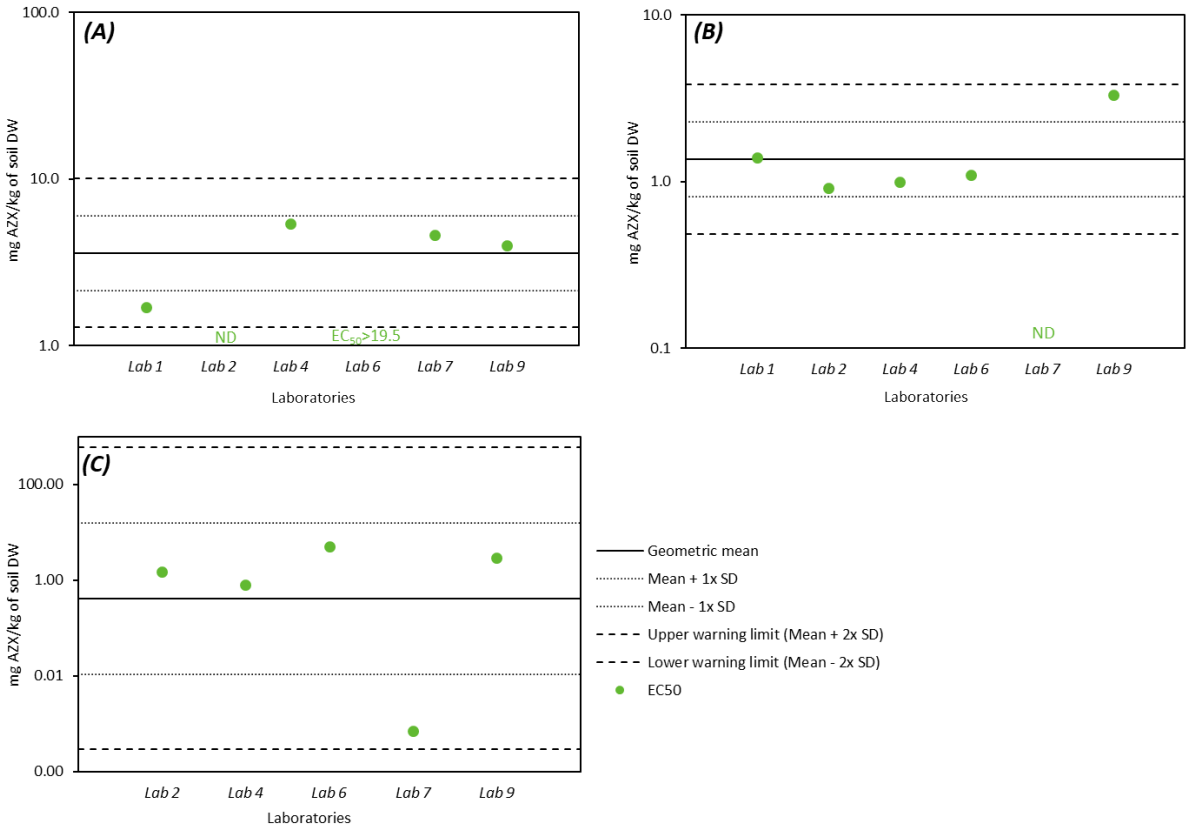
are described in the respective graph. ND – data did not allow the estimation of EC10.



Source: own illustration, CloverStrategy.

Figure 97: Results (EC50 as mg of Azoxystrobin/kg of soil DW) for (A) total colonization, (B) arbuscular colonization and (C) extra-radicular hyphae length from the experiments of the symbiotic phase ring test using inoculant of *R. irregularis* and

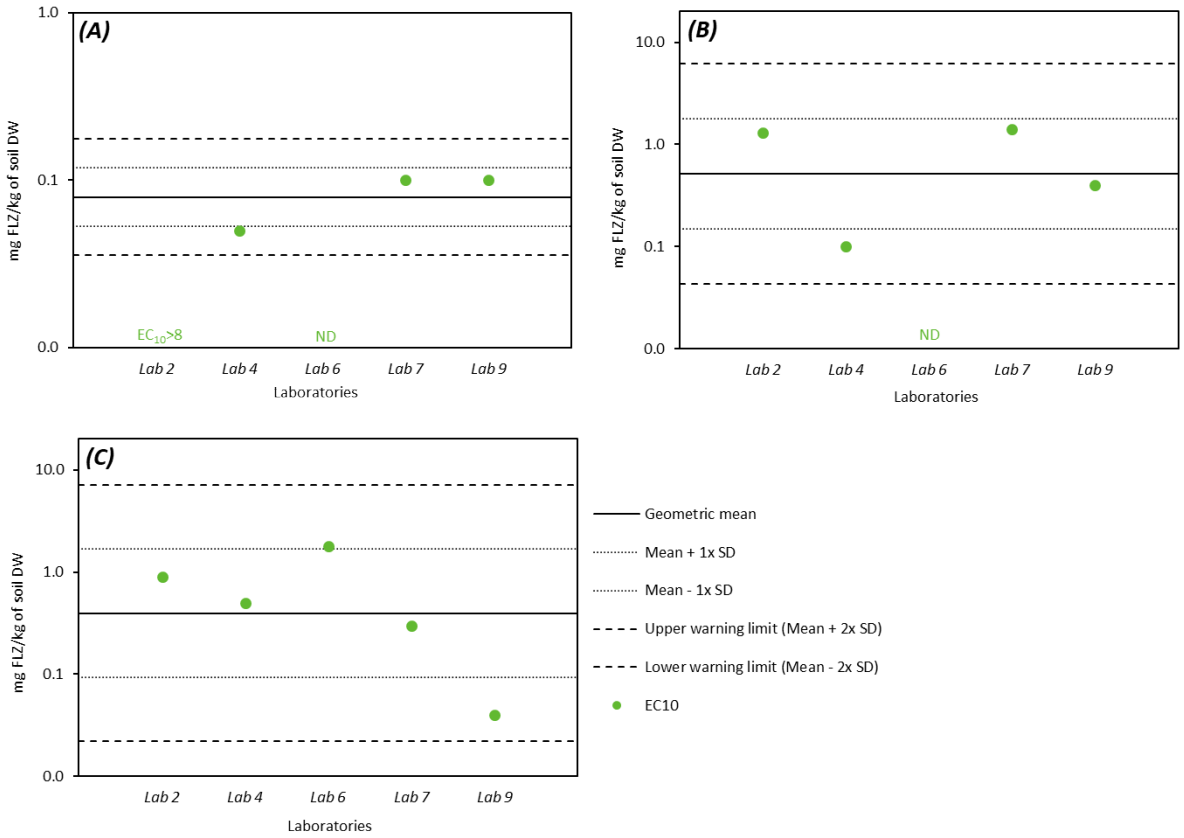
OECD 2.5% artificial soil. EC50 higher than the highest concentration tested are described in the respective graph. ND – data did not allow the estimation of EC50.



Source: own illustration, CloverStrategy.

Figure 98: Results (EC10 as mg of Fluazinam/kg of soil DW) for (A) total colonization, (B) arbuscular colonization and (C) extra-radicular hyphae length from the experiments of the symbiotic phase ring test using inoculant of *R. irregularis* and

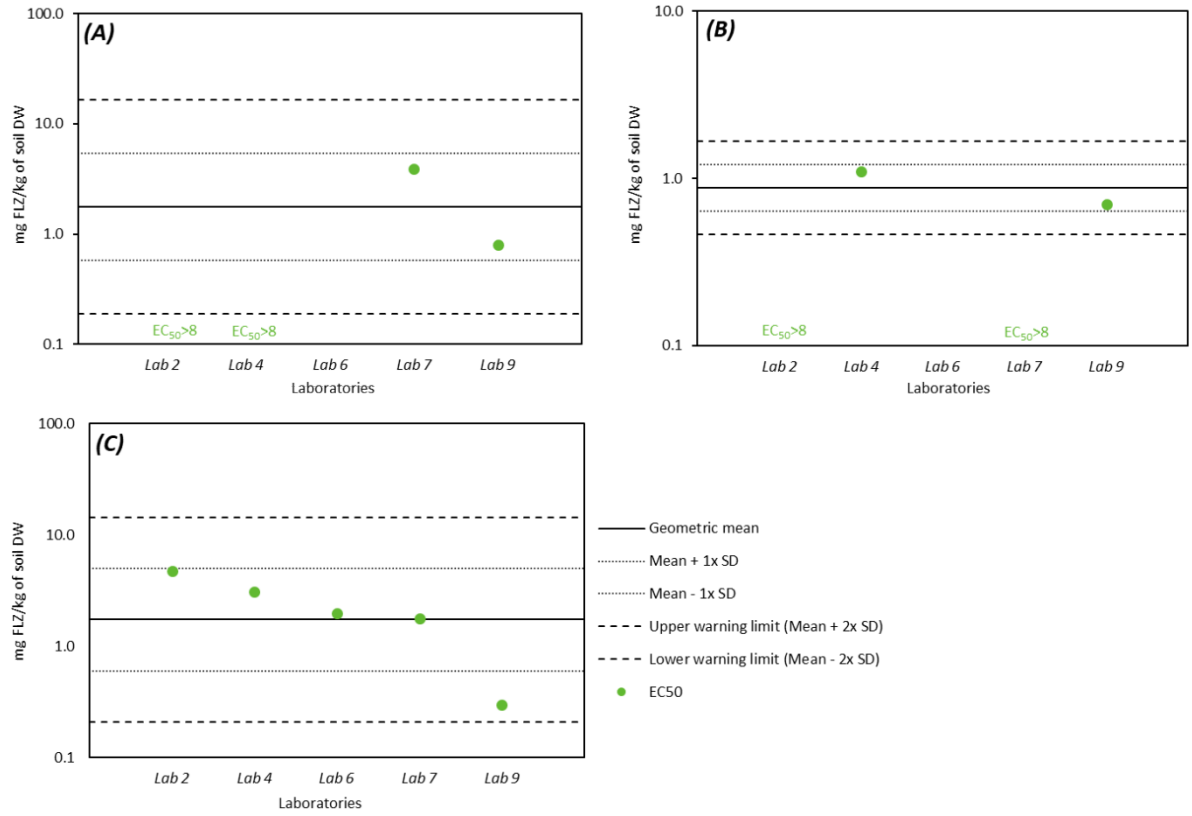
OECD 2.5% artificial soil. EC10 higher than the highest concentration tested are described in the respective graph. ND – data did not allow the estimation of EC10.



Source: own illustration, CloverStrategy.

Figure 99: Results (EC50 as mg of Fluazinam/kg of soil DW) for (A) total colonization, (B) arbuscular colonization and (C) extra-radicular hyphae length from the experiments of the symbiotic phase ring test using inoculant of *R. irregularis* and

OECD 2.5% artificial soil. EC50 higher than the highest concentration tested are described in the respective graph. ND – data did not allow the estimation of EC50.



Source: own illustration, CloverStrategy.

ECx values within the warning limits (upper warning limit= geometric mean + 2x standard deviation (SD); lower warning limit= geometric mean – 2x SD) are considered satisfactory/precise and any ECx values standing outside the warning limits are considered outliers. In this case, all ECx values estimated for the ring test are within the warning limits. However, for example in Figure 97 (graph C), the warning limits are very wide and, therefore, all ECx are within the satisfactory zone. This "distortion" of the warning limits resulted from the low number of laboratories/ECx values and is considered in the analysis. This issue will be discussed further ahead.

The variation on the ECx values must also be subject of analysis. According to Environment Canada (2005), a "reasonable" variation of the ECx data set should correspond to a 30% coefficient of variation and a "preferred" variation to 20% coefficient of variation, and these percentages correspond to standard deviations of 0.132 and 0.03, respectively. The standard deviations estimated for the symbiotic phase ring test are presented in Table 63.

Table 63: Standard deviation (SD) of the data sets obtained in the symbiotic phase ring test.

Test substance	Parameter	SD
Azoxystrobin	EC10 TC	0.81

Test substance	Parameter	SD
	EC10 AC	0.33
	EC10 EHL	1.88
	EC50 TC	0.22
	EC50 AC	0.23
	EC50 EHL	1.58
Fluazinam	EC10 TC	0.17
	EC10 AC	0.54
	EC10 EHL	0.63
	EC50 TC	0.49
	EC50 AC	0.14
	EC50 EHL	0.46

TC – total colonization; AC – arbuscular colonization; EHL – extra-radicular hyphae length.

The estimated ECx values of the ring test are more variable than those considered in the Environment Canada (2005) as reasonable, since all the standard deviations are higher than 0.132. This could be due to the high variability of the estimated endpoints between laboratories that was associated to a low number of valid data sets considered or due to natural intraspecific variability.

In fact, according to the ISO guideline 5594 (2021), a minimum of 6 valid experiments must be obtained from laboratories of 3 different countries to validate a methodology. On this ring test, 11 valid experiments of 4 countries were valid - 6 valid experiments of 4 countries for Azoxystrobin and 5 valid experiments of 4 countries for Fluazinam. Therefore, the general requirements for the number of valid tests in different countries defined in ISO 5594 are met for Azoxystrobin, indicating a potential of the method to be standardized in the future.

However, due to the high variability of the data obtained during the ring test and due to the impossibility of estimating the reproductibility and repeatability of the method, more data would be needed to strengthen validation. Therefore, more laboratories (and more valid experiments) would be needed to narrow the warning limits and the standard deviations estimated for the ECx data sets, allowing the visualization of outliers and a more accurate interpretation of the results for the validation of the reproducibility and repeatability of the method.

Some adaptations to the proposed methodology, such as the increase of the number of replicates in the control treatment (e.g. a minimum of 8 replicates), the use of a source of propagules other than trap cultures (e.g. *in-vitro* cultivations) and the possibility of providing a more customized training to participant laboratories could be important steps to increase the number of valid tests. This would particularly help reduce the coefficient of variation in control replicates, which was the validity criterion that led to the invalidation of most of the data sets produced during the ring test.

3.3 Pre-symbiotic phase ring test

From the 9 laboratories available to the symbiotic phase experiments (including the laboratories from the consortium), 6 were also available to carry out the pre-symbiotic phase experiments (Table 57).

The methodologies for the performance of the pre-symbiotic experiments were described in SOP Test 1 (Appendix A.17) and shared with the participating laboratories to guide them during the ring test. As for the symbiotic phase tests, an Excel spreadsheet with the calculations for the test setup and a spreadsheet for registration of the results was prepared and shared with the participants (Appendix A.28).

In September 2024, an online training coordinated by UDESC (Brazil), was carried out with the laboratories of the pre-symbiotic phase ring test to explore, step-by-step, the methodologies described on SOP Test 1 and to clarify participants' doubts.

Spores of the same AMF species used in the symbiotic phase ring test (i.e. *Rhizophagus irregularis* SAF 22) and the OECD 2.5% artificial soil were selected for the performance of the experiments of the pre-symbiotic phase ring test using Azoxystrobin and Fluazinam as test substances. The gradients of concentrations selected for the experiments were based in the concentrations used on the symbiotic phase ring test and assuming an expected lower sensitivity of spores to the presence of contaminants (according to what is described in literature). Therefore, the selected gradients were:

- 0; 1.25; 3.125; 7.8; 19.5; 39.0 and 78.0 mg Azoxystrobin/kg of soil DW;
- 0; 1.0; 2.0; 4.0; 8.0; 16.0 and 32.0 mg Fluazinam/kg of soil DW;
- A solvent control (CTsol) was also included in each test.

The scheme of soil sampling defined for chemical analysis was based on the following strategy: in each experiment, three soil samples were collected per test treatment at the beginning and at the end of the tests so that the following samples were collected:

- 6 treatments x 2 dates (beginning and end of the test) x 3 samples x 2 substances x 1 soil x 6 laboratories x 1 extractant
- One sample of the control treatment was also collected at the beginning of each test per laboratory.

The determination of initial measured concentrations of the test substances on soil samples of the pre-symbiotic ring test were conducted by Tentamus LAB S.L.U. (Spain), following the same procedures described on the experiments conducted during the 2nd phase of WP2.

3.3.1 Results of the pre-symbiotic phase experiments of the ring test

The pre-symbiotic phase experiments of the ring test started in October 2024. The number of recovered and germinated spores of each replicate was recorded after 14 days of exposure in test treatments and the percentage of germinated spores was estimated.

The validity of the pre-symbiotic phase experiments was evaluated considering the following criteria (described on SOP Test 1 and according to ISO 10832):

- Mean number of recovered spores in all treatments ≥ 25;
- Mean percentage of germinated spores in control treatment ≥ 75% at the end of the test.

Statistical analysis of the data produced in the pre-symbiotic phase experiments to estimate the respective endpoints was similar to that applied in the symbiotic phase experiments.

Normality and homogeneity of data were assessed through Shapiro and Bartlett tests, respectively. When these assumptions were confirmed, a one-way ANOVA was performed to assess differences among the treatments (considering treatments as independent variable and the percentage of spores' germination as dependent variable).

When assumptions of data normality and/or homogeneity were not confirmed, data were logarithm (i.e., log (x+1)) or square root (i.e., $\sqrt{x}$) transformed. When the transformed data complied with the assumptions, a one-way ANOVA was performed, following the methodology described above. When the transformed data did not comply with the assumptions, a non-parametric Kruskal-Wallis' test was performed, using the non-transformed data, to assess the differences among treatments.

When significant differences were found (either through one-way ANOVA or Kruskal-Wallis' test), differences between each treatment and the respective control were assessed through a post-hoc Dunnett test or a post-hoc Dunn test (with Bonferroni correction). The No-Observed Effect Concentration (NOEC) and the Lowest-Observed Effect Concentration (LOEC) were estimated for both approaches. All the statistical analyses were performed considering a significance level of 5%.

Data regarding the percentage of spores' germination between test treatments was analysed using R Studio (version 2023.09.1), with packages drc (v. 3.0-1). car (v. 3.1-2), dunn.test (v. 1.3.6), DescTools (v. 0.99.52) and PMCMRplus (v. 1.9.10).

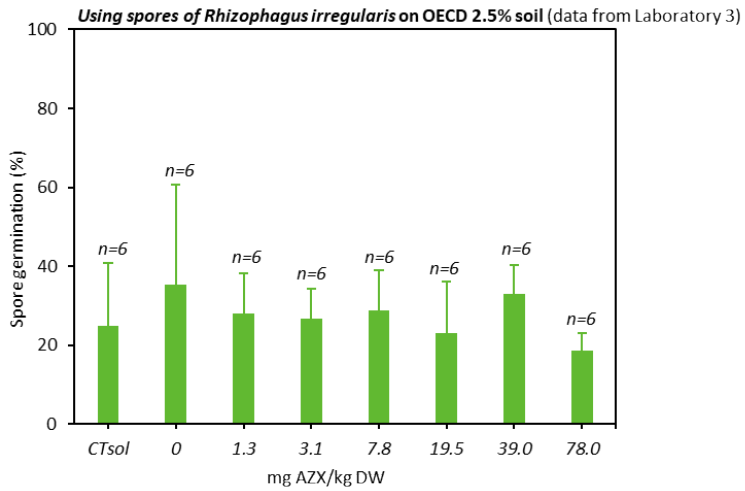
When possible, and only for valid experiments, Inhibition Concentration values for 10% and 50% inhibition of spore germination when compared to control were estimated (IC10 and IC50, respectively) trough non-linear regression models (using spore germination as dependent variable and concentrations as independent variable; models applied were logistic, exponential or Hormesis). The 95% confidence intervals were also estimated when data allowed. The most adequate model was selected through the highest coefficient of regression (r^2) and best fit to the data. The IC10 and IC50 estimations were performed in R Studio (version 2023.09.1), using packages drc (v. 3.0-1), tidyverse (v. 2.0.0), readxl (v. 1.4.3) and PMCMRplus (v. 1.9.10).

Results of the pre-symbiotic phase experiments obtained in the ring test are presented in the next sections. The same code assigned to each participating laboratory in the ring test of the symbiotic phase test was considered in the ring test for the pre-symbiotic phase tests.

3.3.1.1 AMF pre-symbiotic phase experiments of the ring test with Azoxystrobin

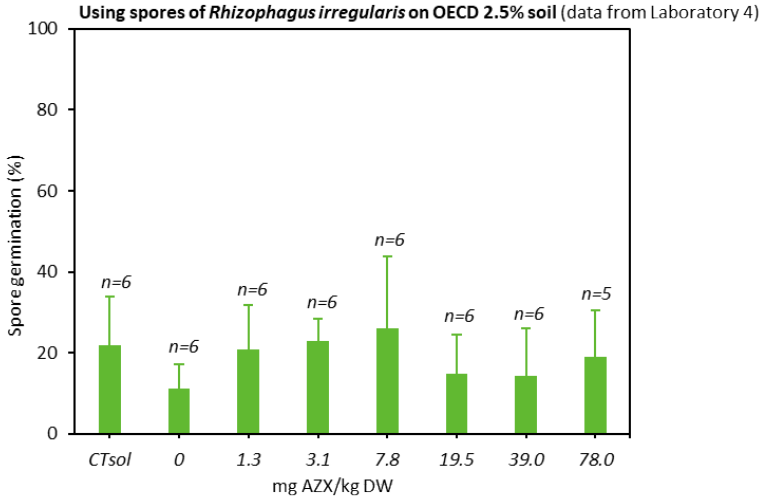
The results obtained by the six laboratories of the pre-symbiotic phase test experiments with Azoxystrobin are presented in Figures 100 to 105 and raw data are presented in Appendix A.29.

Figure 100: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 3.



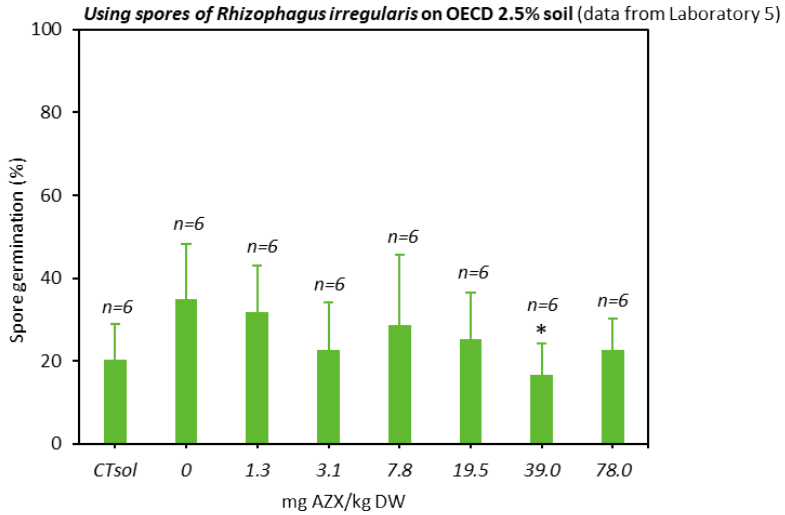
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 64). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 101: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 4.



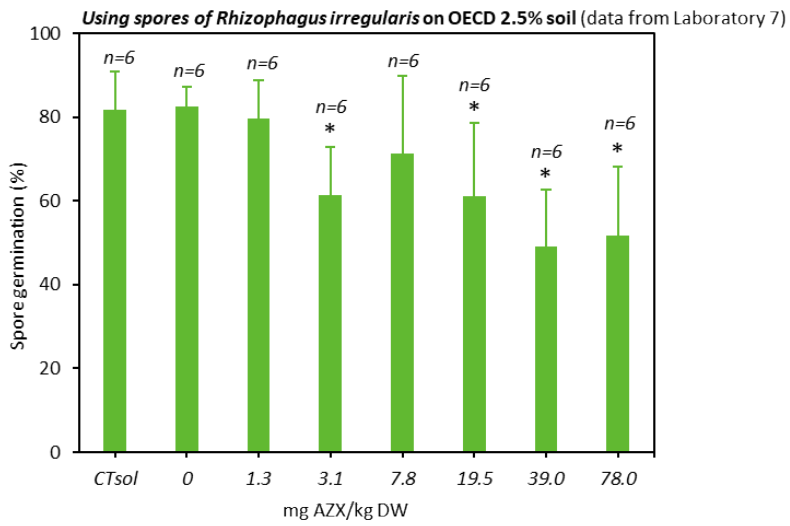
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 64). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 102: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 5.



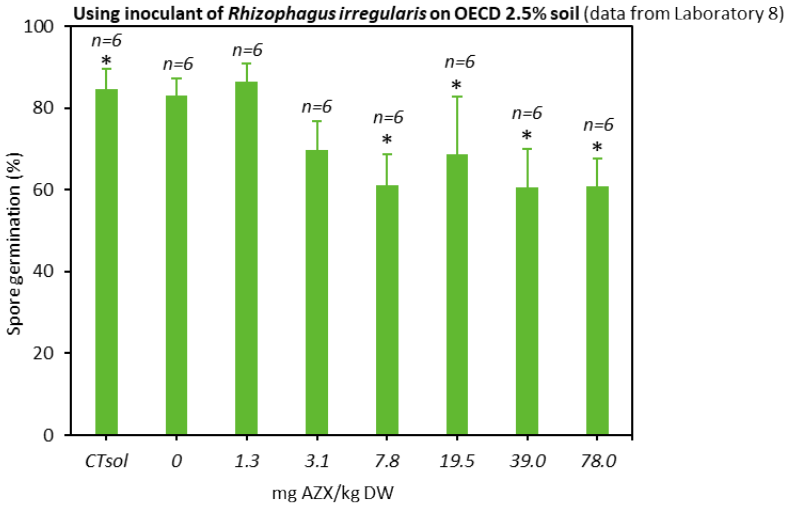
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 64). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 103: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 7.



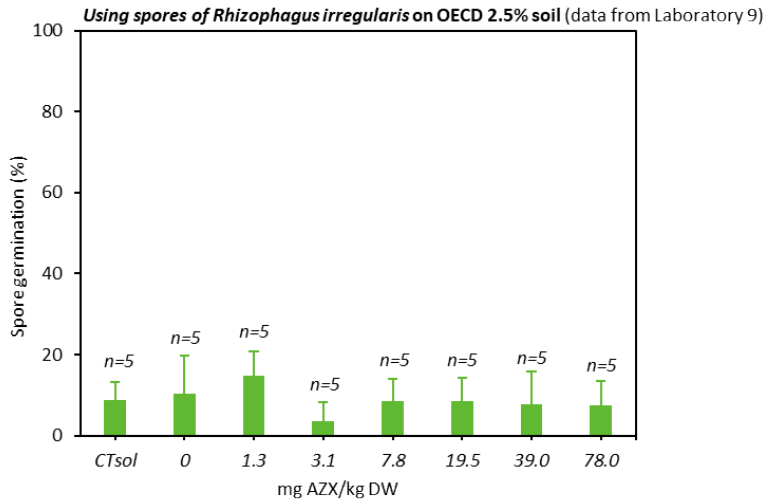
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is valid (Table 64). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 104: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 8.



Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is valid (Table 64). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 105: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Azoxystrobin; Performing lab: Laboratory 9.



Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 64). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

3.3.1.1.1 Summary of the results obtained in AMF pre-symbiotic phase experiments of the ring test with Azoxystrobin

From the six experiments performed, only two were valid (experiments of laboratories 7 and 8). The compliance of each experiment with the validity criteria is summarized in Table 64.

Table 64: Summary of the compliance with the validity criteria defined in SOP Test 1 of the pre-symbiotic phase experiments of the ring test carried out exposing *R. irregularis* to Azoxystrobin in OECD 2.5% artificial soil.

Lab	Nº recovered spores/treatment ≥ 25								Germinated spores in C0 ≥ 75%	Test validity
	Ctsoil	C0	C1	C2	C3	C4	C5	C6		
L3	27.5	**23.8**	27.5	26.7	25.8	25.8	25.7	**24.8**	**35.3%**	**Invalid**
L4	27.2	27.8	29.3	27.7	25.8	26.7	25.2	25.3	**11.3%**	**Invalid**
L5	26.5	25.2	25.0	26.2	27.7	26.8	28.8	29.2	**35.1%**	**Invalid**
L7	27.7	28.5	28.8	28.0	28.0	28.5	27.7	28.5	82.5%	Valid
L8	27.7	28.7	28.2	26.5	28.2	27.0	28.7	27.3	83.0%	Valid
L9	29.4	28.4	27.2	28.4	28.4	28.0	27.8	27.8	**10.3%**	**Invalid**

Chemical analyses to determine the actual contamination of Azoxystrobin in soil samples collected at the beggining and end of the experiments were performed following the modified QuEChERS methodology (Perestrelo et al., 2019). Results are presented on Appendix A.31.

The endpoints estimated for the effects of Azoxystrobin on the spore germination of *R. irregularis* in OECD 2.5% artificial soil are presented on Table 65.

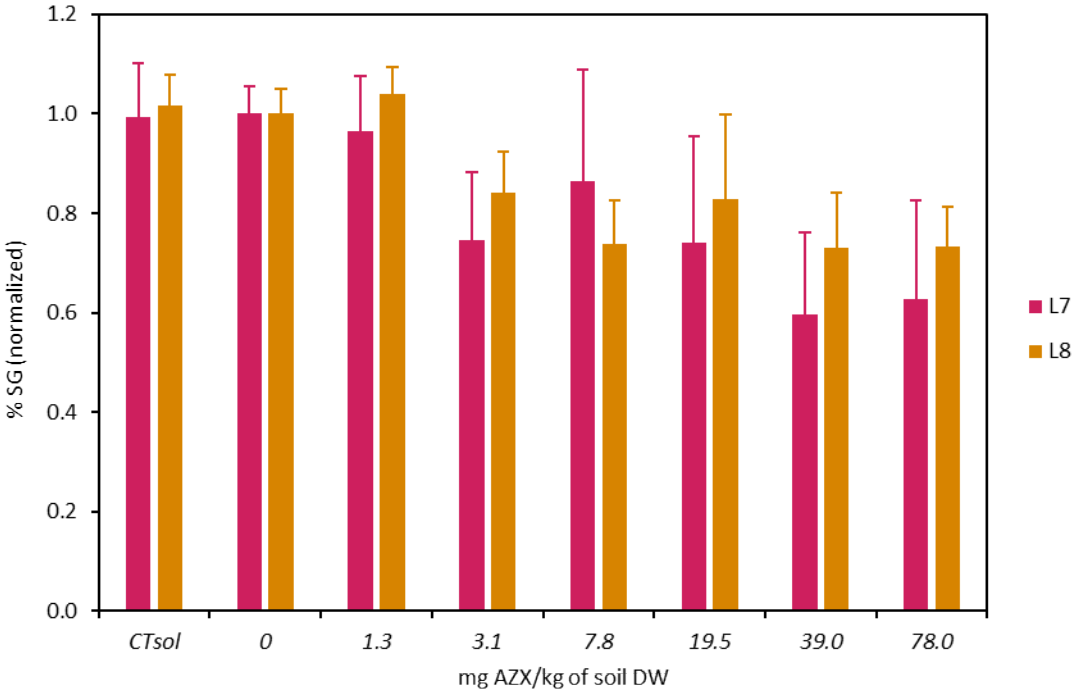
Table 65: **Summary of the experiments performed in the pre-symbiotic phase ring test with Azoxystrobin in OECD 2.5% artificial soil. Nominal concentrations used in the experiments were: 0; 1.3; 3.1; 7.8; 19.5; 39.0 and 78.0 mg AZX/kg of soil DW. No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Inhibition Concentrations for 10% and 50% (IC10 and IC50) reduction of spore germination (SG) were estimated in valid experiments. ICx values were estimated based on nominal and on initial measured concentrations. Results are expressed in mg AZX/kg of soil dry weight and presented with the respective 95% confidence interval, when data allowed. Non-linear models applied and the respective r^2 values are presented in Appendix A.32.**

Laboratory	State (valid/invalid)	Parameters	Nominal concentrations				Initial measured concentrations			
			NOEC	LOEC	IC10	IC50	NOEC	LOEC	IC10	IC50
Lab 3	Invalid	SG	-	-	-	-	-	-	-	-
Lab 4	Invalid	SG	-	-	-	-	-	-	-	-
Lab 5	Invalid	SG	-	-	-	-	-	-	-	-
Lab 7	Valid	SG	1.3	3.1	1.6*	10.2*	1.0	1.3	1.5 (0.9-2.2)	10.2 (5.8-14.5)
Lab 8	Valid	SG	3.1	7.8	0.4 (0.1-0.6)	2.5 (0.8-4.2)	3.0	8.0	0.4 (0.1-0.8)	2.9 (0.6-5.2)
Lab 9	Invalid	SG	-	-	-	-	-	-	-	-

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of IC10 and IC50.

The percentage of spores' germination obtained on the experiments described above was normalized by dividing the percentage of spores' germination obtained in each replicate by the germination observed in the negative control (0 mg test AZX/kg of soil DW). Normalization of data makes easier the comparability of the data among laboratories as the response obtained in each experiment (and therefore, by each laboratory) should be analysed in relation to its own control response. The normalized percentage of spores' germination for the pre-symbiotic experiments conducted with Azoxystrobin is presented in Figure 106.

Figure 106: Percentage of spores germination (on average and normalized to the respective control response) observed in valid pre-symbiotic phase experiments of the ring test exposing spores of the AMF *R. irregularis* to OECD 2.5% artificial soil spiked with Azoxystrobin.



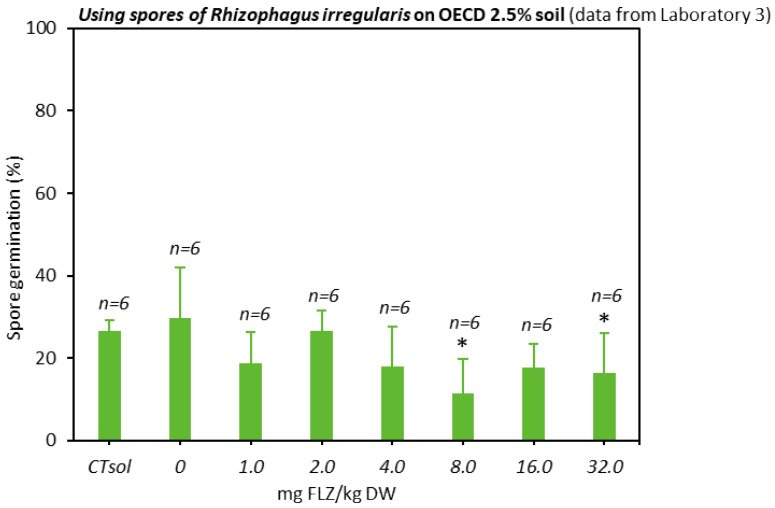
Source: own illustration, CloverStrategy.

The percentages of spores germination in the valid experiments were similar among laboratories. Results suggest a slight tendency (although without statistically significant evidence) for the reduction of spores germination among the increasing concentrations of Azoxystrobin in both laboratories.

3.3.1.2 AMF pre-symbiotic phase experiments of the ring test with Fluazinam

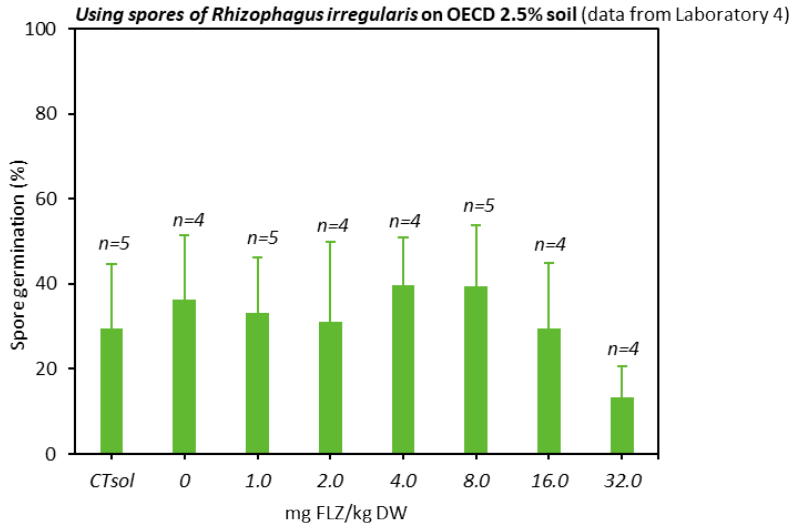
The results obtained by the six laboratories of the pre-symbiotic phase experiments with Fluazinam are presented in Figures 107 to 112 and raw data are presented in Appendix A.30.

Figure 107: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 3.



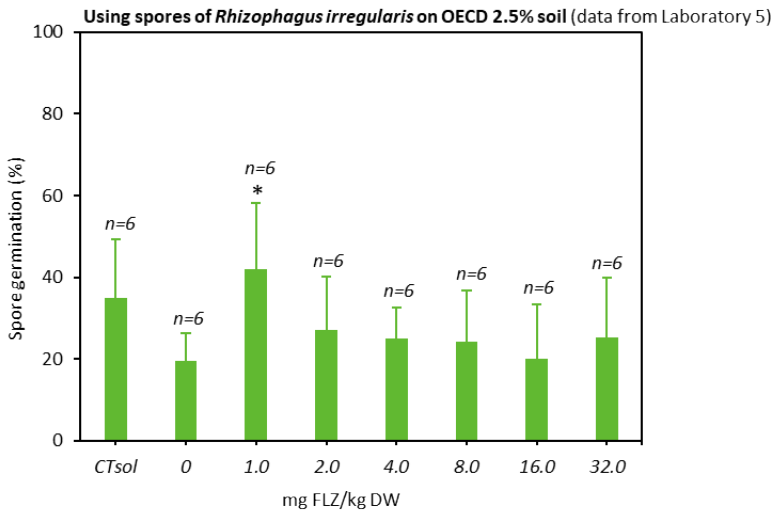
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 66). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 108: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 4.



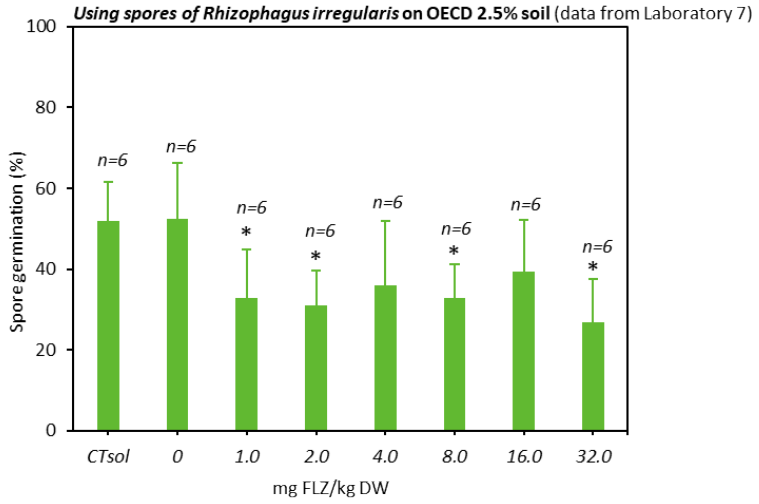
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is valid (Table 66). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars.
Source: own illustration, CloverStrategy.

Figure 109: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 5.



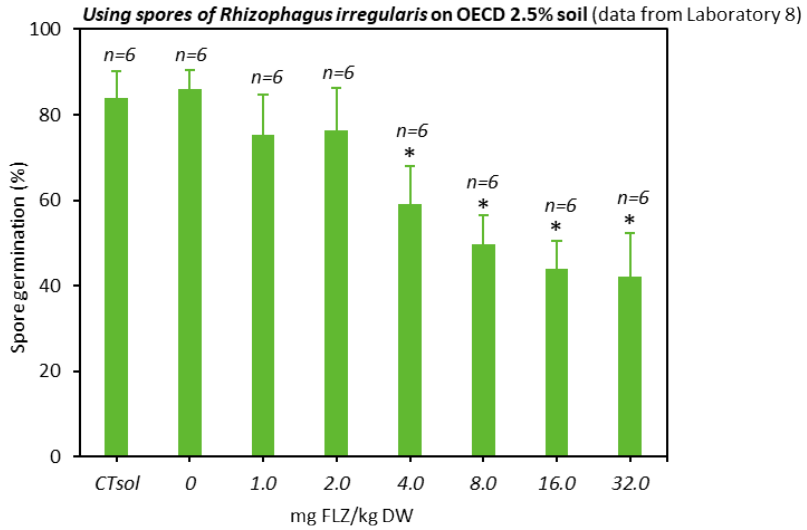
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 66). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 110: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 7.



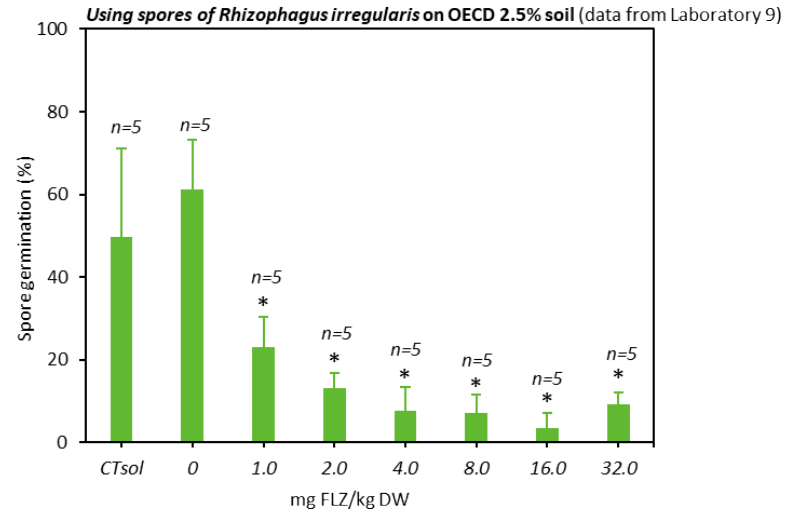
Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 66). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 111: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 8.



Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is valid (Table 66). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

Figure 112: Spores: *Rhizophagus irregularis*; Test soil: OECD 2.5%; Test substance: Fluazinam; Performing lab: Laboratory 9.



Percentage of germinated spores (average ± standard deviation) after 14 days of exposure. Test is invalid (Table 66). *n* values considered in each treatment for each parameter are expressed in the top of the respective bars; * - means significantly different compared to control after one-way ANOVA/Kruskal-Wallis followed by Dunnett/Dunn post hoc test for $p \leq 0.05$.
Source: own illustration, CloverStrategy.

3.3.1.2.1 Summary of the results obtained in AMF pre-symbiotic phase experiments of the ring test with Fluazinam

From the six experiments conducted during this phase, only one was valid (experiment of laboratory 8) according to the validity criteria defined on SOP Test 1 (Table 66).

Table 66: Summary of the compliance with the validity criteria defined in SOP Test 1 of the pre-symbiotic phase experiments of the ring test carried out exposing *R. irregularis* to Fluazinam in OECD 2.5% artificial soil.

Lab ID	Nº recovered spores/treatment ≥ 25								Germinated spores in C0 ≥ 75%	Test validity
	Ctsol	C0	C1	C2	C3	C4	C5	C6		
L3	27.0	26.8	27.8	26.8	28.8	29.3	27.5	29.2	**29.8%**	**Invalid**
L4	28.0	29.0	28.6	27.3	27.0	27.6	28.0	25.5	**36.2%**	**Invalid**
L5	26.5	26.2	28.0	28.7	27.7	26.3	27.7	26.5	**19.5%**	**Invalid**
L7	29.0	26.8	**24.5**	27.7	29.3	28.7	25.7	25.3	**52.5%**	**Invalid**
L8	27.7	28.7	27.5	27.0	27.0	27.8	27.3	27.5	86.0%	Valid
L9	25.6	27.4	29.6	27.4	28.8	28.8	28.0	28.2	**61.3%**	**Invalid**

Chemical analyses to determine the actual contamination of Fluazinam in soil samples collected at the beginning and end of the experiments were performed following the modified QuEChERS methodology (Perestrelo et al., 2019). Results are presented on Appendix A.31.

The endpoints estimated for the effects of Fluazinam on the germination of *R. irregularis* spores in the valid tests are presented on Table 67.

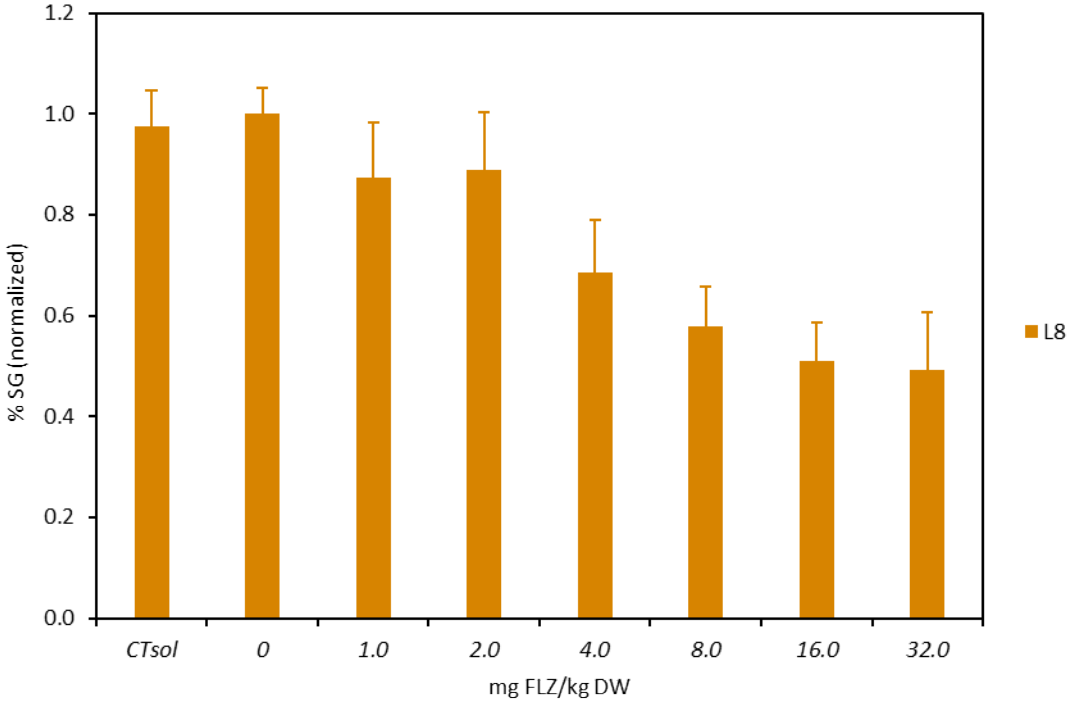
Table 67: **Summary of the experiments performed in the pre-symbiotic phase ring test with Fluazinam in OECD 2.5% artificial soil. Nominal concentrations used in the experiments were: 0; 1.0; 2.0; 4.0; 8.0; 16.0 and 32.0 mg FLZ/kg of soil DW. No-Observed Effect Concentration (NOEC), Lowest Observed Effect Concentration (LOEC) and Inhibition Concentrations for 10% and 50% (IC_{10} and IC_{50}) reduction of spores' germination (SG) were estimated in valid experiments only. ICx values were estimated based on nominal and initial measured concentrations. Results are expressed in mg FLZ/kg of soil dry weight and presented with the respective 95% confidence interval. Non-linear models applied and the respective r^2 values are presented in Appendix A.32.**

Laboratory	State (valid/invalid)	Parameters	Nominal concentrations				Actual concentrations			
			NOEC	LOEC	IC10	IC50	NOEC	LOEC	IC10	IC50
Lab 3	Invalid	SG	-	-	-	-	-	-	-	-
Lab 4	Invalid	SG	-	-	-	-	-	-	-	-
Lab 5	Invalid	SG	-	-	-	-	-	-	-	-
Lab 7	Invalid	SG	-	-	-	-	-	-	-	-
Lab 8	Valid	SG	2.0	4.0	0.5 (0.3-0.7)	3.3 (1.9-4.7)	1.3	2.8	0.3 (0.2-0.5)	2.2 (1.3-3.1)
Lab 9	Invalid	SG	-	-	-	-	-	-	-	-

* - Data did not allow to estimate the 95% confidence intervals. ND - the fit of the model did not allow the estimation of IC10 and IC50.

As performed with the data of Azoxystrobin, the percentage of spores' germination obtained on the pre-symbiotic experiments with Fluazinam was normalized by dividing the percentage of spores' germination of each replicate by the mean response observed on the negative control (0 mg FLZ/kg of soil DW). The normalized data for spores' germination after exposure to Fluazinam are presented on Figure 113.

Figure 113: Data obtained in the valid pre-symbiotic phase experiment of the ring test exposing spores of the AMF *R. irregularis* in OECD 2.5% artificial soil spiked with Fluazinam.



Source: own illustration, CloverStrategy.

There was a significant reduction in the percentage of spores' germination compared to the control in concentrations higher or equal to 4 mg FLZ/kg of soil.

3.3.2 Conclusions in pre-symbiotic phase testing

The pre-symbiotic phase ring test was conducted to evaluate the validity of the adapted method suggested by the ERAMYC consortium for the assessment of effects of chemicals based on the germination of spores of the AMF *Rhizophagus irregularis* (an AMF species different than that suggested by ISO 10832).

From the 12 pre-symbiotic phase experiments performed (including both test substances), 3 complied with the validity criteria. This corresponds to the validation of 25% of the experiments performed. From the 7 laboratories participating on the ring test, only two validated at least one of the experiments performed (either with Azoxystrobin or with Fluazinam). The results obtained during the pre-symbiotic phase ring test do not confirm the results obtained in the ISO document 10832 using *F. mosseae* (for which at least 4 data sets/laboratories were considered). Difficulties in handling and/or selection spores of *R.*

irregularis, which are considerably smaller than those of *F. mosseae*, may be one of the reasons for the low number of valid tests obtained in the pre-symbiotic phase experiments.

Due to the lack of valid experiments in this phase, the number of data sets obtained was insufficient to evaluate the reproducibility and/or precision of the method, according to the approaches described in ISO 5594 (2021), ISO 5725-2 (1994) and Environment Canada EPS 1/RM/46 (2005). More participating laboratories and valid experiments would be needed do draw further conclusions regarding the validity of the method proposed. Moreover, some adaptations to the methodologies proposed could be important for obtaining more valid experiments, such as increasing the number of replicates in the control treatment (e.g. minimum of 8 replicates) and providing more costumized and intensive training for the technicians performing the methods (e.g. in isolation and handling of AMF spores).

The endpoints estimated for the pre-symbiotic experiments conducted with Azoxystrobin and Fluazinam are within the same order of magnitude as the generality of the endpoints estimated in the symbiotic phase experiments. This suggests that AMF spores' germination is as sensitive to the exposure to Azoxystrobin or Fluazinam as the symbiotic parameters measured (i.e. total and arbuscular colonization and extra-radicular hyphae length). Data from literature do not provide clarity on whether pre-symbiotic or symbiotic parameters are the most sentitive, if exposed to chemicals. Moreover, as concluded in WP1, it should be noted that the relative sensitivity of pre-symbiotic parameters/symbiotic parameters may depend on the test substance. More data (with more AMF species, test soils and test substances) would be needed to further assess the relative sensitivity of both AMF phase methods.

4 Section 4 – WP4: Preparation of OECD documents

The ERAMYC consortium worked in the development of two drafts for OECD documents for ecotoxicological testing with AMF, one for the AMF pre-symbiotic phase and one for AMF symbiotic phase. These documents are presented in Appendixes A.33 and A.34, respectively for pre-symbiotic and symbiotic phase testing.

These draft documents, constituting the deliverables of the WP4 of the ERAMYC project, took into consideration the methodologies discussed and agreed during the ERAMYC project that were based on the information available in existing literature, with special attention to the ISO document 10832 (ISO, 2009) for the pre-symbiotic phase test, and to the documentation fascicule from AFNOR for the effects of pollutants on the symbiotic phase of AMF (AFNOR, 2014).

Despite the relatively high variability of the data collected during the ring tests, the information provided about the methodologies developed during the project constitute a highly relevant contribution for the standardization of a method to measure the toxicity of chemicals in soil for the establishment of AMF-plant symbiosis and for pre-symbiotic phase testing.

5 Final remarks

The ERAMYC project raised from the necessity of filling some of the gaps identified on EFSA's scientific opinion from 2017 (EFSA, 2017) for the inclusion of microorganisms on ERA schemes for PPPs.

Concerning AMF testing, these gaps included the lack of a standardized method to evaluate the effects of contaminants on the AMF symbiotic phase of the development of these organisms, despite the technical specification document from AFNOR, and the refinement of the methodologies described on ISO 10832 for the effects on the AMF pre-symbiotic development.

The ERAMYC project comprised an extensive literature review on the state of the art of the use of AMF on ecotoxicological studies, including both phases of the AMF life cycle. Then, based on the information collected during the literature review and on the experimental knowledge of a group of experts that made part of the project consortium, a series of experiments was carried out to define and refine methodologies to be used in laboratory experiments to evaluate the effects of contaminants on AMF development.

The performance of the ring test for pre-symbiotic and symbiotic AMF phases revealed that, despite the important developments made towards standardization and the feasibility of the methodologies described, these could not be considered reproductible due to the high variance observed among the results obtained.

The broad genetic variability of AMF associated with the existing gap of knowledge about the AMF structures that comprise the propagules of the AMF species, could have been at the origin of the difficulties in standardizing the method for these organisms. According to Purin et al. (2012), AMF from the same species and origin can be genetically different, which can cause genetic incompatibility and impair the formation of anastomosis. According to Mallman et al. (2018), the multinucleate spores from the same species but with different origins can originate different results when submitted to testing. Because of that, these authors suggest that different culturing/test conditions may be needed for AMF with different origins, which makes it more difficult to standardize a procedure for AMF testing. Some experts even defend that the use of AMF propagules from *in-vitro* cultivations instead of trap cultures may promote a considerable reduction in variability of test results.

Another aspect that can contribute to the variability of data is the technical skills required to the adequate measurement of the AMF parameters of both phases by the technicians that carry out the tests. Actually, little experience of a technician in the measurements of AMF parameters, can make him/her take faulty decisions, resulting in a high variance in the generated data. From this point of view, the selection of parameters less prone to be influenced by the technician procedures/criteria can contribute to reduce the variability of data.

In the ring-test for the AMF symbiotic phase method, the high variability of data did not allow the estimation of endpoints from the data of six laboratories, which is the minimum number advised to confirm the reproducibility of the method. Despite the online training sessions ministered by the consortium to the laboratories of the ring test, the inexperience of the laboratories could have contributed to the variability of the data. Actually, data analysis of the experiments of the 2nd phase of WP2 evidenced that there are AMF parameters more prone to be influenced by laboratory technicians (e.g. the measurements of the percentage of arbuscular colonization). Data of ring test also showed a higher variability in measurements of arbuscular

colonization and extra-radicular hyphae length than in total colonization measurements. Another aspect that was not achieved in this method was the definition of a reference substance. Although the experiments carried out in WP2 have indicated that Azoxystrobin and Fluazinam could be adequate for reference substances, the experiments of the ring test did not confirm that, mainly due to the high data variability. Despite that, beyond Azoxystrobin and Fluazinam, other substances like Terbuthylazine and even Copper sulphate showed to have potential to be used as reference substances, but further tests would be needed to confirm that. Taking these considerations into account, further research steps are needed to substantiate the standardization of the AMF symbiotic phase testing. From this perspective, the following aspects should be considered in the future work for the standardization of the symbiotic phase testing:

- The measurement of AMF parameters less dependent on the technical skills/criteria of the technician (e.g. percentage of total colonization) should be prioritized;
- Investment in testing Copper sulphate and/or Terbuthylazine should be made to confirm if any of these substances are adequate to be used as reference chemical in AMF symbiotic phase method;
- The use of AMF propagules from in-vitro cultivations instead of trap cultures should be checked with the aim of reducing data variability.

In the ring test of pre-symbiotic phase method, the low number of valid tests resulted in an insufficient amount of data to confirm the reproducibility of the method. The reduced number of valid tests can be related to the AMF species (*R. irregularis* spores may be too sensitive not being viable after handling for testing) or to a dormant state of the spores previously collected from the inoculants used in the experiments. Therefore, for the standardization of the pre-symbiotic phase method, further experiments are needed, and the following aspects should be considered:

- Spores from AMF species other than *R. irregularis* should be tested;
- Especial care must be taken when handling spores to be used in testing.

Despite the impossibility of supporting the validation of the methods applied during ERAMYC, the effect data produced are relevant to support the importance and urgency of including AMF sensitivity on ERA schemes.

The comparison between the endpoints obtained for in-soil non-target standard species considered in the current ERA (i.e. *Folsomia candida, Eisenia andrei/fetida, Hypoaspis aculeifer*, among plant species and microorganisms) and those estimated for AMF showed that, for three of the seven substances tested in WP2, AMF are more than 5 times more sensitive to the test substances than the other groups. Therefore, the data suggest that AMF are not sufficiently protected in the current ERA schemes.

Moreover, the results obtained in this project show that initial measured concentrations of the majority of tested substances were below 80 % already at test start. Constant exposure during the whole test was hampered due to degradation of test substances. Although the endpoints estimated based on nominal concentrations are generally within a factor of 5 compared to those estimated from initial measured concentrations, differences up to factor 50000 were observed. Therefore, ERA for in-soil organisms based strictly on nominal values may not be sufficiently protective. Performing chemical analyses and estimating endpoints based on

measured concentrations can provide more relevant data to complement and confirm the accuracy of the procedures applied during the tests, leading to a more accurate basis for regulation.

6 Dissemination of the ERAMYC project

The ERAMYC project was disseminated in various occasions:

- SETAC Europe 32nd Annual Meeting, between May 15th and 19th 2022 in Copenhagen, Denmark. The project was presented by Tiago Natal da Luz as an oral platform in the session "4.10 – Linking soil ecology, ecotoxicology and risk assessment to the impact of multiple stressors on soil organism communities in the field" chaired by Silvia Pieper, M. Nazaret Gonzalez-Alcaraz, Susana Loureiro and Tiago Natal da Luz that was held on May 17th, 2022. The extended abstract submitted to this congress is presented in Appendix A.35.
- SETAC Europe 33rd Annual Meeting, between April 30th and May 4th, 2023, in Dublin, Ireland. The project was presented by Tiago Natal da Luz as an oral platform in the session "6.14 – "Getting the Soil Loop Rolling: from Ecotoxicology to Risk Prediction to Monitoring and Back to Better Protect Soils Under the European Green Deal" chaired by Silvia Pieper, Amy C. Brooks, Gregor Ernst and Paola Grenni that was held on May 1st, 2023. The extended abstract submitted to this congress is presented in Appendix A.36.
- Summer School of the Aristo Project (a project focused on the development of tools, methods and procedures to assess in the most comprehensive and robust way the toxicity of pesticides on soil microorganisms) by Tiago Natal da Luz on May 30th, 2024, at the invitation of Prof Dimitrios Karpoyzas from Thessaly University, Greece.
- An abstract was accepted for poster presentation on SETAC Europe 35th Annual Meeting that will be held on Viena, Austria, between 11th and 15th May 2025. The submitted extended abstract is presented in Appendix A.37.
- An abstract was submitted for an oral presentation on SETAC Latin America 16th Biennial Meeting that will be held on Lima, Peru, between the 26th and 29th August 2025. The submitted abstract is presented in Appendix A.38.

7 List of references

Abarenkov, K., Tedersoo, L., Nilsson, R. H., Vellak, K., Saar, I., Veldre, V., Parmasto, E., Prous, M., Aan, A., Ots, M., Kurina, O., Ostonen, I., Jõgeva, J., Halapuu, S., Põldmaa, K., Toots, M., Truu, J., Larsson, K.-H., & Kõljalg, U. (2010). PlutoF—a Web Based Workbench for Ecological and Taxonomic Research, with an Online Implementation for Fungal ITS Sequences. Evolutionary Bioinformatics, 6. https://doi.org/10.4137/EBO.S6271

Akiyama, K. (2007). Chemical Identification and Functional Analysis of Apocarotenoids Involved in the Development of Arbuscular Mycorrhizal Symbiosis. *Bioscience, Biotechnology, and Biochemistry*, *71*(6), 1405–1414. https://doi.org/10.1271/bbb.70023

Allen, E. B., & West, N. E. (1993). Nontarget effects of the herbicide tebuthiuron on mycorrhizal fungi in sagebrush semidesert. *Mycorrhiza*, *3*(2), 75–78. https://doi.org/10.1007/BF00210696

Alotaibi, M. O., Saleh, A. M., Sobrinho, R. L., Sheteiwy, M. S., El-Sawah, A. M., Mohammed, A. E., & Elgawad, H. A. (2021). Arbuscular Mycorrhizae Mitigate Aluminum Toxicity and Regulate Proline Metabolism in Plants Grown in Acidic Soil. *Journal of Fungi*, *7*(7), 531. https://doi.org/10.3390/jof7070531

AMF PHYLOGENY (2021): Phylogeny and taxonomy of Glomeromycota ('arbuscular mycorrhizal [AM] and related fungi'). Glomeromycota SPECIES LIST. http://www.amf-phylogeny.com/amphylo_species.html. (20.04.2021)

Amorim, M. J. B., Natal-da-Luz, T., Sousa, J. P., Loureiro, S., Becker, L., Römbke, J., & Soares, A. M. V. M. (2012). Boric acid as reference substance: pros, cons and standardization. *Ecotoxicology*, *21*(3), 919–924. https://doi.org/10.1007/s10646-011-0832-9

Antunes, P. M., & Koyama, A. (2017). Mycorrhizas as Nutrient and Energy Pumps of Soil Food Webs. In *Mycorrhizal Mediation of Soil* (pp. 149–173). Elsevier. https://doi.org/10.1016/B978-0-12-804312-7.00009-7

Association Française de Normalisation (AFNOR). (2014): Qualité du sol - Effets des polluants vis-à-vis des champignons mycorhizogènes - Partie 2 : essai de mycorhization racinaire. In Normes nationales et documents normatifs nationaux. Association Française de Normalisation. https://www.boutique.afnor.org/fr-fr/norme/fd-x312052/qualite-du-sol-effets-des-polluants-visavis-des-champignons-mycorhizogenes-/fa174745/44258

Association Française de Normalisation (AFNOR) (2024): NF X 31 – 120 – Soil quality - Determination of copper, iron, manganese and zinc contents - Extraction by means of ammonium acetate in presence of EDTA. https://www.boutique.afnor.org/fr-fr/norme/nf-x31120/qualite-des-sols-determination-du-cuivre-du-fer-du-manganese-et-du-zinc-ext/fa203589/415783

Azevedo, G., & Souza, F. (2007). Crescimento, ontogenia e dinâmica de esporulação de uma estirpe dismórfica de fungo micorrízico arbuscular (Glomeromycota) do gênero *Glomus* em cultivo *in vitro*. *Anais do 5º Congresso Brasileiro de Micologia*, UFPE, Recife.

Bártíková, H., Podlipná, R., & Skálová, L. (2016). Veterinary drugs in the environment and their toxicity to plants. *Chemosphere*, *144*, 2290–2301. https://doi.org/10.1016/j.chemosphere.2015.10.137

Basile, A. (2008): Desenvolvimento de teste ecotoxicológico com o fungo *Alternaria cassiae*: toxicidade aguda de agrotóxicos e avaliação de risco ambiental. Dissertação (Mestrado em Produção Vegetal), Faculdade de Ciências Agrárias e Veterinárias – UNESP. URI: http://hdl.handle.net/11449/96877

Beauregard, M. S., Gauthier, M.-P., Hamel, C., Zhang, T., Welacky, T., Tan, C. S., & St-Arnaud, M. (2013). Various forms of organic and inorganic P fertilizers did not negatively affect soil- and root-inhabiting AM fungi in a maize–soybean rotation system. *Mycorrhiza*, *23*(2), 143–154. https://doi.org/10.1007/s00572-012-0459-6

Becard, G., & Fortin, J. A. (1988). Early events of vesicular–arbuscular mycorrhiza formation on Ri T-DNA transformed roots. *New Phytologist*, *108*(2), 211–218. https://doi.org/10.1111/j.1469-8137.1988.tb03698.x

Becker, L., Scheffczyk, A., Förster, B., Oehlmann, J., Princz, J., Römbke, J., & Moser, T. (2011). Effects of boric acid on various microbes, plants, and soil invertebrates. *Journal of Soils and Sediments*, *11*(2), 238–248. https://doi.org/10.1007/s11368-010-0282-7

Begum, N., Qin, C., Ahanger, M. A., Raza, S., Khan, M. I., Ashraf, M., Ahmed, N., & Zhang, L. (2019). Role of Arbuscular Mycorrhizal Fungi in Plant Growth Regulation: Implications in Abiotic Stress Tolerance. *Frontiers in Plant Science*, *10*. https://doi.org/10.3389/fpls.2019.01068

Belden, J. B., Phillips, T. A., Clark, B. W., & Coats, J. R. (2005). Toxicity of Pendimethalin to Nontarget Soil Organisms. *Bulletin of Environmental Contamination and Toxicology*, *74*(4), 769–776. https://doi.org/10.1007/s00128-005-0648-5

Bentivenga, S. P., Kumar, T. K. A., Kumar, L., Roberson, R. W., & McLaughlin, D. J. (2013). Cellular organization in germ tube tips of *Gigaspora* and its phylogenetic implications. *Mycologia*, *105*(5), 1087–1099. https://doi.org/10.3852/12-291

Berbara, R. L. L., Souza, F. A., & Fonseca, H. M. A. (2006). Fungos micorrízicos arbusculares: Muito além da nutrição. In M. Fernandes (Ed.), *Nutrição mineral de plantas* (pp. 53–88). Lavras: Sociedade Brasileira de Ciência do Solo.

Bossio, D. A., Girvan, M. S., Verchot, L., Bullimore, J., Borelli, T., Albrecht, A., Scow, K. M., Ball, A. S., Pretty, J. N., & Osborn, A. M. (2005). Soil Microbial Community Response to Land Use Change in an Agricultural Landscape of Western Kenya. *Microbial Ecology*, *49*(1), 50–62. https://doi.org/10.1007/s00248-003-0209-6

Bressan, W. (2001): The interactive effect of phosphorus and nitrogen on "in vitro" spore germination of *Glomus etunicatum* Becker & Gerdemann, root growth and mycorrhizal colonization. *Brazilian Journal of Microbiology*, *32*(4). https://doi.org/10.1590/S1517-83822001000400004

Brundrett, M. C., & Tedersoo, L. (2018). Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist*, *220*(4), 1108–1115. https://doi.org/10.1111/nph.14976

Buysens, C., Dupré de Boulois, H., & Declerck, S. (2015). Do fungicides used to control Rhizoctonia solani impact the non-target arbuscular mycorrhizal fungus *Rhizophagus irregularis*? *Mycorrhiza*, *25*(4), 277–288. https://doi.org/10.1007/s00572-014-0610-7

Calonne, M., Sahraoui, A. L.-H., Campagnac, E., Debiane, D., Laruelle, F., Grandmougin-Ferjani, A., & Fontaine, J. (2012). Propiconazole inhibits the sterol 14α-demethylase in *Glomus irregulare* like in phytopathogenic fungi. *Chemosphere*, *87*(4), 376–383. https://doi.org/10.1016/j.chemosphere.2011.12.027

Campos, A. A. B., Scotton, J. C., Costa, W. L. F., Giassi, V., Pinto, D. F. P., & Homma, S. K. (2015). Seleção de fungicidas visando à preservação de fungos micorrízicos arbusculares nativos no cultivo do feijoeiro. *Revista Brasileira de Engenharia Agrícola e Ambiental*, *19*(9), 898–902. https://doi.org/10.1590/1807-1929/agriambi.v19n9p898-902

Cardenas-Flores, A., Cranenbrouck, S., Draye, X., Guillet, A., Govaerts, B., & Declerck, S. (2011). The sterol biosynthesis inhibitor molecule fenhexamid impacts the vegetative compatibility of Glomus clarum. *Mycorrhiza*, *21*(5), 443–449. https://doi.org/10.1007/s00572-011-0385-z

Cardinale, B. J., Duffy, J. E., Gonzalez, A., Hooper, D. U., Perrings, C., Venail, P., Narwani, A., Mace, G. M., Tilman, D., Wardle, D. A., Kinzig, A. P., Daily, G. C., Loreau, M., Grace, J. B., Larigauderie, A., Srivastava, D. S., & Naeem, S. (2012). Biodiversity loss and its impact on humanity. *Nature*, *486*(7401), 59–67. https://doi.org/10.1038/nature11148

Cardoso, E. J. B. N., Navarro, R. B., & Nogueira, M. A. (2002). Manganês e germinação de esporos de fungos micorrízicos arbusculares in vitro. *Revista Brasileira de Ciência Do Solo*, *26*(3), 795–799. https://doi.org/10.1590/S0100-06832002000300025

Cavagnaro, T. R., Bender, S. F., Asghari, H. R., & Heijden, M. G. A. van der. (2015). The role of arbuscular mycorrhizas in reducing soil nutrient loss. *Trends in Plant Science*, *20*(5), 283–290. https://doi.org/10.1016/j.tplants.2015.03.004

Carvalho, F., de Souza, F. A., Carrenho, R., Moreira, F. M. de S., Jesus, E. da C., & Fernandes, G. W. (2012). The mosaic of habitats in the high-altitude Brazilian rupestrian fields is a hotspot for arbuscular mycorrhizal fungi. *Applied Soil Ecology*, *52*, 9–19. https://doi.org/10.1016/j.apsoil.2011.10.001

Chen, E. C. H., Morin, E., Beaudet, D., Noel, J., Yildirir, G., Ndikumana, S., Charron, P., St-Onge, C., Giorgi, J., Krüger, M., Marton, T., Ropars, J., Grigoriev, I. v., Hainaut, M., Henrissat, B., Roux, C., Martin, F., & Corradi, N. (2018). High intraspecific genome diversity in the model arbuscular mycorrhizal symbiont *Rhizophagus irregularis*. *New Phytologist*, *220*(4), 1161–1171. https://doi.org/10.1111/nph.14989

Chen, J., Saleem, M., Wang, C., Liang, W., & Zhang, Q. (2018). Individual and combined effects of herbicide tribenuron-methyl and fungicide tebuconazole on soil earthworm Eisenia fetida. *Scientific Reports*, *8*(1), 2967. https://doi.org/10.1038/s41598-018-21288-y

CICG. (2021). International Culture Collection of Glomeromycota. Available in: https://sites.google.com/view/cicg-furb-english/home. Access in: 08.06.2021.

Clark, R. B. (1997). Arbuscular mycorrhizal adaptation, spore germination, root colonization, and host plant growth and mineral acquisition at low pH. *Plant and Soil*, *192*(1), 15–22. https://doi.org/10.1023/A:1004218915413

Colozzi-Filho, A., Siqueira, J., Oliveira, E. (1994): Desinfestação superficial de esporos de fungos micorrízicos vesicular-arbusculares. *Pesquisa Agropecuária Brasileira*, 29(7), 1119–1127. https://doi.org/10.1590/S1678-3921.pab1994.v29.4156

Costa, F. A., Haddad, L. S. M., Kasuya, M. C. M., Otoni, W. C., Costa, M. D., & Borges, A. C. (2013). In vitro culture of Gigaspora decipiens and Glomus clarum in transformed roots of carrot: the influence of temperature and pH. *Acta Scientiarum. Agronomy*, *35*(3), 315–323. https://doi.org/10.4025/actasciagron.v35i3.16581

Cranenbrouck, S., Voets, L., Bivort, C., Renard, L., Strullu, D.-G., & Declerck, S. (2005). *Methodologies for in Vitro Cultivation of Arbuscular Mycorrhizal Fungi with Root Organs* (pp. 341–375). https://doi.org/10.1007/3-540-27331-X_18

Croll, D., Giovannetti, M., Koch, A. M., Sbrana, C., Ehinger, M., Lammers, P. J., & Sanders, I. R. (2009). Nonself vegetative fusion and genetic exchange in the arbuscular mycorrhizal fungus *Glomus intraradices*. *New Phytologist*, *181*(4), 924–937. https://doi.org/10.1111/j.1469-8137.2008.02726.x

Cruz-Paredes, C., Diera, T., Davey, M., Rieckmann, M. M., Christensen, P., dela Cruz, M., Laursen, K. H., Joner, E. J., Christensen, J. H., Nybroe, O., & Jakobsen, I. (2021). Disentangling the abiotic and biotic components of AMF suppressive soils. *Soil Biology and Biochemistry*, *159*, 108305. https://doi.org/10.1016/j.soilbio.2021.108305

Daniels, B. A., & Trappe, J. M. (1980). Factors Affecting Spore Germination of the Vesicular-Arbuscular Mycorrhizal Fungus, *Glomus Epigaeus*. *Mycologia*, *72*(3), 457–471. https://doi.org/10.1080/00275514.1980.12021207

Davison, J., Moora, M., Öpik, M., Adholeya, A., Ainsaar, L., Bâ, A., Burla, S., Diedhiou, A. G., Hiiesalu, I., Jairus, T., Johnson, N. C., Kane, A., Koorem, K., Kochar, M., Ndiaye, C., Pärtel, M., Reier, Ü., Saks, Ü., Singh, R., … Zobel, M. (2015). Global assessment of arbuscular mycorrhizal fungus diversity reveals very low endemism. *Science*, *349*(6251), 970–973. https://doi.org/10.1126/science.aab1161

de La Providencia, I. E., de Souza, F. A., Fernández, F., Delmas, N. S., & Declerck, S. (2005). Arbuscular mycorrhizal fungi reveal distinct patterns of anastomosis formation and hyphal healing mechanisms between different phylogenic groups. *New Phytologist*, *165*(1), 261–271. https://doi.org/10.1111/j.1469-8137.2004.01236.x

Declerck, S., D'or, D., Cranenbrouck, S., & Boulengé, L. E. (2001). Modelling the sporulation dynamics of arbuscular mycorrhizal fungi in monoxenic culture. *Mycorrhiza*, *11*(5), 225–230. https://doi.org/10.1007/s005720100124

Delgado-Baquerizo, M., Maestre, F. T., Reich, P. B., Jeffries, T. C., Gaitan, J. J., Encinar, D., Berdugo, M., Campbell, C. D., & Singh, B. K. (2016). Microbial diversity drives multifunctionality in terrestrial ecosystems. *Nature Communications*, *7*(1), 10541. https://doi.org/10.1038/ncomms10541

Demenois, J., Carriconde, F., Bonaventure, P., Maeght, J.-L., Stokes, A., & Rey, F. (2018). Impact of plant root functional traits and associated mycorrhizas on the aggregate stability of a tropical Ferralsol. *Geoderma*, *312*, 6–16. https://doi.org/10.1016/j.geoderma.2017.09.033

Dodd, J. C., & Jeffries, P. (1989a). Effects of herbicides on three vesicular-arbuscular fungi associated with winter wheat (*Riticum aestivum* L.). *Biology and Fertility of Soils*, *7*(2), 113–119. https://doi.org/10.1007/BF00292568

Dodd, J. C., & Jeffries, P. (1989b). Effect of fungicides on three vesicular-arbuscular mycorrhizal fungi associated with winter wheat (Triticum aestivum L.). *Biology and Fertility of Soils*, *7*(2), 120–128. https://doi.org/10.1007/BF00292569

Dong, B. (2024). A comprehensive review on toxicological mechanisms and transformation products of tebuconazole: Insights on pesticide management. *Science of The Total Environment*, *908*, 168264. https://doi.org/10.1016/j.scitotenv.2023.168264

Druille, M., Omacini, M., Golluscio, R. A., & Cabello, M. N. (2013). Arbuscular mycorrhizal fungi are directly and indirectly affected by glyphosate application. *Applied Soil Ecology*, *72*, 143–149. https://doi.org/10.1016/j.apsoil.2013.06.011

Egnér, H., Riehm,H. & Domingo, W. R. (1960): Investigations on chemical soil analysis as the basis for estimating the nutrient status of soils. II. Chemical methods of extraction for phosphorus and potassium determinations. *Kungliga Lantbrukshogskolans Annaler*, 26, 199–215.

Ehinger, M. O., Croll, D., Koch, A. M., & Sanders, I. R. (2012). Significant genetic and phenotypic changes arising from clonal growth of a single spore of an arbuscular mycorrhizal fungus over multiple generations. *New Phytologist*, *196*(3), 853–861. https://doi.org/10.1111/j.1469-8137.2012.04278.x

Ellatif, S. A., M. Ali, E. A., Senousy, H. H., & Razik, E. S. A. (2019). Production of Arbuscular Mycorrhizal Fungi using In vitro Root Organ Culture and Phenolic Compounds. *Journal of Pure and Applied Microbiology*, *13*(4), 1985–1994. https://doi.org/10.22207/JPAM.13.4.10

Entry, J. A., Rygiewicz, P. T., Watrud, L. S., & Donnelly, P. K. (2002). Influence of adverse soil conditions on the formation and function of Arbuscular mycorrhizas. *Advances in Environmental Research*, *7*(1), 123–138. https://doi.org/10.1016/S1093-0191(01)00109-5

European Commission (EC) (2003): Communication from the Commission to the Council, the European Parliament, the Economic and Social Committee and the Committee of the Regions - Towards a Thematic Strategy for Soil Protection. COM 179 Final, Brussels. *Official Journal of the European Union*, 128, 43–48. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:52002DC0179

European Communities (EC) (2005): Soil Atlas of Europe, European Soil Bureau Network European Commission, 2005, 128 pp Office for Official Publications of the European Communities, L-2995 Luxembourg. https://esdac.jrc.ec.europa.eu/content/soil-atlas-europe

European Food Safety Authority (EFSA) (2008): Conclusion regarding the peer review of the pesticide risk assessment of the active substance fluazinam. *EFSA Scientific Report*, 137, 1-82. https://doi.org/10.2903/j.efsa.2008.137r

European Food Safety Authority (EFSA) (2010): Conclusion on the peer review of the pesticide risk assessment of the active substance azoxystrobin. *EFSA Journal* 2010; 8(4):1542. https://doi.org/10.2903/j.efsa.2010.1542

European Food Safety Authority (EFSA) (2010): Scientific Report of EFSA: selection of scenarios for exposure of soil organisms to plant protection products. In support of Revision of the Guidance Document on Persistence in Soil under Council Directive 91/414/EEC and Council Regulation 11/07/2009 (SANCO/9188/VI/97 rev. 8, 12.07.2000). *EFSA Journal* 2010; 8(6):1642. https://doi.org/10.2903/j.efsa.2010.1642

European Food Safety Authority (EFSA) (2011): Conclusion on the peer review of the pesticide risk assessment of the active substance terbuthylazine. *EFSA Journal* 2011, 9(1):1969. https://doi.org/10.2903/j.efsa.2011.1969

European Food Safety Authority (EFSA) (2014): Conclusion on the peer review of the pesticide risk assessment of the active substance tebuconazole. *EFSA Journal* 2014, 12(1):3485. https://doi.org/10.2903/j.efsa.2014.3485

European Food Safety Authority (EFSA) (2016): Peer review of the pesticide risk assessment of the active substance pendimethalin. *EFSA Journal* 2016, 14(3):4420. doi:10.2903/j.efsa.2016.4420

European Food Safety Authority (EFSA) (2017): Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. *EFSA Journal*, 15(2), 4690. https://doi.org/10.2903/j.efsa.2017.4690

European Food Safety Authority (EFSA) (2021): Statement of the PPR Panel on a framework for conducting the environmental exposure and risk assessment for transition metals when used as active substances in plant protection products (PPP). *EFSA Journal* 2021, 19(3): 6498. https://doi.10.2903/j.efsa.2021.6498

European Union (EU) (2012): Regulation (EU) No 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products. *Official Journal of the European Union*, 167, 1–123. https://eur-lex.europa.eu/eli/reg/2012/528/oj/eng

Food and Agriculture Organization of the United Nations (FAO) & Intergovernmental Technical Panel on Soils (ITPS) (2017): Global assessment of the impact of plant protection products on soil functions and soil ecosystems. Rome, FAO, 40. https://openknowledge.fao.org/handle/20.500.14283/i8168en

Frew, A. (2019). Arbuscular mycorrhizal fungal diversity increases growth and phosphorus uptake in C3 and C4 crop plants. *Soil Biology and Biochemistry*, *135*, 248–250. https://doi.org/10.1016/j.soilbio.2019.05.015

Gavito, M. E., Olsson, P. A., Rouhier, H., Medina-Peñafiel, A., Jakobsen, I., Bago, A., & Azcón-Aguilar, C. (2005). Temperature constraints on the growth and functioning of root organ cultures with arbuscular mycorrhizal fungi. *New Phytologist*, *168*(1), 179–188. https://doi.org/10.1111/j.1469-8137.2005.01481.x

Giovannetti, M., Avio, L., & Sbrana, C. (2010). Fungal Spore Germination and Pre-symbiotic Mycelial Growth – Physiological and Genetic Aspects. In *Arbuscular Mycorrhizas: Physiology and Function* (pp. 3–32). Springer Netherlands. https://doi.org/10.1007/978-90-481-9489-6_1

Giovannetti, M., Turrini, A., Strani, P., Sbrana, C., Avio, L., & Pietrangeli, B. (2006). Mycorrhizal fungi in ecotoxicological studies: soil impact of fungicides, insecticides and herbicides. *Prevention today*, *2*(1-2), 47-61. https://www.beyondpesticides.org/assets/media/documents/Mycorrhizal_fungi_in_ecotoxicological_studies_Soil.pdf

Gomes, S. I. L., Ammendola, A., Casini, S., & Amorim, M. J. B. (2021). Toxicity of fungicides to terrestrial non-target fauna – Formulated products versus active ingredients (azoxystrobin, cyproconazole, prothioconazole, tebuconazole) – A case study with *Enchytraeus crypticus* (Oligochaeta). *Science of The Total Environment*, *754*, 142098. https://doi.org/10.1016/j.scitotenv.2020.142098

Göransson, P., Olsson, P. A., Postma, J., & Falkengren-Grerup, U. (2008). Colonisation by arbuscular mycorrhizal and fine endophytic fungi in four woodland grasses – variation in relation to pH and aluminium. *Soil Biology and Biochemistry*, *40*(9), 2260–2265. https://doi.org/10.1016/j.soilbio.2008.05.002

Goto BT & Maia LC. (2006). Glomerospores: a new denomination for the spores of Glomeromycota, a group molecularly distinct from the Zygomycota. *Mycotaxon,* 96:129-132. https://www.periodicos.capes.gov.br/index.php/acervo/buscador.html?task=detalhes&source=all&id=W2906240811

Guimarães, B. M. M. (2018). *MEmO: Multigenerational exposure in ecotoxicological model species – effects, mechanisms and implications* [Dissertation, Universidade de Aveiro].

Hage-Ahmed, K., Rosner, K., & Steinkellner, S. (2019). Arbuscular mycorrhizal fungi and their response to pesticides. *Pest Management Science*, *75*(3), 583–590. https://doi.org/10.1002/ps.5220

Haugen, L. M., & Smith, S. E. (1992). The effect of high temperature and fallow period on infection of mung bean and cashew roots by the vesicular-arbuscular mycorrhizal fungus Glomus intraradices. Plant and Soil, 145(1), 71–80. https://doi.org/10.1007/BF00009543

Hayman, D. S., & Tavares, M. (1985). Plant Growth Responses to Vesicular-Arbuscular Mycorrhiza. XV. Influence of Soil pH on the Symbiotic Efficiency of Different Endophytes. *The New Phytologist*, *100*(3), 367–377. http://www.jstor.org/stable/2432836

Heinemeyer, A., & Fitter, A. H. (2004). Impact of temperature on the arbuscular mycorrhizal (AM) symbiosis: growth responses of the host plant and its AM fungal partner. *Journal of Experimental Botany*, *55*(396), 525–534. https://doi.org/10.1093/jxb/erh049

Hijri, M., & Sanders, I. R. (2005). Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. *Nature*, *433*(7022), 160–163. https://doi.org/10.1038/nature03069

Hillis, D. G., Antunes, P., Sibley, P. K., Klironomos, J. N., & Solomon, K. R. (2008). Structural responses of Daucus carota root-organ cultures and the arbuscular mycorrhizal fungus, *Glomus intraradices*, to 12 pharmaceuticals. *Chemosphere*, *73*(3), 344–352. https://doi.org/10.1016/j.chemosphere.2008.05.063

International Organization for Standardization (ISO) (1994): ISO – 11263: Soil quality — Determination of phosphorus — Spectrometric determination of phosphorus soluble in sodium hydrogen carbonate solution. Genève, Switzerland.

International Organization for Standardization (ISO) (1994): ISO – 11265: Soil quality — Determination of the specific electrical conductivity. Genève, Switzerland.

International Organization for Standardization (ISO) (1995): ISO – 10694: Soil Quality — Determination of Organic and Total Carbon After Dry Combustion (Elementary Analysis). Genève, Switzerland.

International Organization for Standardization (ISO) (1998): ISO – 13878: Soil quality — determination of total nitrogen content by dry combustion (“elemental analysis”). Genève, Switzerland.

International Organization for Standardization (ISO) (2009): ISO – 10832: Soil quality – Effects of pollutants on mycorrhizal fungi – Spore germination test. Genève, Switzerland.

International Organization for Standardization (ISO) (2018): ISO – 11260 – Soil quality - determination of effective cation exchange capacity and base saturation level using barium chloride solution. Genève, Switzerland.

International Organization for Standardization (ISO) (2020): ISO – 11277: Soil quality—Determination of particle size distribution in mineral soil material — Method by sieving and sedimentation. Genève, Switzerland.

International Organization for Standardization (ISO) (2021): ISO – 10390: Soil Quality - Determination of pH. Genève, Switzerland.

International Organization for Standardization (ISO) (2023): ISO-16387: Soil quality — Effects of contaminants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction, Genève, Switzerland.

International Organization for Standardization (ISO) (2023): ISO-11268-2: Soil quality – Effects of pollutants on earthworms (Part 2: Determination of effects on reproduction of *Eisenia fetida/Eisenia andrei* and other earthworm species), Genève, Switzerland.

International Organization for Standardization (ISO) (2023): ISO-11267: Soil quality — Inhibition of reproduction of Collembola (*Folsomia candida*) by soil contaminants, Genève, Switzerland.

INVAM (2021): International Culture Collection of (Vesicular) Arbuscular Mycorrhizal Fungi. Methods/Inocula Storage/Refrigeration Storage. https://invam.wvu.edu/home (08.06.2021).

Ipsilantis, I., Samourelis, C., & Karpouzas, D. G. (2012). The impact of biological pesticides on arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry*, *45*, 147–155. https://doi.org/10.1016/j.soilbio.2011.08.007

Isman, M. B. (2006). Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annual Review of Entomology*, *51*(1), 45–66. https://doi.org/10.1146/annurev.ento.51.110104.151146

Jakobsen, I., Murmann, L. M., & Rosendahl, S. (2021). Hormetic responses in arbuscular mycorrhizal fungi. *Soil Biology and Biochemistry*, *159*, 108299. https://doi.org/10.1016/j.soilbio.2021.108299

Jansa, J., & Treseder, K. K. (2017). Chapter 19 - Introduction: Mycorrhizas and the Carbon Cycle. In *Mycorrhizal Mediation of Soil* (pp. 343–355). Elsevier. https://doi.org/10.1016/B978-0-12-804312-7.00019-X

Jenkinson, D. S., & Powlson, D. S. (1976). The effects of biocidal treatments on metabolism in soil—V. *Soil Biology and Biochemistry*, *8*(3), 209–213. https://doi.org/10.1016/0038-0717(76)90005-5

Jorge-Escudero, G., Pérez Polanco, M., Lagerlöf, J. E., Pérez, C. A., & Míguez, D. (2022). Commercial Fungicide Toxic Effects on Terrestrial Non-Target Species Might Be Underestimated When Based Solely on Active Ingredient Toxicity and Standard Earthworm Tests. *Toxics*, *10*(9), 488. https://doi.org/10.3390/toxics10090488

Jovana, M., Tanja, M., & Mirjana, S. (2014). Effects of three pesticides on the earthworm *Eisenia fetida* (Savigny 1826) under laboratory conditions: Assessment of mortality, biomass and growth inhibition. *European Journal of Soil Biology*, *62*, 127–131. https://doi.org/10.1016/j.ejsobi.2014.03.003

Juge, C., Samson, J., Bastien, C., Vierheilig, H., Coughlan, A., & Piché, Y. (2002). Breaking dormancy in spores of the arbuscular mycorrhizal fungus *Glomus intraradices*: a critical cold-storage period. *Mycorrhiza*, *12*(1), 37–42. https://doi.org/10.1007/s00572-001-0151-8

Kaczala, F., & E. Blum, S. (2016). The Occurrence of Veterinary Pharmaceuticals in the Environment: A Review. *Current Analytical Chemistry*, *12*(3), 169–182. https://doi.org/10.2174/1573411012666151009193108

Karpouzas, D. G., Papadopoulou, E., Ipsilantis, I., Friedel, I., Petric, I., Udikovic-Kolic, N., Djuric, S., Kandeler, E., Menkissoglu-Spiroudi, U., & Martin-Laurent, F. (2014). Effects of nicosulfuron on the abundance and diversity

of arbuscular mycorrhizal fungi used as indicators of pesticide soil microbial toxicity. *Ecological Indicators*, *39*, 44–53. https://doi.org/10.1016/j.ecolind.2013.12.004

Kim, S.-C., & Carlson, K. (2005). LC–MS2 for quantifying trace amounts of pharmaceutical compounds in soil and sediment matrices. *TrAC Trends in Analytical Chemistry*, *24*(7), 635–644. https://doi.org/10.1016/j.trac.2005.04.006

Klugh-Stewart, K., & Cumming, J. R. (2009). Organic acid exudation by mycorrhizal *Andropogon virginicus* L. (broomsedge) roots in response to aluminum. *Soil Biology and Biochemistry*, *41*(2), 367–373. https://doi.org/10.1016/j.soilbio.2008.11.013

Koch, A. M., Croll, D., & Sanders, I. R. (2006). Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. *Ecology Letters*, *9*(2), 103–110. https://doi.org/10.1111/j.1461-0248.2005.00853.x

Koch, A. M., Kuhn, G., Fontanillas, P., Fumagalli, L., Goudet, J., & Sanders, I. R. (2004). High genetic variability and low local diversity in a population of arbuscular mycorrhizal fungi. *Proceedings of the National Academy of Sciences*, *101*(8), 2369–2374. https://doi.org/10.1073/pnas.0306441101

Kovačević, M., Hackenberger, D. K., & Hackenberger, B. K. (2021). Effects of strobilurin fungicides (azoxystrobin, pyraclostrobin, and trifloxystrobin) on survival, reproduction and hatching success of *Enchytraeus crypticus*. *Science of The Total Environment*, *790*, 148143. https://doi.org/10.1016/j.scitotenv.2021.148143

Kuhn, G., Hijri, M., & Sanders, I. R. (2001). Evidence for the evolution of multiple genomes in arbuscular mycorrhizal fungi. *Nature*, *414*(6865), 745–748. https://doi.org/10.1038/414745a

Leitão, S., Cerejeira, M. J., van den Brink, P. J., & Sousa, J. P. (2014). Effects of azoxystrobin, chlorothalonil, and ethoprophos on the reproduction of three terrestrial invertebrates using a natural Mediterranean soil. *Applied Soil Ecology*, *76*, 124–131. https://doi.org/10.1016/j.apsoil.2013.12.013

Lerat, S., Gauci, R., Catford, J. G., Vierheilig, H., Piché, Y., & Lapointe, L. (2002). 14C transfer between the spring ephemeral *Erythronium americanum* and sugar maple saplings via arbuscular mycorrhizal fungi in natural stands. *Oecologia*, *132*(2), 181–187. https://doi.org/10.1007/s00442-002-0958-9

Li, S., Yuan, Y., Wang, X., Cai, L., Wang, J., Zhao, Y., Jiang, L., & Yang, X. (2023). Bioaccumulation and toxicity of terbuthylazine in earthworms (*Eisenia fetida*). *Environmental Toxicology and Pharmacology*, *97*, 104016. https://doi.org/10.1016/j.etap.2022.104016

Li, X., Miao, W., Gong, C., Jiang, H., Ma, W., & Zhu, S. (2013). Effects of prometryn and acetochlor on arbuscular mycorrhizal fungi and symbiotic system. *Letters in Applied Microbiology*, *57*(2), 122–128. https://doi.org/10.1111/lam.12084

Liu, A., Wang, B., & Hamel, C. (2004). Arbuscular mycorrhiza colonization and development at suboptimal root zone temperature. *Mycorrhiza*, *14*(2), 93–101. https://doi.org/10.1007/s00572-003-0242-9

Liu, W., Zhang, Y., Jiang, S., Deng, Y., Christie, P., Murray, P. J., Li, X., & Zhang, J. (2016). Arbuscular mycorrhizal fungi in soil and roots respond differently to phosphorus inputs in an intensively managed calcareous agricultural soil. *Scientific Reports*, *6*(1), 24902. https://doi.org/10.1038/srep24902

Louarn, J., Carbonne, F., Delavault, P., Bécard, G., & Rochange, S. (2012). Reduced Germination of Orobanche cumana Seeds in the Presence of Arbuscular Mycorrhizal Fungi or Their Exudates. *PLoS ONE*, *7*(11), e49273. https://doi.org/10.1371/journal.pone.0049273

Louis, I., & Lim, G. (1988). Effect of Storage of Inoculum on Spore Germination of a Tropical Isolate of *Glomus Clarum*. *Mycologia*, *80*(2), 157–161. https://doi.org/10.1080/00275514.1988.12025517

Maia, L. C., Silva, F. S. B., & Goto, B. T. (2010). Estrutura, ultraestrutura e germinação de glomerosporos. In J. O. Siqueira, F. A. Souza, E. J. B. N. Cardoso, & S. M. Tsai (Eds.), *Micorrizas: 30 anos de pesquisa no Brasil* (Chap. 3). Lavras: UFLA.

Maia, L. C., & Yano-Melo, A. M. (2001). Germination and germ tube growth of the arbuscular mycorrhizal fungi *Gigaspora albida* in different substrates. *Brazilian Journal of Microbiology*, *32*(4). https://doi.org/10.1590/S1517-83822001000400005

Malfatti, A. de L. R., Mallmann, G. C., Oliveira Filho, L. C. I., Carniel, L. S. C., Cruz, S. P., & Klauberg-Filho, O. (2021). Ecotoxicological test to assess effects of herbicides on spore germination of Rhizophagus clarus and Gigaspora albida. *Ecotoxicology and Environmental Safety*, *207*, 111599. https://doi.org/10.1016/j.ecoenv.2020.111599

Mallmann, G. (2020). *Métodos ecotoxicológicos na avaliação do efeito de agrotóxicos sobre fungos micorrízicos arbusculares* [Thesis, Universidade do Estado de Santa Catarina]. https://www.udesc.br/arquivos/cav/id_cpmenu/2416/Tese_16177154574956_2416.pdf

Mallmann, G. C., Sousa, J. P., Sundh, I., Pieper, S., Arena, M., da Cruz, S. P., & Klauberg-Filho, O. (2018). Placing arbuscular mycorrhizal fungi on the risk assessment test battery of plant protection products (PPPs). *Ecotoxicology*, *27*(7), 809–818. https://doi.org/10.1007/s10646-018-1946-0

Malty, J. dos S., Siqueira, J. O., & Moreira, F. M. de S. (2006). Efeitos do glifosato sobre microrganismos simbiotróficos de soja, em meio de cultura e casa de vegetação. *Pesquisa Agropecuária Brasileira*, *41*(2), 285–291. https://doi.org/10.1590/S0100-204X2006000200013

Martin, C. A., & Stutz, J. C. (2004). Interactive effects of temperature and arbuscular mycorrhizal fungi on growth, P uptake and root respiration of *Capsicum annuum* L. *Mycorrhiza*, *14*(4), 241–244. https://doi.org/10.1007/s00572-003-0261-6

Martínez-García, L. B., de Dios Miranda, J., & Pugnaire, F. I. (2012). Impacts of changing rainfall patterns on mycorrhizal status of a shrub from arid environments. *European Journal of Soil Biology*, *50*, 64–67. https://doi.org/10.1016/j.ejsobi.2011.12.005

Mathieu, S., Cusant, L., Roux, C., & Corradi, N. (2018). Arbuscular mycorrhizal fungi: intraspecific diversity and pangenomes. *New Phytologist*, *220*(4), 1129–1134. https://doi.org/10.1111/nph.15275

Matsubara, Y., & Harada, T. (1996). Effect of Constant and Diurnally Fluctuating Temperatures on Arbuscular Mycorrhizal Fungus Infection and the Growth of Infected Asparagus (*Asparagus officinalis* L.) Seedlings. *Journal of the Japanese Society for Horticultural Science*, *65*(3), 565–570. https://doi.org/10.2503/jjshs.65.565

McGowan, A. R., Nicoloso, R. S., Diop, H. E., Roozeboom, K. L., & Rice, C. W. (2019). Soil Organic Carbon, Aggregation, and Microbial Community Structure in Annual and Perennial Biofuel Crops. *Agronomy Journal*, *111*(1), 128–142. https://doi.org/10.2134/agronj2018.04.0284

McGuire, K. L., Henkel, T. W., Granzow de la Cerda, I., Villa, G., Edmund, F., & Andrew, C. (2008). Dual mycorrhizal colonization of forest-dominating tropical trees and the mycorrhizal status of non-dominant tree and liana species. *Mycorrhiza*, *18*(4), 217–222. https://doi.org/10.1007/s00572-008-0170-9

Medeiros, C. A. B., Clark, R. B., & Ellis, J. R. (1994). Growth and nutrient uptake of sorghum cultivated with vesicular-arbuscular mycorrhiza isolates at varying pH. *Mycorrhiza*, *4*(5), 185–191. https://doi.org/10.1007/BF00206778

Miao, F., Wang, S., Yuan, Y., Chen, Y., Guo, E., & Li, Y. (2023). The Addition of a High Concentration of Phosphorus Reduces the Diversity of Arbuscular Mycorrhizal Fungi in Temperate Agroecosystems. *Diversity*, *15*(10), 1045. https://doi.org/10.3390/d15101045

Moreira, F. M. S., & Siqueira, J. O. (2006). *Microbiologia e bioquímica do solo* (2ª ed.). Lavras: UFLA.

Moser, H., & Römbke, J. (2009). *Ecotoxicological Characterization of Waste* (H. Moser & J. Römbke, Eds.). Springer New York. https://doi.org/10.1007/978-0-387-88959-7

Nara, K. (2006). Ectomycorrhizal networks and seedling establishment during early primary succession. *New Phytologist*, *169*(1), 169–178. https://doi.org/10.1111/j.1469-8137.2005.01545.x

Niemeyer, J. C., Carniel, L. S. C., de Santo, F. B., Silva, M., & Klauberg-Filho, O. (2018). Boric acid as reference substance for ecotoxicity tests in tropical artificial soil. *Ecotoxicology*, *27*(4), 395–401. https://doi.org/10.1007/s10646-018-1915-7

Nogueira, M. A., & Cardoso, E. J. B. N. (2003). Mycorrhizal effectiveness and manganese toxicity in soybean as affected by soil type and endophyte. *Scientia Agricola*, *60*(2), 329–335. https://doi.org/10.1590/S0103-90162003000200018

Nogueira, M. A., Magalhães, G. C., & Cardoso, E. J. B. N. (2004). Manganese Toxicity in Mycorrhizal and Phosphorus-Fertilized Soybean Plants. *Journal of Plant Nutrition*, *27*(1), 141–156. https://doi.org/10.1081/PLN-120027552

Novais, C. B., Avio, L., Giovannetti, M., de Faria, S. M., Siqueira, J. O., & Sbrana, C. (2019a). Interconnectedness, length and viability of arbuscular mycorrhizal mycelium as affected by selected herbicides and fungicides. *Applied Soil Ecology*, *143*, 144–152. https://doi.org/10.1016/j.apsoil.2019.06.013

Novais, C. B., Giovannetti, M., de Faria, S. M., & Sbrana, C. (2019b). Two herbicides, two fungicides and spore-associated bacteria affect *Funneliformis mosseae* extraradical mycelium structural traits and viability. *Mycorrhiza*, *29*(4), 341–349. https://doi.org/10.1007/s00572-019-00901-6

Novais, C. B., Borges, W. L., Jesus, E. da C., Júnior, O. J. S., & Siqueira, J. O. (2014). Inter- and intraspecific functional variability of tropical arbuscular mycorrhizal fungi isolates colonizing corn plants. *Applied Soil Ecology*, *76*, 78–86. https://doi.org/10.1016/j.apsoil.2013.12.010

Oehl, F., Laczko, E., Oberholzer, H.-R., Jansa, J., & Egli, S. (2017). Diversity and biogeography of arbuscular mycorrhizal fungi in agricultural soils. *Biology and Fertility of Soils*, *53*(7), 777–797. https://doi.org/10.1007/s00374-017-1217-x

Office of Environmental Health Hazard Assessment (OEHHA) (2009): Soil toxicity and bioassessment test methods for ecological risk assessment, California. https://oehha.ca.gov/sites/default/files/media/downloads/ecotoxicology/document/soiltox120208.pdf

Öpik, M., Moora, M., Liira, J., & Zobel, M. (2006). Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. *Journal of Ecology*, *94*(4), 778–790. https://doi.org/10.1111/j.1365-2745.2006.01136.x

Öpik, M., Vanatoa, A., Vanatoa, E., Moora, M., Davison, J., Kalwij, J. M., Reier, Ü., & Zobel, M. (2010). The online database Maarj *AM* reveals global and ecosystemic distribution patterns in arbuscular mycorrhizal fungi (Glomeromycota). *New Phytologist*, *188*(1), 223–241. https://doi.org/10.1111/j.1469-8137.2010.03334.x

OECD (2000a), *Test No. 216: Soil Microorganisms: Nitrogen Transformation Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070226-en

OECD (2000b), *Test No. 217: Soil Microorganisms: Carbon Transformation Test*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264070240-en

OECD (2005), *Guidance Document on the Validation and International Acceptance of New or Updated Test Methods for Hazard Assessment*, OECD Series on Testing and Assessment, No. 34, OECD Publishing, Paris, https://doi.org/10.1787/e1f1244b-en.

OECD (2016), *Test No. 222: Earthworm Reproduction Test (Eisenia fetida/Eisenia andrei)*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264264496-en

OECD (2016), *Test No. 226: Predatory mite (Hypoaspis (Geolaelaps) aculeifer) reproduction test in soil*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264264557-en

OECD (2016), *Test No. 232: Collembolan Reproduction Test in Soil*, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris, https://doi.org/10.1787/9789264264601-en

Owojori, O. J., Waszak, K., & Roembke, J. (2013). Avoidance and reproduction tests with the predatory mite *Hypoaspis aculeifer* : Effects of different chemical substances. *Environmental Toxicology and Chemistry*, *33*(1), 230–237. https://doi.org/10.1002/etc.2421

Pánková, H., Dostálek, T., Vazačová, K., & Münzbergová, Z. (2018). Slow recovery of arbuscular mycorrhizal fungi and plant community after fungicide application: An eight-year experiment. *Journal of Vegetation Science*, *29*(4), 695–703. https://doi.org/10.1111/jvs.12656

Pasaribu, A., Mohamad, R., Awang, Y., Othman, R. & Puteh, A. (2011): Growth and development of symbiotic Arbuscular mycorrhizal fungi, *Glomus mossea* (Nicol. and Gerd.), in alachlor and glyphosate treated soils. *African Journal of Biotechnology*, 10(55), 11520–11526. https://www.ajol.info/index.php/ajb/article/view/96173

Pawlowska, T. E., & Taylor, J. W. (2004). Organization of genetic variation in individuals of arbuscular mycorrhizal fungi. *Nature*, *427*(6976), 733–737. https://doi.org/10.1038/nature02290

Pawlowska, T. E., Douds, D. D., & Charvat, I. (1999). In vitro propagation and life cycle of the arbuscular mycorrhizal fungus *Glomus etunicatum*. *Mycological Research*, *103*(12), 1549–1556. https://doi.org/10.1017/S0953756299008801

Pedersen, M. B., van Gestel, C. A., & Elmegaard, N. (2000). Effects of copper on reproduction of two collembolan species exposed through soil, food, and water. *Environmental Toxicology and Chemistry*, *19*(10), 2579–2588. https://doi.org/10.1002/etc.5620191026

Peña, R., Robbins, C., Corella, J. C., Thuita, M., Masso, C., Vanlauwe, B., Signarbieux, C., Rodriguez, A., & Sanders, I. R. (2020). Genetically Different Isolates of the Arbuscular Mycorrhizal Fungus *Rhizophagus irregularis* Induce Differential Responses to Stress in Cassava. *Frontiers in Plant Science*, *11*. https://doi.org/10.3389/fpls.2020.596929

Perestrelo, R., Silva, P., Porto-Figueira, P., Pereira, J. A. M., Silva, C., Medina, S., & Câmara, J. S. (2019). QuEChERS - Fundamentals, relevant improvements, applications and future trends. *Analytica Chimica Acta*, *1070*, 1–28. https://doi.org/10.1016/j.aca.2019.02.036

Pesticide Properties DataBase (PPDB) (2024). *University of Hertfordshire*. Available in: https://sitem.herts.ac.uk/aeru/ppdb/en/index.htm

Põlme, S., Abarenkov, K., Henrik Nilsson, R., Lindahl, B. D., Clemmensen, K. E., Kauserud, H., Nguyen, N., Kjøller, R., Bates, S. T., Baldrian, P., Frøslev, T. G., Adojaan, K., Vizzini, A., Suija, A., Pfister, D., Baral, H.-O., Järv, H., Madrid, H., Nordén, J., … Tedersoo, L. (2020). FungalTraits: a user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Diversity*, *105*(1), 1–16. https://doi.org/10.1007/s13225-020-00466-2

Powell, J. R., & Rillig, M. C. (2018). Biodiversity of arbuscular mycorrhizal fungi and ecosystem function. *New Phytologist*, *220*(4), 1059–1075. https://doi.org/10.1111/nph.15119

Pretty, J. (2018). Intensification for redesigned and sustainable agricultural systems. *Science*, *362*(6417). https://doi.org/10.1126/science.aav0294

Prosser, R. S., Lissemore, L., Shahmohamadloo, R. S., & Sibley, P. K. (2015). Effect of biosolids-derived triclosan and triclocarban on the colonization of plant roots by arbuscular mycorrhizal fungi. *Science of The Total Environment*, *508*, 427–434. https://doi.org/10.1016/j.scitotenv.2014.12.014

PubChem (2024). *National Library of Medicine*. Available in: https://pubchem.ncbi.nlm.nih.gov/

Purin, S., & Rillig, M. C. (2007). The arbuscular mycorrhizal fungal protein glomalin: Limitations, progress, and a new hypothesis for its function. *Pedobiologia*, *51*(2), 123–130. https://doi.org/10.1016/j.pedobi.2007.03.002

Purin, S., & Morton, J. B. (2013). Anastomosis behavior differs between asymbiotic and symbiotic hyphae of *Rhizophagus clarus*. *Mycologia*, *105*(3), 589–602. https://doi.org/10.3852/12-135

Püschel, D., Kolaříková, Z., Šmilauer, P., & Rydlová, J. (2019). Survival and long-term infectivity of arbuscular mycorrhizal fungi in peat-based substrates stored under different temperature regimes. *Applied Soil Ecology*, *140*, 98–107. https://doi.org/10.1016/j.apsoil.2019.04.020

Rabab, A. M., & Reda, E. A. (2019). Impact of Ridomil, Bavistin and Agrothoate on arbuscular mycorrhizal fungal colonization, biochemical changes and potassium content of cucumber plants. *Ecotoxicology*, *28*(5), 487–498. https://doi.org/10.1007/s10646-019-02042-0

Radha, V., Marimuthu, R. P., & Kumutha, K. (2015). Sporulation of arbuscular mycorrhizal fungus, *Glomus intraradices* , through root organ culture. *Applied Biological Research*, *17*(1), 38. https://doi.org/10.5958/0974-4517.2015.00006.3

Rahman, S. F. S. A., Singh, E., Pieterse, C. M. J., & Schenk, P. M. (2018). Emerging microbial biocontrol strategies for plant pathogens. *Plant Science*, *267*, 102–111. https://doi.org/10.1016/j.plantsci.2017.11.012

Rajput, P., Thakur, A., & Devi, P. (2020). Emerging agrochemicals contaminants: current status, challenges, and technological solutions. In *Agrochemicals Detection, Treatment and Remediation* (pp. 117–142). Elsevier. https://doi.org/10.1016/B978-0-08-103017-2.00005-2

Rico, A., Sabater, C., & Castillo, M.-Á. (2016). Lethal and sub-lethal effects of five pesticides used in rice farming on the earthworm Eisenia fetida. *Ecotoxicology and Environmental Safety*, *127*, 222–229. https://doi.org/10.1016/j.ecoenv.2016.02.004

Rillig, M. C. (2004). Arbuscular mycorrhizae and terrestrial ecosystem processes. *Ecology Letters*, *7*(8), 740–754. https://doi.org/10.1111/j.1461-0248.2004.00620.x

Rillig, M. C., Aguilar-Trigueros, C. A., Bergmann, J., Verbruggen, E., Veresoglou, S. D., & Lehmann, A. (2015). Plant root and mycorrhizal fungal traits for understanding soil aggregation. *New Phytologist*, *205*(4), 1385–1388. https://doi.org/10.1111/nph.13045

Rivera-Becerril, F., van Tuinen, D., Chatagnier, O., Rouard, N., Béguet, J., Kuszala, C., Soulas, G., Gianinazzi-Pearson, V., & Martin-Laurent, F. (2017). Impact of a pesticide cocktail (fenhexamid, folpel, deltamethrin) on the abundance of Glomeromycota in two agricultural soils. *Science of The Total Environment*, *577*, 84–93. https://doi.org/10.1016/j.scitotenv.2016.10.098

Robert, V., Stegehuis, G., and Stalpers, J. (2005): The MycoBank engine and related databases. https://www.MycoBank.org/

Rodrigues, K. M., & Rodrigues, B. F. (2015). Endomycorrhizal association of *Funneliformis mosseae* with transformed roots of *Linum usitatissimum* : germination, colonization, and sporulation studies. *Mycology*, *6*(1), 42–49. https://doi.org/10.1080/21501203.2015.1024777

Rohyadi, A. (2008). Growth Responses of External Hyphae of Arbuscular Mycorrhizal Fungi to Acidic Soil Conditions and their Effects on Cowpea Growth. *Microbiology Indonesia*, *2*(1), 22–26. https://doi.org/10.5454/mi.2.1.5

Rohyadi, A., Smith, F. A., Murray, R. S., & Smith, S. E. (2004). Effects of pH on mycorrhizal colonisation and nutrient uptake in cowpea under conditions that minimise confounding effects of elevated available aluminium. *Plant and Soil*, *260*(1/2), 283–290. https://doi.org/10.1023/B:PLSO.0000030183.87228.0b

Römbke, J., Bauer, C., & Marschner, A. (1996). Hazard assessment of chemicals in soil. Proposed Ecotoxicological Test Strategy. *Environmental Science and Pollution Research*, *3*(2), 78–82. https://doi.org/10.1007/BF02985494

Rouphael, Y., Cardarelli, M., & Colla, G. (2015). Role of arbuscular mycorrhizal fungi in alleviating the adverse effects of acidity and aluminium toxicity in zucchini squash. *Scientia Horticulturae*, *188*, 97–105. https://doi.org/10.1016/j.scienta.2015.03.031

Sanders, I. R., & Croll, D. (2010). Arbuscular Mycorrhiza: The Challenge to Understand the Genetics of the Fungal Partner. *Annual Review of Genetics*, *44*(1), 271–292. https://doi.org/10.1146/annurev-genet-102108-134239

Sanyal, S., Dasgupta, R., Kaviraj, A., & Chakravorty, P. (2015). Comparative toxicity studies of four pesticides on the epigeic earthworm *Perionyx excavatus* in two different soil media. *Pollution Research*. 34. 47-51. https://mcconline.org.in/download/publication-rnd/PUB43.pdf

Säle, V., Palenzuela, J., Azcón-Aguilar, C., Sánchez-Castro, I., da Silva, G. A., Seitz, B., Sieverding, E., van der Heijden, M. G. A., & Oehl, F. (2021). Ancient lineages of arbuscular mycorrhizal fungi provide little plant benefit. *Mycorrhiza*, *31*(5), 559–576. https://doi.org/10.1007/s00572-021-01042-5

Sbrana, C., Strani, P., Pepe, A., de Novais, C. B., & Giovannetti, M. (2018). Divergence of *Funneliformis mosseae* populations over 20 years of laboratory cultivation, as revealed by vegetative incompatibility and molecular analysis. *Mycorrhiza*, *28*(4), 329–341. https://doi.org/10.1007/s00572-018-0830-3

Schenck, N. C., Graham, S. O., & Green, N. E. (1975). Temperature and Light Effect on Contamination and Spore Germination of Vesicular-Arbuscular Mycorrhizal Fungi. *Mycologia*, *67*(6), 1189. https://doi.org/10.2307/3758840

Schenck, N. C., & Smith, G. S. (1982). Responses of Six Species of Vesicular-Arbuscular Mycorrhizal Fungi and Their Effects on Soybean at Four Soil Temperatures. *New Phytologist*, *92*(2), 193–201. https://doi.org/10.1111/j.1469-8137.1982.tb03376.x

Schoonmaker, A. L., Teste, F. P., Simard, S. W., & Guy, R. D. (2007). Tree proximity, soil pathways and common mycorrhizal networks: their influence on the utilization of redistributed water by understory seedlings. *Oecologia*, *154*(3), 455–466. https://doi.org/10.1007/s00442-007-0852-6

Schreiner, R. P., & Bethlenfalvay, G. J. (1997). Mycorrhizae, biocides, and biocontrol 3. Effects of three different fungicides on developmental stages of three AM fungi. *Biology and Fertility of Soils*, *24*(1), 18–26. https://doi.org/10.1007/BF01420215

Selosse, M. -A., & Duplessis, S. (2006). More complexity in the mycorrhizal world. *New Phytologist*, *172*(4), 600–604. https://doi.org/10.1111/j.1469-8137.2006.01929.x

Shahmohamadloo, R. S., Lissemore, L., Prosser, R. S., & Sibley, P. K. (2017). Comparative evaluation of four biosolids formulations on the effects of triclosan on plant-arbuscular mycorrhizal fungal interactions in three crop species. *Science of The Total Environment*, *583*, 292–299. https://doi.org/10.1016/j.scitotenv.2017.01.067

Silva, F. S. B. da, Yano-Melo, A. M., & Maia, L. C. (2007). Production and infectivity of inoculum of arbuscular mycorrhizal fungi multiplied in a substrate supplemented with Tris-HCl buffer. *Brazilian Journal of Microbiology*, *38*(4), 752–755. https://doi.org/10.1590/S1517-83822007000400030

Simard, S. W., Beiler, K. J., Bingham, M. A., Deslippe, J. R., Philip, L. J., & Teste, F. P. (2012). Mycorrhizal networks: Mechanisms, ecology and modelling. *Fungal Biology Reviews*, *26*(1), 39–60. https://doi.org/10.1016/j.fbr.2012.01.001

Singla, P., & Garg, N. (2017). Plant Flavonoids: Key Players in Signaling, Establishment, and Regulation of Rhizobial and Mycorrhizal Endosymbioses. In *Mycorrhiza - Function, Diversity, State of the Art* (pp. 133–176). Springer International Publishing. https://doi.org/10.1007/978-3-319-53064-2_8

Siqueira, J. O., Safir, G. R., & Nair, M. G. (1991). Stimulation of vesicular-arbuscular mycorrhiza formation and growth of white clover by flavonoid compounds. *New Phytologist*, *118*(1), 87–93. https://doi.org/10.1111/j.1469-8137.1991.tb00568.x

Siqueira, J. O., Hubbell, D. H., & Mahmud, A. W. (1984). Effect of liming on spore germination, germ tube growth and root colonization by vesicular-arbuscular mycorrhizal fungi. *Plant and Soil*, *76*(1–3), 115–124. https://doi.org/10.1007/BF02205572

Siqueira, J. O., Sylvia, D. M., Gibson, J., & Hubbell, D. H. (1985). Spores, germination, and germ tubes of vesicular–arbuscular mycorrhizal fungi. *Canadian Journal of Microbiology*, *31*(11), 965–972. https://doi.org/10.1139/m85-183

Smith, S.E. and Read, D.J. (1997) Mycorrhizal Symbiosis. 2nd Edition, Academic Press, London.

Smith, S.E. and Read, D.J. (2008) Mycorrhizal Symbiosis. 3rd Edition, Academic Press, London.

Soares, S., Mariano, R., Cavalcante, U. & Maia, L. (2009): Efeito de bactérias na germinação de fungos micorrízicos arbusculares e co-inoculação em mudas de abacaxizeiro. *Revista Caatinga*, 22(2), 31–38. https://periodicos.ufersa.edu.br/caatinga/article/view/162/565

Solomon, K. R., Hillis, D. G., Lissemore, L., & Sibley, P. K. (2010). *Risks of agricultural pharmaceuticals in surface water systems and soils* (pp. 191–204). https://doi.org/10.1021/bk-2009-1018.ch013

Song, Y. Y., Zeng, R. sen, Xu, J. F., Li, J., Shen, X., & Yihdego, W. G. (2010). Interplant Communication of Tomato Plants through Underground Common Mycorrhizal Networks. *PLoS ONE*, *5*(10), e13324. https://doi.org/10.1371/journal.pone.0013324

Soudzilovskaia, N. A., Vaessen, S., Barcelo, M., He, J., Rahimlou, S., Abarenkov, K., Brundrett, M. C., Gomes, S. I. F., Merckx, V., & Tedersoo, L. (2020). FungalRoot: global online database of plant mycorrhizal associations. *New Phytologist*, *227*(3), 955–966. https://doi.org/10.1111/nph.16569

Souza, F. A., Silva, I. R., & Berbara, R. L. L. (2008). Fungos micorrízicos arbusculares: Muito mais diversos do que se imaginava. In F. M. S. Moreira, J. O. Siqueira, & L. Brussaard (Eds.), *Biodiversidade do solo em ecossistemas brasileiros* (pp. 213–242). Lavras: UFLA.

Souza, F. A., Stürmer, S. L., Carrenho, R., & Trufem, S. F. B. (2010). Classificação e taxonomia de fungos micorrízicos arbusculares, sua diversidade e ocorrência no Brasil. In J. O. Siqueira, F. A. Souza, E. J. B. N. Cardoso, & S. M. Tsai (Eds.), *Micorrizas: 30 anos de pesquisa no Brasil* (Chap. 2). Lavras: UFLA.

Stürmer, S. L., Bever, J. D., & Morton, J. B. (2018). Biogeography of arbuscular mycorrhizal fungi (Glomeromycota): a phylogenetic perspective on species distribution patterns. *Mycorrhiza*, *28*(7), 587–603. https://doi.org/10.1007/s00572-018-0864-6

Stürmer, S. L., & Siqueira, J. O. (2008). Diversidade de fungos micorrízicos arbusculares em ecossistemas brasileiros. In F. M. S. Moreira, J. O. Siqueira, & L. Brussaard (Eds.), *Biodiversidade do solo em ecossistemas brasileiros* (pp. 243–272). Lavras: UFLA.

Tamm, L., Thuerig, B., Apostolov, S., Blogg, H., Borgo, E., Corneo, P. E., Fittje, S., de Palma, M., Donko, A., Experton, C., Alcázar Marín, É., Morell Pérez, Á., Pertot, I., Rasmussen, A., Steinshamn, H., Vetemaa, A., Willer, H., & Herforth-Rahmé, J. (2022). Use of Copper-Based Fungicides in Organic Agriculture in Twelve European Countries. *Agronomy*, *12*(3), 673. https://doi.org/10.3390/agronomy12030673

Teste, F. P., Simard, S. W., Durall, D. M., Guy, R. D., Jones, M. D., & Schoonmaker, A. L. (2009). Access to mycorrhizal networks and roots of trees: importance for seedling survival and resource transfer. *Ecology*, *90*(10), 2808–2822. https://doi.org/10.1890/08-1884.1

Thomas, G. W. (1961): Forms of aluminum in cation exchangers. *Transactions of the International Congress of Soil Science*. 7th Madison. Vol. ll: 364-369.

Tisserant, E., Malbreil, M., Kuo, A., Kohler, A., Symeonidi, A., Balestrini, R., Charron, P., Duensing, N., Frei dit Frey, N., Gianinazzi-Pearson, V., Gilbert, L. B., Handa, Y., Herr, J. R., Hijri, M., Koul, R., Kawaguchi, M., Krajinski, F., Lammers, P. J., Masclaux, F. G., … Martin, F. (2013). Genome of an arbuscular mycorrhizal fungus provides insight into the oldest plant symbiosis. *Proceedings of the National Academy of Sciences*, *110*(50), 20117–20122. https://doi.org/10.1073/pnas.1313452110

Tommerup, I. C. (1983). Temperature relations of spore germination and hyphal growth of vesicular-arbuscular mycorrhizal fungi in soil. *Transactions of the British Mycological Society*, *81*(2), 381–387. https://doi.org/10.1016/S0007-1536(83)80090-4

Torsvik, V., & Øvreås, L. (2007). Microbial phylogeny and diversity in soil. In J. D. van Elsas, J. K. Jansson, & J. T. Trevors (Eds.), *Modern soil microbiology* (pp. 23–54). Boca Raton, USA: CRC Press.

Twanabasu, B. R., Smith, C. M., Stevens, K. J., Venables, B. J., & Sears, W. C. (2013a). Triclosan inhibits arbuscular mycorrhizal colonization in three wetland plants. *Science of The Total Environment*, *447*, 450–457. https://doi.org/10.1016/j.scitotenv.2012.12.085

Twanabasu, B. R., Stevens, K. J., & Venables, B. J. (2013). The effects of triclosan on spore germination and hyphal growth of the arbuscular mycorrhizal fungus Glomus intraradices. *Science of The Total Environment*, *454–455*, 51–60. https://doi.org/10.1016/j.scitotenv.2013.02.036

United States Environmental Protection Agency (USEPA) (1995): Fact Sheet: Reregistration Eligibility Decision: (RED): Terbuthylazine. https://www3.epa.gov/pesticides/chem_search/reg_actions/reregistration/fs_PC-080814_1-Jun-95.pdf

Vahter, T., Bueno, C. G., Davison, J., Herodes, K., Hiiesalu, I., Kasari-Toussaint, L., Oja, J., Olsson, P. A., Sepp, S., Zobel, M., Vasar, M., & Öpik, M. (2020). Co-introduction of native mycorrhizal fungi and plant seeds accelerates restoration of post-mining landscapes. *Journal of Applied Ecology*, *57*(9), 1741–1751. https://doi.org/10.1111/1365-2664.13663

van der Heijden, M. G. A., Martin, F. M., Selosse, M., & Sanders, I. R. (2015). Mycorrhizal ecology and evolution: the past, the present, and the future. *New Phytologist*, *205*(4), 1406–1423. https://doi.org/10.1111/nph.13288

van Groenigen, K.-J., Bloem, J., Bååth, E., Boeckx, P., Rousk, J., Bodé, S., Forristal, D., & Jones, M. B. (2010). Abundance, production and stabilization of microbial biomass under conventional and reduced tillage. *Soil Biology and Biochemistry*, *42*(1), 48–55. https://doi.org/10.1016/j.soilbio.2009.09.023

Velicogna, J. R., Schwertfeger, D., Jesmer, A., Beer, C., Kuo, J., DeRosa, M. C., Scroggins, R., Smith, M., & Princz, J. (2021). Soil invertebrate toxicity and bioaccumulation of nano copper oxide and copper sulphate in soils, with and without biosolids amendment. *Ecotoxicology and Environmental Safety*, *217*, 112222. https://doi.org/10.1016/j.ecoenv.2021.112222

Verlinden, M. S., Ven, A., Verbruggen, E., Janssens, I. A., Wallander, H., & Vicca, S. (2018). Favorable effect of mycorrhizae on biomass production efficiency exceeds their carbon cost in a fertilization experiment. *Ecology*, *99*(11), 2525–2534. https://doi.org/10.1002/ecy.2502

Vos, C., Schouteden, N., van Tuinen, D., Chatagnier, O., Elsen, A., de Waele, D., Panis, B., & Gianinazzi-Pearson, V. (2013). Mycorrhiza-induced resistance against the root–knot nematode *Meloidogyne incognita* involves priming of defense gene responses in tomato. *Soil Biology and Biochemistry*, *60*, 45–54. https://doi.org/10.1016/j.soilbio.2013.01.013

Wagg, C., Bender, S. F., Widmer, F., & van der Heijden, M. G. A. (2014). Soil biodiversity and soil community composition determine ecosystem multifunctionality. *Proceedings of the National Academy of Sciences*, *111*(14), 5266–5270. https://doi.org/10.1073/pnas.1320054111

Wagner, S. C., Skipper, H. D., Walley, F., & Bridges, W. B. (2001). Long-term survival of *Glomus claroideum* propagules from soil pot cultures under simulated conditions. *Mycologia*, *93*(5), 815–820. https://doi.org/10.1080/00275514.2001.12063216

Wang, X., Lu, Z., Miller, H., Liu, J., Hou, Z., Liang, S., Zhao, X., Zhang, H., & Borch, T. (2020). Fungicide azoxystrobin induced changes on the soil microbiome. *Applied Soil Ecology*, *145*, 103343. https://doi.org/10.1016/j.apsoil.2019.08.005

Wilkes, T. I., Warner, D. J., Edmonds-Brown, V., Davies, K. G., & Denholm, I. (2021). Zero Tillage Systems Conserve Arbuscular Mycorrhizal Fungi, Enhancing Soil Glomalin and Water Stable Aggregates with Implications for Soil Stability. *Soil Systems*, *5*(1), 4. https://doi.org/10.3390/soilsystems5010004

Wubs, E. R. J., van der Putten, W. H., Bosch, M., & Bezemer, T. M. (2016). Soil inoculation steers restoration of terrestrial ecosystems. *Nature Plants*, *2*(8), 16107. https://doi.org/10.1038/nplants.2016.107

Xu, H., Demetriades, A., Reimann, C., Jiménez, J. J., Filser, J., Zhang, C., & GEMAS Project Team. (2019). Identification of the co-existence of low total organic carbon contents and low pH values in agricultural soil in north-central Europe using hot spot analysis based on GEMAS project data. *Science of The Total Environment*, *678*, 94–104. https://doi.org/10.1016/j.scitotenv.2019.04.382

Xu, Y., Li, B., Hou, K., Du, Z., Allen, S. C., Zhu, L., Li, W., Zhu, L., Wang, J., & Wang, J. (2021). Ecotoxicity evaluation of azoxystrobin on *Eisenia fetida* in different soils. *Environmental Research*, *194*, 110705. https://doi.org/10.1016/j.envres.2020.110705

Zaller, J. G., Frank, T., & Drapela, T. (2011). Soil sand content can alter effects of different taxa of mycorrhizal fungi on plant biomass production of grassland species. *European Journal of Soil Biology*, *47*(3), 175–181. https://doi.org/10.1016/j.ejsobi.2011.03.001

Zaller, J. G., Heigl, F., Ruess, L., & Grabmaier, A. (2014). Glyphosate herbicide affects belowground interactions between earthworms and symbiotic mycorrhizal fungi in a model ecosystem. *Scientific Reports*, *4*(1), 5634. https://doi.org/10.1038/srep05634

Zhang, S., Lehmann, A., Zheng, W., You, Z., & Rillig, M. C. (2019). Arbuscular mycorrhizal fungi increase grain yields: a meta-analysis. *New Phytologist*, *222*(1), 543–555. https://doi.org/10.1111/nph.15570

Zocco, D., Fontaine, J., Lozanova, E., Renard, L., Bivort, C., Durand, R., Grandmougin-Ferjani, A., & Declerck, S. (2008). Effects of two sterol biosynthesis inhibitor fungicides (fenpropimorph and fenhexamid) on the development of an arbuscular mycorrhizal fungus. *Mycological Research*, *112*(5), 592–601. https://doi.org/10.1016/j.mycres.2007.11.010

A Appendix

A.1 Global frequency of occurrence of *Gigaspora margarita* by country, considering data from 4 databases: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010) and FungalTraits (Põlme et al., 2021). Numbers within each country represent the total number of entries recorded.

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Argentina	-	2	-	-
Australia	-	3	78	19
Brazil	-	41	6	8
Burkina Faso	-	1	-	-
Cameroon	-	1	-	12
Canada	-	1	-	-
China	-	3	-	-
Germany	6	-	1	-
India	-	12	-	-
Japan	2	11	6	1
Kenya	-	2	-	-
Korea	-	1	-	-
Malaysia	-	2	-	-
Mexico	-	6	-	-
Namibia	-	1	-	-
New Zealand	-	2	5	45
Pakistan	-	1	-	-
Phillippines	-	1	1	-
Portugal	-	-	72	16
Scotland	-	-	1	-
Senegal	-	1	-	-
South Korea	1	-	-	-
Spain	-	4	-	-
Switzerland	15	1	-	15
Thailand	-	1	-	-
Uganda	-	-	2	-

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
United States of America	-	50	3	2
Venezuela	-	1	1	-

A.2 Global frequency of occurrence of *Rhizophagus intraradices* by country, considering data from 4 databases: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010) and FungalTraits (Põlme et al., 2021). Numbers within each country represent the total number of entries recorded.

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Algeria	-	1	-	-
Argentina	-	5	2	-
Australia	-	4	-	3
Bangladesh	-	-	2	-
Belgium	-	-	3	-
Benin	-	11	-	-
Bolivia	-	-	-	1
Brazil	-	14	-	-
Burkina Faso	-	1	-	-
Canada	5	9	8	13
Canary Island	-	2	-	-
Chile	-	1	-	-
China	-	18	24	17
Colombia	-	1	-	-
Costa Rica	-	-	2	3
Croatia	-	2	-	-
Czech Republic	8	6	-	3
Denmark	3	3	2	-
Ecuador	-	1	-	-
England	-	1	-	-
Estonia	-	1	2	-
France	1	3	2	2
Germany	-	3	44	50
Greece	-	1	-	-
India	-	25	1	1
Iran	-	3	10	12
Israel	-	1	-	-
Italy	-	3	2	-

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Japan	3	4	2	3
Kenya	-	1	2	3
Korea	-	1	-	-
Libya	-	-	-	1
Mali	-	1	-	-
Mexico	-	9	-	-
Namibia	-	3	2	3
Netherlands	3	2	2	3
New Zealand	-	-	4	-
Nigeria	-	1	-	-
Peru	-	1	-	-
Philippines	-	-	1	-
Poland	-	3	1	-
Republic of Korea	-	1	-	-
Russian Federation	-	-	1	-
Scotland	-	3	3	-
Senegal	-	1	-	-
Slovenia	-	-	1	-
South Africa	-	3	-	-
Spain	2	14	10	-
Sweden	-	1	-	-
Switzerland	27	7	9	84
Syria	-	1	-	-
Thailand	-	1	-	-
Tibet	-	7	-	-
Tunisia	-	2	-	-
United States of America	7	91	100	71
Venezuela	-	1	-	-

A.3 Global frequency of occurrence of *Rhizophagus irregularis* by country, considering data from 4 databases: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010) and FungalTraits (Põlme et al., 2021). Numbers within each country represent the total number of entries recorded.

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Canada	22	-	26	170
China	-	-	1	2
Czech Republic	-	-	-	1
Denmark	3	-	1	-
Estonia	-	-	1	-
Iran	-	-	-	3
Lithuania	-	-	-	1
Poland	-	-	-	2
Russian Federation	-	-	8	8
South Korea	1	-	-	-
Ukraine	-	-	1	1
USA	-	-	1	-

A.4 Global frequency of occurrence of *Rhizophagus claurs* by country, considering data from 4 databases: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010) and FungalTraits (Põlme et al., 2021). Numbers within each country represent the total number of entries recorded.

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Argentina	-	3	1	-
Australia	-	3	62	-
Benin	-	1	-	-
Brazil	-	16	11	3
Cameroon	-	1	-	-
Canada	-	7	4	-
China	-	14	-	-
Colombia	-	5	3	2
Croatia	-	1	-	-
Cuba	-	-	5	-
Denmark	-	-	-	7
Finland	-	-	4	-
Germany	-	1	8	-
Iceland	-	1	20	9
India	-	8	-	-
Indonesia	-	-	6	-
Japan	-	3	2	-
Korea	-	1	-	-
Madagascar	-	1	-	-
Malaysia	-	1	-	-
Mexico	1	5	-	-
Namibia	-	5	1	4
Nicaragua	-	1	-	-
Nigeria	-	2	-	-
Poland	-	1	-	-
Portugal	-	-	47	-
Republic of Korea	-	1	-	-
Scotland	-	-	5	-

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Singapore	-	1	-	-
South Korea	-	1	-	-
Spain	-	3	-	-
Switzerland	-	1	-	3
Syria	-	1	-	-
Thailand	-	2	3	-
Tibet	-	7	-	-
Tunisia	-	1	-	-
USA	1	46	11	-
Venezuela	-	1	-	-

A.5 Global frequency of occurrence of *Funneliformis mosseae* by country, considering data from 4 databases: MaarjAM (Öpik et al., 2010), Stürmer et al. (2018), PlutoF (Abarenkov et al., 2010) and FungalTraits (Põlme et al., 2021). Numbers within each country represent the total number of entries recorded.

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Algeria	-	1	-	-
Antarctica	-	-	16	-
Argentina	-	3	1	-
Australia	21	15	1118	23
Austria	-	-	14	-
Belgium	-	-	3	-
Benin	-	3	-	-
Bolivia	2	-	2	-
Brazil	-	18	-	8
Canada	4	25	6	2
Chile	-	1	-	-
China	14	28	11	29
Christmas Island	-	-	1	-
Colombia	3	1	-	-
Costa Rica	-	2	-	-
Croatia	-	2	-	-
Cuba	-	3	-	5
Czech Republic	-	5	-	-
Denmark	40	4	3	4
Ecuador	-	1	-	-
England	-	4	-	-
Estonia	-	1	-	-
Ethiopia	6	-	-	6
Finland	11	4	14	-
France	1	5	6	-
Germany	15	8	62	64
Greece	-	1	-	-
Guatemala	-	1	-	-

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Honduras	-	1	-	-
Hungary	-	1	-	-
India	-	32	6	10
Indonesia	-	1	4	-
Iran	-	2	6	7
Israel	-	2	-	-
Italy	14	12	-	26
Japan	3	3	2	-
Jordan	-	1	-	-
Kazakhstan	2	-	-	-
Kenya	-	2	-	-
Libya	-	1	2	2
Mexico	-	12	1	-
Namibia	-	3	1	1
Netherlands	-	3	3	-
New Zealand	-	8	3	-
Nicaragua	-	1	2	-
Nigeria	-	2	-	-
Pakistan	-	1	-	-
Philippines	7	2	7	-
Poland	-	11	-	3
Portugal	-	3	4	-
Saudi Arabia	-	1	-	-
Scotland	-	7	63	45
Senegal	-	3	2	-
Slovakia	-	-	-	1
Slovenia	-	1	-	-
Somalia	-	2	-	-
South Africa	-	1	-	-
Spain	7	15	32	-
Sweden	-	1	-	-

Country/Database	Maarj AM	Stürmer	PlutoF	Fungal Traits
Switzerland	19	4	18	20
Syria	-	1	-	-
Thailand	-	1	2	-
Tibet	-	5	-	-
Tunisia	-	1	-	-
Uganda	-	-	4	-
Ukraine	-	-	1	1
United States	32	139	56	20
Venezuela	1	-	6	-

A.6 The classification of biocides in the Biocidal Products Regulation (EU) 528/2012)(BPR) (EU, 2012).

MAIN GROUP 1: Disinfectants

Product-type 1: Human hygiene

Product-type 2: Disinfectants and algaecides not intended for direct application to humans or animals

Product-type 3: Veterinary hygiene

Product-type 4: Food and feed area

Product-type 5: Drinking water

MAIN GROUP 2: Preservatives

Product-type 6: Preservatives for products during storage

Product-type 7: Film preservatives

Product-type 8: Wood preservatives

Product-type 9: Fiber, leather, rubber and polymerised materials preservatives

Product-type 10: Construction material preservatives

Product-type 11: Preservatives for liquid-cooling and processing systems

Product-type 12: Slimicides

Product-type 13: Working or cutting fluid preservatives

MAIN GROUP 3: Pest control

Product-type 14: Rodenticides

Product-type 15: Avicides

Product-type 16: Molluscicides, vermicides and products to control other invertebrates

Product-type 17: Piscicides

Product-type 18: Insecticides, acaricides and products to control other arthropods

Product-type 19: Repellents and attractants

Product-type 20: Control of other vertebrates

MAIN GROUP 4: Other biocidal products

Product-type 21: Antifouling products

Product-type 22: Embalming and taxidermist fluids

A.7 List of chemicals used in ecotoxicological studies until June of 2021. Number in parentheses indicates the number of times they were used in the experiments.

Biocides - Biological pesticide (BP):

Azadirachtin (2)

Bacillus thuringiensis (1)

Fusarium oxysporum (1)

Pyrethrum (2)

Terpens (2)

Spinosad (2)

Biocides - Disinfectant agents (DA):

Chloramine-T (1)

Formaldehyde (1)

Mercury chloride (1)

Sodium hypochlorite (1)

Biocides - Personal Care Products (PCP):

Triclocarban (1)

Triclosan (4)

Biocides - Veterinary and human-use pharmaceuticals (VHP):

17-α-ethynyl estradiol (1)

Arbamazepine (1)

Atorvastatin (1)

Chloramphenicol (1)

Chlortetracycline (1)

Doxycycline (1)

Garamicyn (1)

Levofloxacin (1)

Monensin (1)

Oxytetracycline (1)

Pimaracin (1)

Streptomycin sulfate (1)

Sulfamethoxazole (1)

Tetracycline (1)

Tylosin (1)

Fungicides (F):

Azoxystrobin (5)

Chinosol (1)

Benomyl (9)

Captan (6)

Carbendazim (8)

Chlorothalonil (5)

Ciproconazole (1)

Copper hydroxide (1)

Copper oxychloride (2)

Copper sulfate (1)

Dichlofluanide (1)

Ethirimol (1)

Etridiazole (1)

Fenarimole (1)

Fenhexamid (8)

Fenpropimorph (3)

Flutholanil (4)

Folpel (1)

Fosethyl-aluminium (3)

Hymexazole (1)

Iprodione (2)

Kresoxim-methyl (1)

Mancozeb (5)

Mepanipyrim (1)

Metalaxyl (2)

Metalaxyl-M (2)

Mephenoxan (1)

Miclobutanil (1)

Oxycarboxin (1)

Penconazole (1)

Pencycuron (3)

Pentachloronitrobenzene (5)

Prochloraz (2)

Propamocarb (1)

Propiconazole (2)

Pyraclostrobin (2)

Sedaxane (1)

Tetramethylthiuram-disulfide (1)

Tridemorph (1)

Trifloxystrobin (2)

Ziram (1)

Herbicides (H):

Acetochlor (1)

Alachlor (3)

Aminomethylphosphonic acid (AMPA - metabolite of glyphosate) (1)

Bentazon (1)

Bifenox (1)

Chlorotoluron (1)

Dicamba (3)

Difenzoquat methyl sulphate (1)

Glufosinate ammonium (2)

Glyphosate (11)

MCPA (1)

Nicosulfuron (2)

Pendimethalin (2)

Prometryn (1)

Tebuthiuron (1)

Terbuthylazine (1)

Inseticides (I):

Deltamethrin (1)

Dimethoate (3)

Pesticides mixtures (F – fungicides; I – insecticides; H – herbicides):

Azoxystrobin (F)+Fludioxonil (F)+Mefenoxam (F)+Thiamethoxam (I) (1)

Fludioxonil (F)+Mefenoxam (F)+Thiamethoxam (I) (1)

Mecoprop+Ioxynil+Clopyralid (H) (1)

Prothioconazole+Penflufen+Metalaxyl (F) (2)

Pyraclostrobin+Triticonzole+Metalaxyl (F) (1)

Ciprodinyl+Fludioxonyl (F) (1)

Dimetomorph+Folpet (F) (1)

Diuron+Paraquat (H) (1)

Methalaxyl+Mancozeb (F) (1)

Propiconazole+Tridemorph (F)

Tebuconazole+Trifloxystrobin (F) (1)

Tebuconazole+Metalaxyl (F) (1)

A.8 List of AMF species used in the ecotoxicological studies carried out until June of 2021. Number in parentheses indicates the number of studies where they were used.

Acaulospora sp. (1)

Acaulospora koskei isolate SCT049C (1)

Acaulospora laevis (3)

Claroideoglomus spp. (1)

Claroideoglomus claroideum (1)

Dentiscutata nigra (1)

Funneliformis caledonium (1)

Glomus caledonoim = Funneliformis caledonium (2)

Funneliformis monosporum (1)

Funneliformis mosseae (3)

Glomus mosseae = *Funneliformis mosseae* (19)

Funneliformis mosseae isolates from Finland. Denmark or Spain (1)

Funneliformis mosseae isolate BEG119 from Granada (1)

Funneliformis mosseae isolate IMA1 from Pisa (1)

Funneliformis. mosseae isolate BEG116 from Barcelona (2)

Gigaspora spp. (1)

Gigaspora albida (1)

Gigaspora albida isolate SCT200A (2)

Gigaspora decipiens (1)

Gigaspora margarita (5)

Gigaspora margarita isolate SCT077A (1)

Gigaspora rosea (3)

Glomus sp. (2)

Glomus aggregatum (4)

Glomus etunicatum = *Claroideoglomus etunicatum* (14)

Glomus fascicularum (1)

Glomus geosporum (2)

Glomus leptotichum (1)

Glomus monosporum (3)

Glornus monosporum (2)

Paraglomus brasilianum (2)

Paraglomus occultum

Glomus occultum = *Paraglomus occultum* (1)

Rhizophagus clarus isolate RJN102A (2)

Glomus clarum = *Rhizophagus clarus* (5)

Rhizoglomus clarum = *Rhizophagus clarus* (1)

Rhizophagus intraradices (1)

Glomus intraradices = *Rhizophagus intraradices* (12)

Rhizophagus irregularis (1)

Glomus irregularis = *Rhizophagus irregularis* (1)

Rhizophagus irregularis isolate MUCL 41833 (2)

Scutellospora heterogama (2)

Gigaspora calospora = Scutellospora heterogama (2)

Native AMF species from cultivated fields, pastures, and grassland (9)

A.9 List of host plants used in the ecotoxicological studies carried out until June of 2021. Number in parentheses indicates the number of studies where they were used.

Allium cepa (1)

Allium porrum L. cv. Lancelot (1)

Anthylis vulneraria sub sp. Sampaiana (transformed roots) (1)

Arachis hipogea (1)

Avena sativa (1)

Bromus tectorum (1)

Capsicum annum (2)

Cichorium intybus (1)

Cichorium intybus (transformed roots) (1)

Citrus aurantium (2)

Cucumis sativus (1)

Daucus carota (2)

Daucus carota (transformed roots) (4)

Daucus carota (transformed roots) clone DC1 (1)

Glycine max (4)

Glycine max cv. Hobbit (1)

Lactuca sativa (1)

Leucaena leucocephala (2)

Lolium multiflorum (1)

Lotus tenuis (1)

Medicago truncatula (1)

Paspalum notatum (1)

Phaseolus vulgaris (1)

Phaseolus vulgaris cv. Pérola (1)

Pisum sativum cv. Finale (1)

Pisum sativum cv. Little Marvel (1)

Pisum sativum cv. Solare (1)

Schizolobium parahyba var amazonicum (1)

Sesamum indicum (1)

Sitanion hystrix (1)

Trifolium repens (1)

Trifolium subterraneum (1)

Trifolium subterraneum L. cv. Seaton Park (1)

Triticum aestivum (4)

Xanthium strumarium (1)

Zea mays (5)

Zea mays var. saccharata (1)

A.10 List of soils, respective origin country (Ger- Germany; Por – Portugal; Bra – Brazil; Est – Estonia) and soil properties made available for collection by the elements of the ERAMYC consortium.

Origin	Country	Soil code	pH	OC	Sand (%)	Silt (%)	Clay (%)
Lufa 2.1	Germany	Ger1	4.7	0.71	86.0	11.0	3.0
Lufa 2.2	Germany	Ger2	5.4	1.61	76.2	15.8	8.0
Lufa 2.4	Germany	Ger3	7.4	1.99	33.1	41.1	25.8
Gleidson	Portugal	Por1	3.53	3.8	51.4	35.0	13.6
Vairão	Portugal	Por2	4.4	3.23	54.1	35.6	10.3
FM	Portugal	Por3	5.4	2.5	68.0	24.0	8.0
FB	Portugal	Por4	5.4	2.8	70.7	22.7	6.7
Roxo	Portugal	Por5	4.16	2.49	49.2	24.2	26.5
CM	Portugal	Por6	6.7	2.09	81.0	7.8	11.2
Cambisol (FAO)	Brazil	Bra1	4.2	0.96	46.8	24.2	29.0
Cambisol (FAO)	Brazil	Bra2	4.2	2.2	60.0	19.0	21.0
Cambisol (FAO)	Brazil	Bra3	3.9	1.24	26.0	43.0	31.0
Nitisol (FAO)	Brazil	Bra4	5.0	1.88	35.0	32.0	33.0
Nitisol (FAO)	Brazil	Bra5	5.3	1.68	11.0	39.0	50.0
Nitisol (FAO)	Brazil	Bra6	5.0	1.2	35.0	32.0	33.0
Arenosol (FAO)	Brazil	Bra7	5.3	0.36	37.0	59.0	4.0
Ferralsol (FAO)	Brazil	Bra8	4.1	1.08	12.0	39.0	49.0
Ferralsol (FAO)	Brazil	Bra9	4.4	0.32	24.0	46.0	30.0
Cambisol (FAO)	Brazil	Bra10	5.9	2.93	13.4	24.6	62.0
Cambisol (FAO)	Brazil	Bra11	4.7	2.7	21.0	20.0	59.0
KIK002T	Estonia	Est1	6.3	2.2	44.9	40.0	15.2
KIK005T	Estonia	Est2	5.9	2.2	44.7	40.6	14.7
KIK006T	Estonia	Est3	7.2	2.1	57.9	27.3	14.8
KIK008T	Estonia	Est4	5.5	2.3	52.4	34.3	13.3
KIK009T	Estonia	Est5	5.2	1.9	50.1	38.5	11.4
KIK010T	Estonia	Est6	6.2	1.5	66.5	20.3	13.2
KIK039R	Estonia	Est7	5.4	1.2	68.9	22.3	8.8
KIK041R	Estonia	Est8	5.9	1.6	58.9	29.0	12.1
KIK042R	Estonia	Est9	5.4	1.7	43.2	44.6	12.2

Origin	Country	Soil code	pH	OC	Sand (%)	Silt (%)	Clay (%)
KIK050T	Estonia	Est10	6.4	2	59.5	25.3	15.2
KIK052T	Estonia	Est11	5.9	1.5	63.6	22.7	13.7
KIK054T	Estonia	Est12	5.7	1.8	63.8	23.3	13.0
KIK056T	Estonia	Est13	6.0	1.6	53.6	31.8	14.6
KIK058T	Estonia	Est14	5.2	1.6	75.5	14.4	10.1
KIK060T	Estonia	Est15	7.1	1.8	41.8	34.5	23.7
KIK062T	Estonia	Est16	6.2	1.8	59.7	24.0	16.2
KIK064T	Estonia	Est17	5.2	1.3	66.3	21.7	12.0
KIK065R	Estonia	Est18	5.4	2.3	74.9	15.3	9.8
KIK067T	Estonia	Est19	5.8	1.8	68.2	20.3	11.5
KIK083T	Estonia	Est20	5.4	1.7	67.9	21.1	11.1
KIK086T	Estonia	Est21	6.5	1.7	70.2	18.4	11.4
KIK087R	Estonia	Est22	6.1	2.4	71.5	16.0	12.5
KIK098T	Estonia	Est23	4.7	1.2	88.0	5.2	6.9
KIK100T	Estonia	Est24	7.2	2	46.6	33.5	19.9
KIK102T	Estonia	Est25	7.3	2.1	48.5	32.8	18.7
KIK243T	Estonia	Est26	6.0	1.3	53.0	29.8	17.1
KIK244T	Estonia	Est27	7.0	2.8	58.0	11.0	31.0
KIK245T	Estonia	Est28	6.2	1.5	48.3	38.7	13.1
KIK246T	Estonia	Est29	6.0	1.3	50.4	36.6	13.0
KIK247T	Estonia	Est30	6.4	1.6	37.2	40.4	22.4
KIK248T	Estonia	Est31	6.2	2.8	49.0	35.9	15.2

A.11 List of active ingredients considered in the multi-residue analysis performed to the 4 natural soils (Ger1, Por3, Bra8 and Est32) and the respective quantification limits (QL).

Please refer to the “Supporting information” supplementary Excel file.

A.12 Raw data of the experiments from the 1st phase of WP2.

Table A.12 – 1: Raw data obtained from the experiments with different weights of AMF inoculants (*Funneliformis mosseae*, *Rhizophagus irregularis* and *Rhizophagus clarus*), host plants (tomato – *Solanum lycopersicum* and leek – *Allium ampeloprasum*) with OECD 2.5% and Bra8 soil. Experiments conducted by UC and UDESC for the 1st phase of WP2, objective 1.

Please refer to the "Raw data" supplementary Excel file.

Table A.12 – 2: Raw data obtained from the experiments with different number of spores isolated from AMF inoculants of *Funneliformis mosseae*, *Rhizophagus irregularis* and *Rhizophagus clarus,* using the host plants tomato (*Solanum lycopersicum)* and leek (*Allium ampeloprasum*) with OECD 2.5% soil. Experiments conducted by UC and UDESC for the 1st phase of WP2, objective 1.

Please refer to the "Raw data" supplementary Excel file.

Table A.12 – 3: Raw data obtained from the experiments with different weights of AMF inoculants (*Funneliformis mosseae*, *Gigaspora margarita, Gigaspora albida* and *Rhizophagus clarus*), host plants (tomato – *Solanum lycopersicum* and leek – *Allium ampeloprasum*) with OECD 2.5% and Bra8 soil. Experiments conducted by UT and UDESC for the 1st phase of WP2, objective 2.

Please refer to the "Raw data" supplementary Excel file.

Table A.12 – 4: Raw data obtained from the experiments with different weights of AMF inoculants (*Funneliformis mosseae*, *Gigaspora margarita*, *Rhizophagus irregularis* and *Rhizophagus clarus*), host plants (tomato – *Solanum lycopersicum* and leek – *Allium ampeloprasum*) with OECD 2.5%, Por3, #Lufa 2.1, Est32 and Bra8 soil. Experiments conducted by UC, UT and UDESC for the 1st phase of WP2, objective 3.

Please refer to the "Raw data" supplementary Excel file.

A.13 SOP 2.1 - Assessing the inoculum potential of Arbuscular Mycorrhizal Fungi (AMF) from a soil/substrate inoculant (reproduction of the final version of the document, from June 2023).

Title:

Assessing the inoculum potential of Arbuscular Mycorrhizal Fungi (AMF) from an inoculant.

Aim of this Standard Operating Procedure:

Ensure an adequate assessment of the inoculum potential of Arbuscular Mycorrhizal Fungi (AMF) from an inoculant. This assessment is particularly relevant to know the amount of inoculant needed to perform definitive AMF laboratory tests.

Summary:

This protocol describes a method to evaluate the inoculum potential through an inoculant for leek (*Allium ampeloprasum*) or other plant species. The inoculum potential is intended as the capacity of an inoculant to promote root colonization (through the measurement of the percentage of colonized roots). The method is based on the use of inoculant of the selected AMF species mixed with natural or artificial soil in different amounts in test containers with presoaked seeds of the host plant. After 28 days (4 weeks) of incubation the percentage of AMF colonization in the roots is determined by assessing the intra-radicular colonization in the roots.

NOTE: Mixed inoculum is considered a mixture with more than one source of AMF propagules (i.e., spores, hyphae, and colonized roots). Inoculant is a mixture of the mixed inoculum and the soil (usually a sandy soil) that is used as source of mixed inoculum in the experiment.

NOTE: This protocol was developed for *Funneliformis mosseae*, *Rhizophagus irregularis* and *Rhizophagus clarus* inoculant in leek but can be adapted to inoculants of other AMF species and other host plants.

Equipment:

- Growth chamber (temperature of 25 ± 2 ºC and a photoperiod of 16:8h, light:dark)

- Weighing scale

- Autoclave

Test Materials:

- Distilled water

- 250 mL plastic pots as test vessels

- Modified standard artificial soil or a natural soil sieved at 5 mm

NOTE: Modified artificial soil composition: 2.5% of peat (use mature peat, air dried and sieved at 2 mm - peat can be milled if necessary to make sieving easier), 20% of kaolin clay and 77.5% of sand. Soil pH should be adjusted to 6.0 ± 0.5 with the addition of CaCO3.

NOTE: For tropical conditions, a tropical artificial soil (TAS) (Garcia, 2004) can be used.

Biological Materials:

- Soil/substrate inoculant - can be produced under pot culture conditions according to Stutz & Morton (1996) and kept at 4°C until its use for tests or can be obtained from certified AMF germplasm banks.
- *Allium ampeloprasum* seeds

Test conditions:

- Growth/room chamber with a controlled temperature of 23 ± 2 °C and photoperiod of 16:8h light:dark.

- Soil moisture adjusted to 50 ± 10% of its water holding capacity (WHC). Soil water content should be adjusted twice a week on a weight basis (distilled water should be added to compensate water losses when the weight loss is higher than 2%).
- When possible, a relative air moisture of 60% should be used (a high air moisture reduces water losses).

NOTE: A fertilizer solution can be used in the experiments.

Procedure:

The soil to be used in the test (whether it is artificial soil or natural soil) must be previously sterilized by gamma irradiation or, alternatively, autoclaving (through 2 cycles of 60 min at 121°C and under 1.5 atm; the soil must "rest" at least overnight between cycles). Note that, after autoclaving, the soils must be kept at room temperature for 5-7 days before being used in the tests.

Before the beginning of the tests, the number of spores per gram of inoculant should be estimated (see SOP 1.1). This measurement should be done for each new inoculant batch and inoculants with a minimum of 1 spore g^{-1} of soil should be considered for use in the tests.

Four replicates should be prepared for each test mixture. In each test container, add 100 g of soil with the moisture adjusted to 50% of its WHC in the bottom and at the top a mixture of 100 g of soil + inoculant (Figure 1). As an example, the following gradient of mixtures can be tested (note that the proportion of soil + inoculant can be adjusted depending on the number of spores per g of inoculant):

Bottom	Top	
Soil (g)	Soil (g)	Soil/substrate inoculant (g)
100	100	0
100	95	5
100	90	10
100	80	20
100	70	30
100	60	40
100	50	50

Seeds should be previously disinfected by immersion in a 1% sodium hypochlorite solution for 5 min and subsequently washed several times with distilled water. Seeds should then be placed in filter paper moistened with autoclaved distilled water in Petri dishes for 2 to 4 days. Immediately before seeds germination (note that AMF roots colonization may be reduced when seedlings with radicles length are used instead of seeds) 3 seeds should be sown in each test container. Seeds should be buried at 0.5-1 cm of soil surface. After seeds germination only one plant should remain per replicate during the test and the others should be harvested. Note that the test period starts only after seeds germination in each vessel.

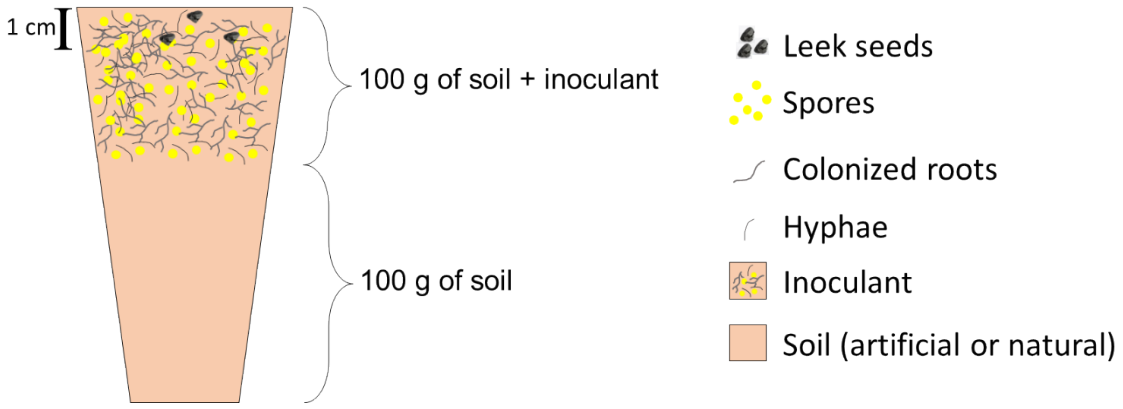


Figure 1. Representation of a test vessel with the respective test mixture. Source: Luís Carlos Iuñes de Oliveira Filho.

After seeds germination, soil surface can be covered with dry and previously washed (e.g. with a 1% HCl solution and/or washed five times with distilled water) and autoclaved glass beads or coarse sand to reduce water losses and contaminations.

Soil moisture content must be corrected twice a week in each replicate, when necessary, by adding water on a weight basis (procedure described in "Test conditions" section).

The test period should be 28 days (4 weeks), as according to Brundrett et al. (1996), seedlings require 2-4 weeks to form AM associations.

Test containers should be randomly distributed within the space used for the test and their positions should be changed once a week.

Test should run in a growth/room chamber at temperature of 25 ± 2 ºC under a photoperiod of 16:8h light:dark.

After the test period (28 days), the total intra-radicular colonization (%) of AMF is evaluated in each replicate. The percentage of root colonization is evaluated in stained roots as proposed by Vierheilig et al. (1998) (see details in SOPs 2.2 and 2.3). Root segments of approximately 1 cm are laid out on glass slides for microscope to allow the observation of hyphae, vesicles, arbuscules and spores inside the roots as described by McGonigle et al. (1990) (see details in SOP 2.3).

NOTE: If necessary, a fertilizer solution can be provided through a Long Ashton solution with 10% Phosphorus (9.3 mg P kg-1 soil) and applying 5 ml per replicate (200 g) 5-7 days after planting or germination. In Annex A the Long Ashton nutrient solution composition is presented.

References:

Brundrett, M.; Bougher, N.; Dell, B.; Grove, T.; Malajczuk, N. 1996. Working with Mycorrhizas in Forestry and Agriculture. ACIAR Monograph 32. 374 + x p.

Garcia, M.V.B. 2004. Effects of pesticides on soil fauna: development of ecotoxicological test methods for tropical regions. Thesis, University of Bonn.

McGonigle, T.P.; Miller, M.H.; Evans, D.G.; Fairchild, G.L; Swan, J.A. 1990. A new method which gives an objective measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. The New Phytologist, 115:495-501.

Stutz, J.C.; Morton, J.B. Successive pot cultures reveal high species richness of arbuscular endomycorrhizal fungi in arid ecosystems. 1996. Canadian Journal of Botany, 74:1883–1889.

Vierheilig, H.; Coughlan, A.P.; Wyss, U.; Piché, Y. 1998. Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi. Applied and Environmental Microbiology, 64:5004-5007.

Annex A
(informative)

Composition of 1 L of a Long Ashton nutrient solution with low P.

Component		Stock solution g L^{-1}	Volume of stock solutions to complete 1 L fertilizer solution
Macronutrients			**100 mL**
KNO_3	Potassium nitrate	4.04	
$Ca(NO_3)_2•4H_2O$	Calcium nitrate tetrahydrate	9.44	
$NaH_2PO_4•4H_2O$	Sodium Dihydrogen Phosphate Tetrahydrate	1.84	
$MgSO_4•7H_2O$	Magnesium sulfate heptahydrate	3.68	
Micronutrients			**10 mL**
$MnSO_4•H_2O$	Manganese (II) sulfate monohydrate	1.68	
$CuSO_4•5H_2O$	Copper sulfate pentahydrate	0.25	
$ZnSO_4•7H_2O$	Zinc sulfate heptahydrate	0.29	
H_3BO_3	Boric acid	2.23	
Molybdenum			**1 mL**
$(NH_4)_6O_{24}Mo_7•4H_2O$	Ammonium molybdate tetrahydrate	0.35	
Iron			**10 mL**
$FeSO_4•7H_2O$	Iron(II) sulfate heptahydrate	3.05	
Ferric sodium EDTA	Ethylenediaminetetraacetic Acid ferric sodium salt	4.09	
Distilled water			**879 mL**

A.14 SOP 2.2: Staining method for colonization structures of Arbuscular Mycorrhizal Fungi (AMF) in root tissues (reproduction of the final version of the document, from January 2024).

Aim of this Standard Operating Procedure

Ensure an adequate staining of roots to facilitate the observation of hyphae, vesicles, arbuscules in root tissues resulting from the colonization of AMF.

Summary

This protocol describes a method for staining root tissues to allow the observation of the presence of colonization structures of Arbuscular Mycorrhizal Fungi (AMF) on leek (*Allium ampeloprasum*). Plant roots are first clarified, acidified and then colored to facilitate the visualization of AMF structures.

Equipment

- Water bath.

Test Materials

- Tap water;
- Distilled water;
- 50 ml Erlenmeyers. test tubes or biopsy specimen cassettes;
- Fine mesh sieve (e.g. 300 µm mesh);
- 10% potassium hydroxide solution;
- 5% acetic acid solution or 4% hydrochloric acid solution;
- Black ink or 0.05% trypan blue;
- Acidic glycerol (optional).

Procedure

The methodologies described for root staining are adapted from Vierheilig et al. (1998) and Kormanik and McGraw (1982).

At the end of the 56 days of test, remove the covering sand or glass beads from the top of the replicate. Collect soil samples for chemical analysis, extra-radicular mycelium length, soil final pH and soil final moisture as described in SOP 2.4 and Test 2.

Collect the maximum of leek roots possible: the objective is to collect at least the sufficient amount to prepare one slide with 10 root segments of 1 cm each per replicate. However, it is recommended to collect an extra quantity of roots from each replicate as a backup for eventual problems in the staining process. Thin roots must be selected (Figure 1A) and old lignified roots can be rejected.

When it is not possible to stain the roots immediately after the end of the test, these can be stored for a maximum of 5 days in distilled water until staining.

The staining of AMF structures inside the roots comprises 3 steps: **root clearing**, **root acidification** and **staining of fungi structures**. These must be followed in the presented sequential order.

To **clear** the collected roots, follow the steps below:

1. Wash the roots collected from the replicate with tap water to remove soil and organic residues;
2. Place the roots in an Erlenmeyer flask (or other type of vessel);
3. Fully immerse the roots in 10% KOH overnight (~14 hours) at room temperature. For thicker roots. the 10% KOH solution may be changed at least once over this period;
4. Place the flasks in a water-bath at 90°C and incubate for 5 minutes. This step removes the cytoplasmic content from the cells. At the end of this period, the solution is expected to present a brownish color. Note that if roots are left in hot KOH for too long, the cortex will disintegrate;
5. Remove the flasks from the water bath and pour their content (roots and KOH) in a fine sieve (e.g.. 300 µm mesh) so that the roots are retained while the KOH is removed by runoff;

6. Wash the roots with tap water to remove KOH residue and place them back into the empty flasks. Roots should look like those presented in Figure 1B.

Alternatively, roots can be cleared in a 2.5% KOH solution by placing cassettes with roots in a beaker that is inside a water bath at 90ºC in a fume cupboard, for 15 minutes. Periodically, cassettes must be agitated so that the KOH is refreshed inside the cassettes. After the 15 minutes period, cassettes are drained and washed thoroughly under tap water as described previously.

The next step is **root acidification**. For that follow the steps presented below, which are based on the methodologies described by Gerdemann (1955) and Phillips & Hayman (1970):

1. Immerse the previously cleared root in a 5% acetic acid solution for 50 minutes at room temperature. Acetic acid opens cortex cell membranes, which improves the staining process of fungal structures. Alternatively, 4% hydrochloric acid may be used for 60 minutes;
2. Remove the acetic acid (or hydrochloric acid) from the flasks. There is no need of washing the roots before following to the next steps.

After clearing and acidifying. the roots are ready for **staining of fungi structures**:

1. Immerse the roots. inside a flask, in a 1:1 solution of 5% black ink solution and 5% acetic acid;
2. Place the flasks in a water bath at 90°C and incubate for 5 minutes;
3. Remove the flasks from the water bath;
4. Pour the flask content (roots and ink-acetic acid solution) in a fine sieve (e.g. 300 µm mesh) so that the roots are retained while the ink-acetic acid solution is removed by runoff;
5. Wash the roots with tap water to remove ink excess;
6. Roots should look like those presented in Figure 1C.

Alternatively, cleared roots inside the cassettes, may be immersed in 0.05% trypan blue at 90ºC for 15 minutes in a fume cupboard. Periodically, cassettes should be agitated so that the dye is refreshed inside the cassettes. Then, roots should be drained and washed thoroughly under tap water and lastly immersed with acid glycerol (50 mL 1% HCl + 450 mL distilled water + 500 mL glycerol) for 10-20 minutes in a water bath at 90ºC or overnight at room temperature to remove excessive coloration from empty root cells.

If necessary, stained roots can be stored, at room temperature, in flasks or Falcon tubes with distillated water for a period not longer than 6 weeks (Vierheilig et al., 1998) or in acidified glycerol (50 mL 1% HCl + 450 mL distilled water + 500 mL glycerol or 70 mL 5% acetic acid + 230 mL distilled water + 700 mL glycerol, depending on the acid used in the acidification step of the staining process).

Note 1: According to Brundrett et al. (1996), depending on the flask used, clearing procedures generally do not work well with root samples with more than 2 g.

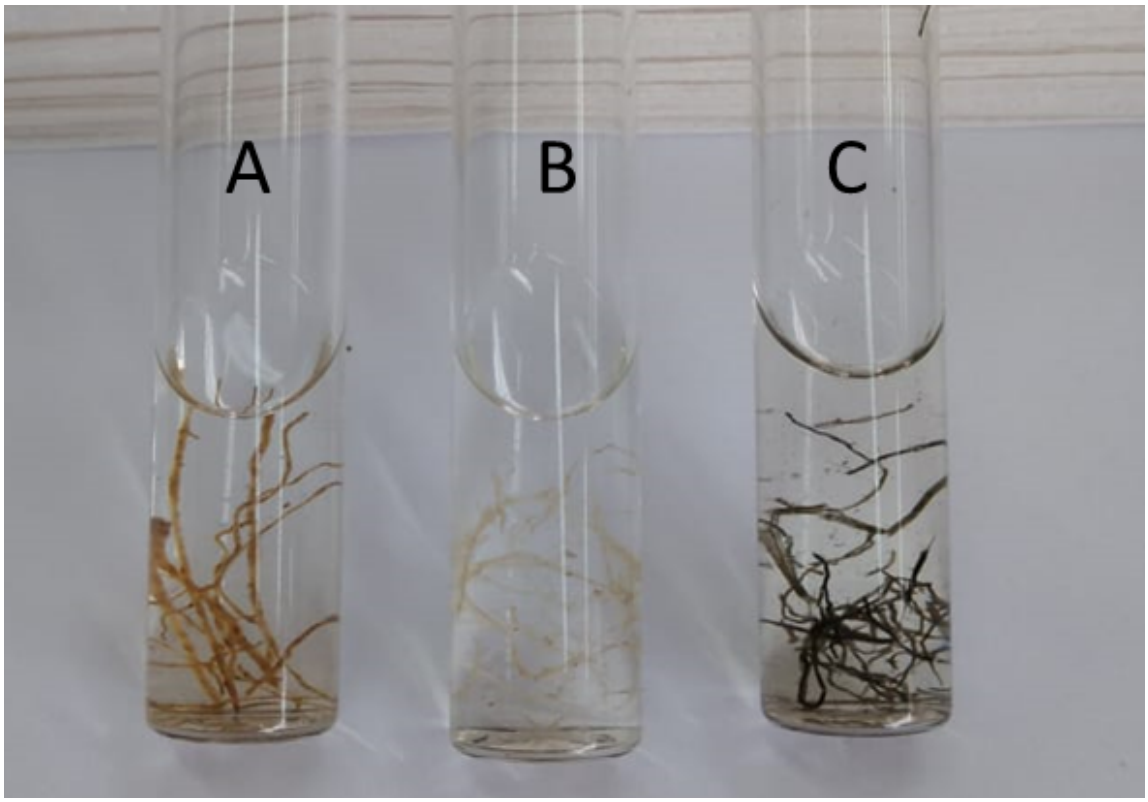


Figure 1. Different steps to root staining: (A) freshly collected roots, (B) cleared roots and (C) stained roots. Source: Luís Carlos Iuñes de Oliveira Filho.

References

Brundrett. M.; Bougher. N.; Dell. B.; Grove. T.; Malajczuk. N. 1996. Working with Mycorrhizas in Forestry and Agriculture. ACIAR Monograph 32. 374 + x p.

Gerdemann. J.W. 1955. Relation of a large soil-borne spore to phycomycetous mycorrhizal infections. Mycologia. 47:619-632.

Kormanik P. McGraw. 1982. Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: Methods and principles of mycorrhizal research. Edited by NC Schenck. The American Phytopathological Society. St. Paul. MN.

Phillips. J. M.; Hayman. D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans. Br. Mycol. Soc.. 55:158-161.

Vierheilig. H.; Coughlan. A.P.; Wyss. U.; Piché. Y. 1998. Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi. Applied and Environmental Microbiology. 64:5004-5007.

A.15 SOP 2.3: Assessing the percentage of total and arbuscular root colonization of Arbuscular Mycorrhizal Fungi (AMF) (reproduction of the final version of the document, from January 2024).

Aim of this Standard Operating Procedure

Ensure an adequate measurement of the percentage of total and arbuscular root colonization of leek.

Summary

This protocol describes a method to determine the total and arbuscular colonization of AMF in roots of leek (*Allium ampeloprasum*). The method is based on the observation of the presence or absence of fungal structures (i.e., hyphae, vesicles and arbuscules) in root fragments (previously stained according to the SOP 2.2).

Equipment

- Microscope 400x magnification (10x ocular × 40x objective).

Test Materials

- Microscope slides and coverslips.

Procedure

After staining the AMF structures on the roots (see SOP 2.2), one microscope slide is mounted per replicate as soon as possible:

1. Cut the roots from the replicate (preferably, the straightest and thinnest roots) into fragments of approximately 1 cm;
2. Place the fragments parallelly on a microscope slide (Figure 1). At least one slide with 10 root fragments should be prepared for each replicate;
3. Cover with a coverslip and observe at the microscope.

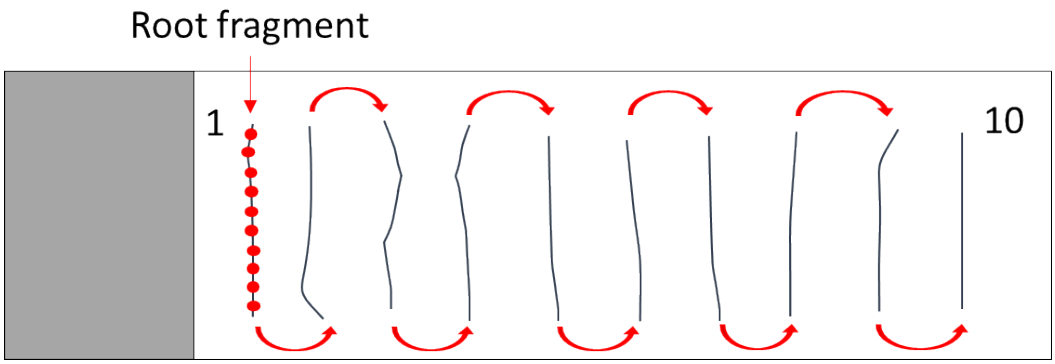


Figure 1. Disposition of root fragments on the microscope slide. Black lines represent stained root fragments; red dots represent the microscope field of view in the observation positions (see below); red arrows indicate the reading sequence of the roots. Source: Luís Carlos Iuñes de Oliveira Filho.

Total and arbuscular root colonization are determined following the method based on McGonigle et al. (1990). An observation point is considered colonized when there is the presence of at least one type of the four fungal structures: vesicles, arbuscles and/or hyphae (Figure 3).

To assess intra-radicular colonization, follow the steps bellow:

1. Place the microscope slide under a microscope at 400x magnification (10x ocular and 40x objective);
2. Observe a randomly selected observation point in a root fragment;
3. Observe and record the presence or absence of any of the four AMF structures (total colonization) but individually record the presence of arbuscules (arbuscular colonization);
4. Assess 9 more randomly selected observation points in the same root fragment. A total of 10 observation points must be assessed per root fragment. Observation points must not overlap (Figure 1 and 2);
5. Repeat for the 10 root fragments present in the microscope slide;
6. Register, in the Excel file provided by the project coordination, the total number of observation points in which you observed the presence of AMF structures (total and/or arbuscular colonization) per replicate.

NOTE 2: register the presence/absence of AMF structures, not the number of structures observed.

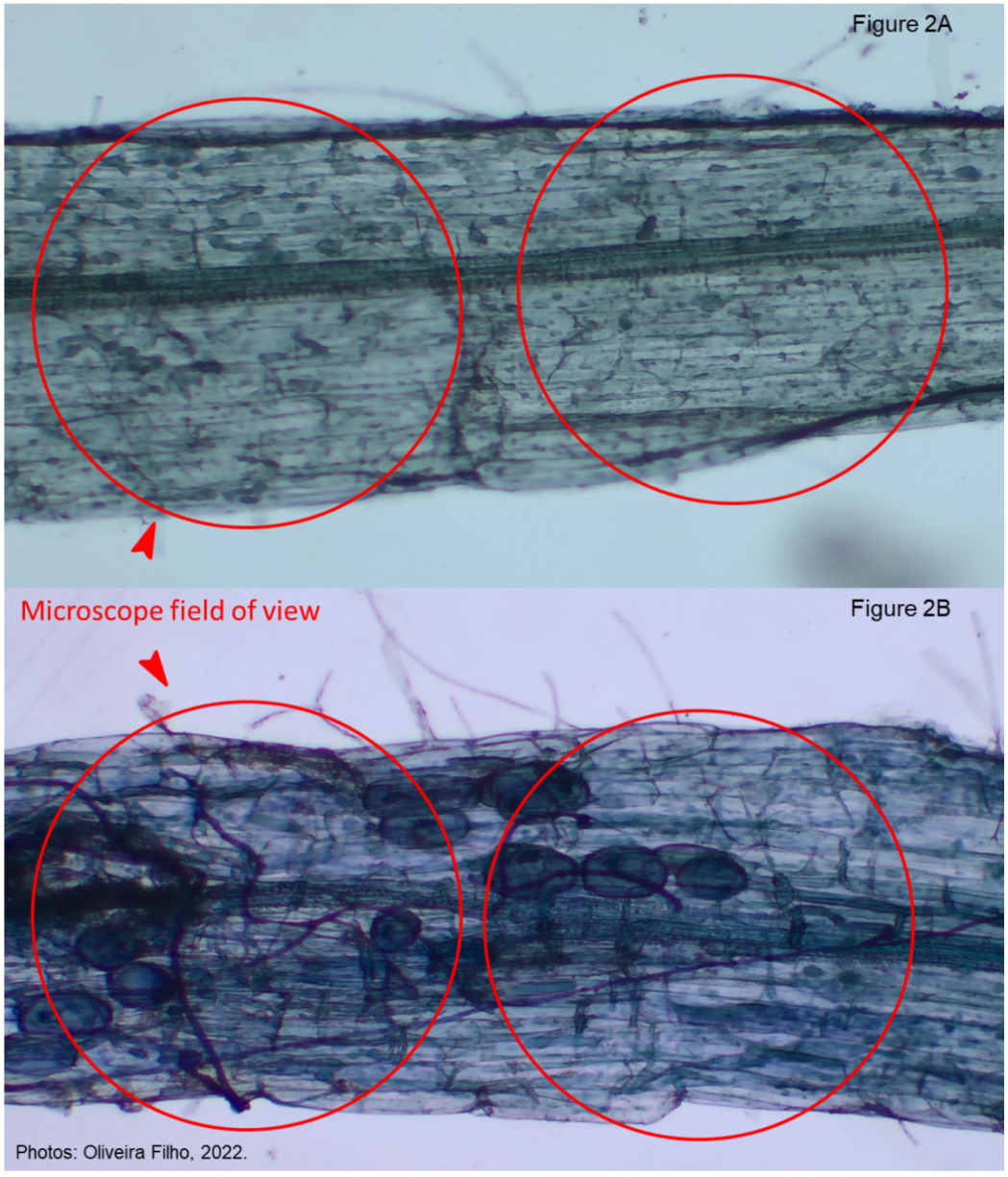


Figure 2. Microscopic examination of non-colonized (2A) and colonized (2B) roots. Red circles represent the observation positions (i.e., microscope field of view).

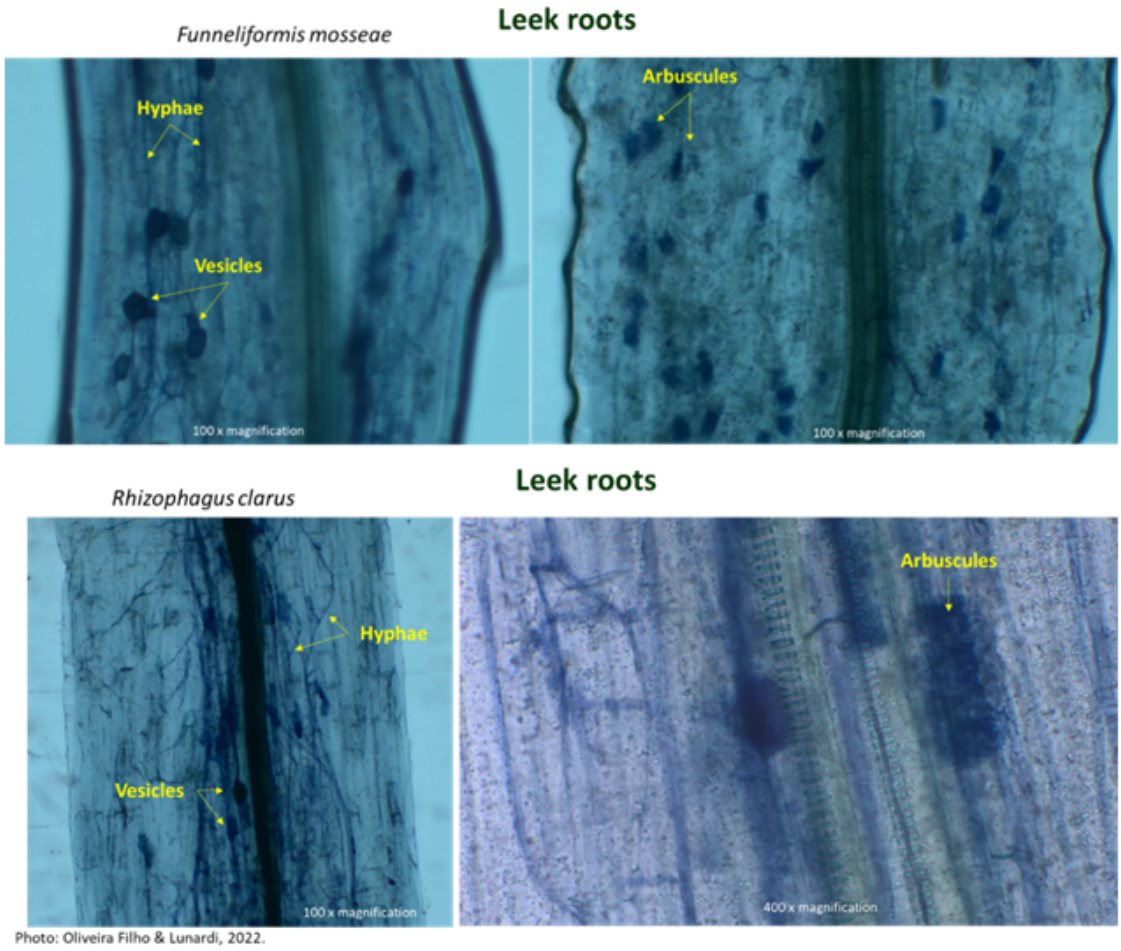


Figure 3. Fungal structures of *Funneliformis mosseae* and *Rhizophagus clarus* in root fragments of leek (*Allium ampeloprasum*).

Calculation and expression of results

The percentage of total colonization and arbuscular colonization is calculated considering the number of observation points where at least one fungal structure (vesicles, arbuscles or hyphae) and one arbuscule is observed, respectively. As the total number of observation points per replicate is 100, the number of observation positions with one or more presences represents the percentage of colonization in each slide or replicate.

References

McGonigle. T.P.; Miller. M.H.; Evans. D.G.; Fairchild. G.L; Swan. J.A. 1990. A new method which gives an objective measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. The New Phytologist. 115:495-501.

A.16 SOP 2.4: Assessing the total extra-radicular mycelium length of Arbuscular Mycorrhizal Fungi (AMF) (reproduction of the final version of the document, from January 2024).

Aim of the Standard Operating Procedure

Ensure an adequate measurement of the length of extra-radicular hyphae in the soil.

Summary

This protocol describes a method to determine the length of extra-radicular hyphae in the soil. The method is based on the extraction of hyphae from soil in a filter holder system where the hyphae retained in a cellulose nitrate membrane are measured through a grid line intersect method.

Equipment

- Blender (with capacity for, at least, 1.5 L);
- Kitasato flask;
- Microscope with magnification of 400x (10x ocular and 40x objective), preferably;
- Polycarbonate filter holder;
- Vacuum pump.

Materials

- Tap water;
- 1 L beaker;
- 2 L beaker;
- Sieve 1 mm mesh;
- Sieve 0.25 mm mesh;
- Sieve 45 µm mesh;
- Cellulose nitrate membranes (4.7 cm diameter, 0.45 µm porosity, 3 mm grid);
- Eye piece Whipple disc - 10 x 10 grid (1 mm^2);

- Wash bottle with distilled water;
- Glass slides;
- Glass rods;
- Spoon or spatula;
- Almond oil.

Procedure

The method for the measurement of the total extra-radicular mycelium length (TEML) in soil is based on the procedures described by Melloni (1996) and Melloni & Cardoso (1999).

At the end of the 56 days of test, remove the covering sand or glass beads from the top of the replicate. Collect soil for final pH and chemical analysis as described in Test 2.

After that, homogenise the remaining soil (both the top and bottom layer) and collect 3 sub-samples of 10 g of soil per experimental unit:

1. One sub-sample will be used to measure hyphae extra-radicular length (expressed in m of hyphae per g of dry soil);
2. One sub-sample will be used to determine the soil final moisture (see Test 2, Annex C). Note that soil moisture must be assessed before the assessment of the extra-radicular mycelium length of AMF. Use the spreadsheet provided by the project coordination to register the results;
3. One sub-sample must be stored at -20ºC to use if further measurements are needed.

NOTE 1: When the assessment of the AMF hyphae length cannot be done immediately after the experiment, soil samples can be stored using one of the two following methods: (i) soil samples can be stored fresh (i.e., with the moisture they had when collected) or (ii) previously air-dried. In both cases, soil samples for mycelium assessment can be stored at 4°C or -20°C.

To **extract extra-radicular hyphae,** follow the steps described below:

1. Place one of the sub-samples (i.e., 10 g of soil) in a 1 L beaker with 500 ml of tap water;
2. Stir vigorously with a glass rod for 30 seconds and leave to settle down for 1 minute;
3. Stack the 1 mm (top sieve) and 0.25 mm (bottom sieve) sieves above a 2 L beaker;
4. Sieve the tap water with soil and collect it in the beaker placed under the sieves;
5. Repeat steps 2, 3 and 4 twice to reach a total sieved tap water volume of about 1.5 L;
6. Stir the 1.5 L of water for 30 seconds in a blender at the lowest speed. Let the water settle down for 2 minutes;
7. In a cellulose nitrate membrane (4.7 cm diameter, 0.45 µm porosity, 3 mm grid) mark, with a pen, a square of 8 x 8 grid (see Figure 1). Place the membrane in the filtration compartment of the vacuum pump;
8. Sieve 500 ml of the supernatant in a 45 µm sieve;
9. With a wash bottle, wash the materials retained on the 45 µm sieve to the filtration compartment of the vacuum pump;
10. Filter the retained materials until there is no water in the compartment with the membrane;
11. The materials retained in the membrane (composed of soil/substrate residue, mycelium, and other particles) are submitted to evaluation.

For the **measurement of the TEML**:

1. Air dry the membrane with the sample (i.e., the filtered material) and place it on a glass slide, lubricated with a drop of almond oil to improve visualization at the microscope;
2. Place the glass slide under a microscope with magnification of 400x (other magnification can be used, see NOTE 3);
3. Place the *Eye piece Whipple disc* inside the microscope's ocular;

4. Observe and register the number of intersections of the hyphae with horizontal and vertical lines of the *Eye piece Whipple disc* grid. Note that when the point of intersection of the hyphae with the grid is the corner of the grid (i.e., the intersection of a vertical and a horizontal line), it only counts as one intersection;
5. Assess one sampling site inside each one of the 64 fields (8 x 8 grid) of the cellulose nitrate membrane (Figure 1);
6. Register the number of intersections observed on the sample in the spreadsheet provided by the project coordination.

NOTE 2: Hyphae are generally colorless and have a diameter of around 2 µm. Roots are generally thicker (root hairs: Ø 10-20 µm; lateral roots: 100-500 µm) and can be colored (Berbara et al. (2006)).

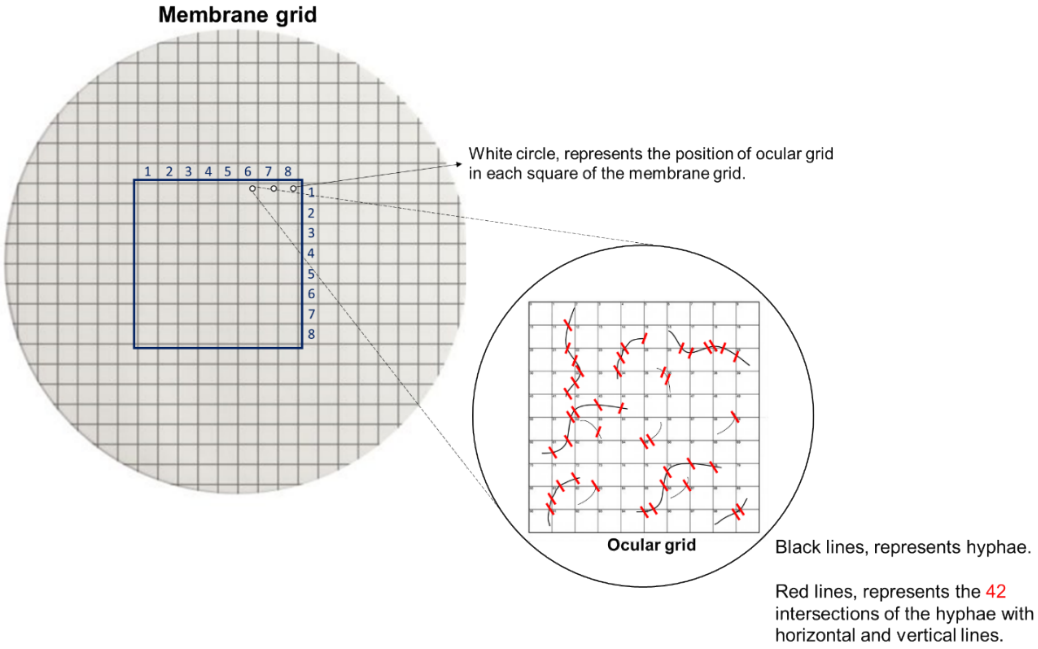


Figure 1. Procedure for quantification of the number of intersections of extra-radicular hyphae. Note that when the point of intersection of the hyphae with the grid is in the intersection of a vertical and horizontal grid line, it only counts as one point of intersection. Source: Luís Carlos Iuñes de Oliveira Filho.

Calculation and expression of results

The number of intersections of the hyphae with horizontal and vertical lines of the *Eye piece Whipple disc* grid is determined and transformed into mycelium length based on information taken from Cardoso-Filho (1994), Melloni (1996), and Nogueira (1997). The TEML is expressed in meters of hyphae per gram of dry soil, after applying the following equation:

$$R = \frac{\mathbf{0.037160233}*n}{10 - \theta}$$

where *R* is the length of the extra-radicular mycelium present in the replicate (in m g^{-1} of soil) evaluated in the 64 membrane fields; *n* is the number of hyphae intersections in the horizontal and vertical lines of the *Eye piece Whipple disc* grid and *θ* is the water content in 10 g of fresh soil (measured using the first sub-sample).

NOTE 3: Changes in the final formula (R), i.e., in the equation correction factor (presented in bold in the equation), must be done when using different microscope magnification (ocular and objective lens) and/or size of Eye piece Whipple disc grid, following the link below:
https://udesc-my.sharepoint.com/:f:/g/personal/48502758934_udesc_br/Epgrsvl8ENJFiLgMbp4K7JIB70r_oMKVNLHk2-lWeZ1FVA?e=WpLSoV

References

Berbara. R.L.L.; Souza. F.A.; Fonseca. H.M.A.C. (2006) Fungos micorrízicos arbusculares: muito além da nutrição. In: FERNANDES. M. S. (Ed.). Nutrição mineral de plantas. Viçosa: SBCS. 2006. p. 53-88.

Cardoso-Filho. J.A. 1994. Quantificação do micélio extramatricial de *Glomus etunicatum* e da sua atividade, em simbiose com o milho. Piracicaba. 121 p. (Dissertação de mestrado) – Escola superior de Agricultura "Luiz de Queiroz". Universidade de São Paulo.

Melloni. R.A. 1996. Quantificação do micélio extrarradicular de fungos micorrízicos arbusculares em plantas cítricas. Piracicaba. 83 p. (Dissertação de mestrado) – Escola superior de Agricultura "Luiz de Queiroz". Universidade de São Paulo.

Melloni. R.; Cardoso. E.J.B.N. 1999. Quantificação de micélio extrarradicular de fungos micorrízicos arbusculares em plantas cítricas e endófitos: I. Método empregado. Rev. Bras. Ciênc. Solo. 23:53-58.

Nogueira. A.N. colonização radicular de produção de micélio externos por duas espécies de fungos micorrízicos arbusculares em soja submetida a doses de fósforo. Piracicaba. 1997. 92 p. (Dissertação de mestrado) – Escola superior de Agricultura "Luiz de Queiroz". Universidade de São Paulo.

A.17 SOP Test 1: Assessing the effects of chemicals on the pre-symbiotic phase of Arbuscular Mycorrhizal Fungi (AMF) (reproduction of the final version of the document, from October 2024).

Aim of this Standard Operating Procedure

Ensure an adequate evaluation of the effects of chemicals on the germination of spores of the arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis*.

Summary

The test assesses the effects of test chemicals present in soil on the percentage of germination of the AMF *Rhizophagus irregularis* mature spores, inside a membrane filter “spore sandwich”, after 14 days of exposure under controlled laboratory conditions. The methodologies described are based on the procedures from ISO 10832 (2009) and Mallmann et al. (2018).

Equipment

- Incubator with controlled temperature (see *Test conditions*);
- Weighing scale;
- Oven;
- Centrifuge, capable of 2000 rpm;
- Stereomicroscope (50x magnification) and/or microscope (100x magnification).

Materials

- Inoculant (must be stored at 4ºC);
- Sterile plastic Petri dishes with 9 cm of diameter;
- Filter paper or paper towel;
- Nitrocellulose membrane filters (47 mm diameter, 0.45 µm porosity, white, 3 mm grid lined);
- Vessels for soil moisture and pH assessment;

- Distilled water with pH ≥ 5.5;
- 50 mL Falcon tubes for spore isolation;
- 50 mL Falcon tubes for soil sampling (provided by the project coordination);
- Spoons;
- Plastic pipettes;
- Watch-glass;
- Ultra-fine tweezers;
- Black or blue ink;
- Sucrose (or regular white sugar);
- Parafilm;
- Microtubes;
- Sieves with mesh of 250 µm and 45 µm;
- 5% acetic acid solution;
- Modified standard artificial soil (see NOTE 1).

NOTE 1: Modified artificial soil composition: 2.5% of peat (use mature peat, air dried and sieved at 2 mm), 20% of kaolin clay (with kaolinite content preferably above 30%) and 77.5% of air-dried industrial sand (with more than 50% of the particles between 50 and 200 microns). Soil pH should be adjusted to 6.0 ± 0.5 with the addition of $CaCO_3$.

Test conditions

Replicates are incubated under controlled conditions: 26 ± 2ºC in the dark (alternatively Petri dishes can be covered with aluminium foil).

Procedure

On the day before the test setup, AMF spores are isolated using a sucrose gradient followed by centrifugation. The methodology for spore isolation is described in Annex A. Test soil aliquots must be dried overnight before the day of soil spiking (see step 1.1).

Test setup

Three types of treatment are considered in each experiment:

Solvent control (CT_{sol})	Replicates with test soil moistened with the organic solvent used in the stock solution but without test substance.
Negative control (C_0)	Replicates with the test soil without the test substance.
Test treatments (C_x = C_1. C_2….C_6)	Treatments with test soil spiked with the test substance in different concentrations.

Each treatment is composed by 6 replicates.

To initiate the experiment, use the Excel spreadsheet (“Test setup”) provided by the consortium and follow the steps below:

1st Step – Aliquot contamination

1.1. Oven-dry one individual portion of fresh test soil per treatment at 70ºC overnight (“Soil aliquot – Fresh weight”);

1.2. Prepare the Stock Solution by diluting the contaminant (Azoxystrobin or Fluazinam) in acetone (“Stock solution preparation”);

1.3. Contaminate the soil portions (“Soil aliquot – Dry weight”) with a mixture of “Stock solution” and “Acetone” in a mortar (in alternative, glass or metallic vessels can be used). Mix very well and let the solvent volatilize totally before following for the 2nd step.

1.4. While waiting for the acetone to evaporate, prepare the “spore sandwiches”:

1.4.1. With a pencil, mark, on a wet nitrocellulose membrane filter, 30 intercalated points as shown in Figure 1a (left panel);

1.4.2. Place the previously isolated spores on a filter paper or paper towel;

1.4.3. Under the stereomicroscope (50x magnification), select 30 viable spores with shiny and undamaged walls. Select spores without

hyphae or, alternatively, remove the hyphae connected to the spore by carefully cutting it with tweezers or a scalpel blade;

1.4.4. Transfer the selected spores, with ultra-fine tweezers, to the points marked on the nitrocellulose membrane filter;

1.4.5. Cover the nitrocellulose membrane filter containing the spores with another previously moisturized membrane, creating a “spore sandwich” (Figure 1a, right panel). The grids of the two membranes must overlap;

1.4.6. Keep the “spore sandwiches” on moist paper towel until inserting them on the replicates (avoid waterlogging as it increases the risk of losing spores from the membranes).

NOTE 2: you can identify the nitrocellulose membranes by carefully writing, with a pencil, the treatment and replicate identification code on their back before preparing the “spore sandwiches”.

NOTE 3: The number of “spore sandwiches” prepared in one day must be the same as the number of replicates to be prepared at the same day because the “spore sandwiches” must be incorporated between the soil layers in the replicates (3rd Step) at the same day of its preparation.

On the day of spiking the soil aliquots and after the acetone has evaporated, replicates of treatments CTsol, C0, C1 and C2 must be prepared following the 2nd and 3rd Steps.

Soil aliquots for treatments C3, C4, C5 and C6 must be sealed and stored at 4ºC overnight after total evaporation of the acetone and replicates of these treatments must be prepared on the following day also following the 2nd and the 3rd Steps.

2nd Step – Contamination of soil by treatment

2.1. Once all solvent has volatilized, mix the “Soil aliquot” with the rest of the soil batch (“Fresh soil”). Add the volume of “Water” to reach 50% of the maximum WHC of the soil.

Each treatment must be prepared individually, from the lowest to the highest concentration.

The 6 replicates composing each treatment are filled after this step, following the steps described in the section "3rd Step – Replicate preparation and sampling".

3rd Step – Replicate preparation and sampling

3.1. Place 40 g DW of soil ("Moistened soil/replicate (g), Bottom layer") on the bottom part of the Petri dish, avoiding soil compaction. Lay the previously prepared "spore sandwich" on the soil and cover it with 40 g DW of soil ("Moistened soil/replicate (g), Top layer") (Figure 1b). Ensure that the "spore sandwich" is in total contact with the two soil layers. Prepare the 6 replicates of the treatment before following to the next treatment;

3.2. For treatments C1 to C6, collect three samples of 50 g of soil FW per treatment for chemical analysis; for C0 collect only one sample of 50 g of soil for chemical analysis (use the Falcons tubes provided by the project coordination). No samples are collected for the solvent control (CTsol). The soil samples must be stored at -20ºC until analysis/shipment;

3.3. Collect one sample of 10 g of soil for determination of soil initial pH and one sample of 10 g of soil for determination of initial soil WC per treatment (see Annex C and D. Results must be registered in the spreadsheet provided by the project coordination);

3.4. Seal each Petri dish with Parafilm;

3.5. Randomly distribute the test replicates in the growth chamber following the described test conditions.

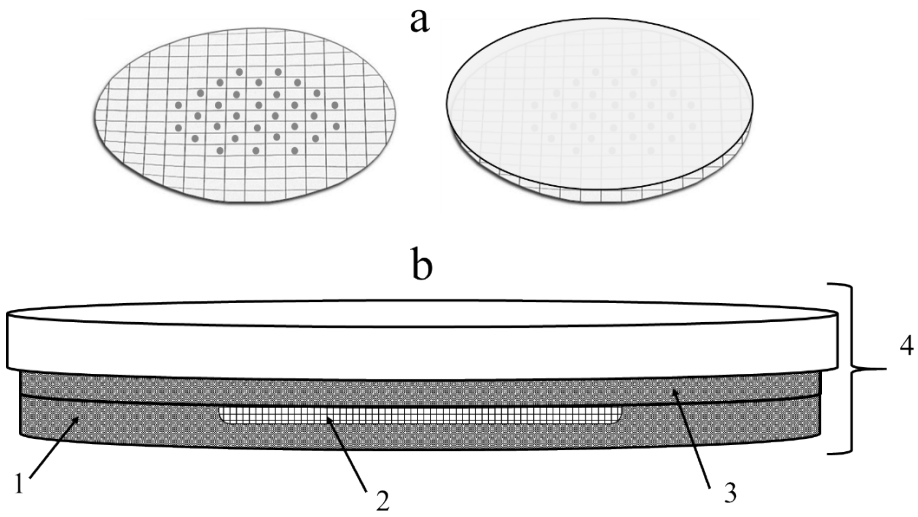


Figure 1 – Representation of an experimental unit: a) spores' arrangement on the grid membrane and "spore sandwich"; b) assembly of the experimental unit for spore germination test with the 1st layer of soil (1), the "spore sandwich" (2), the 2nd layer of soil (3) in the Petri dish (4). Adapted from Mallmann et al. (2018).

Test final measurements

Fourteen days after replicates preparation, disassemble the replicates. Note that replicates of treatments C3, C4, C5 and C6 must be disassembled one day after the replicates of treatments CTsol, C0, C1 and C2 have been disassembled.

Carefully extract the "spore sandwiches" from each replicate and remove the excess of soil on the membranes with a paintbrush or with tweezers. Keep the "spore sandwiches" on a horizontal position to avoid loss of spores. Lay the "spore sandwiches" in a tray with a wet paper towel to avoid excessive loss of moisture until the moment of the assessment (avoid waterlogging).

Using the Falcon tubes provided by the project coordination, collect soil samples (~50 g FW) from three random replicates per treatment for chemical analysis (except for C0 and CTsol. where no replicates need to be collected). These samples must be stored at -20ºC ± 4ºC until analysis/shipment.

Also, one sample of 10 g of soil from a random replicate per treatment must be collected to determine soil final pH and another sample of 10 g of soil must be collected to determine soil final water content at the end of the test. Register the measured pH and soil water content in the spreadsheet provided by the project coordination.

"Spore sandwich" staining and germination assessment

Using a Pasteur pipette, add some drops of 1:1 mixture of 5% ink solution and 5% acetic acid solution on the "spore sandwiches" and let it rest for 10-15 minutes to stain the germinated hyphae.

The number of germinated and recovered spores on both membranes composing the sandwich is determined using a stereomicroscope (50x magnification) or a microscope (100x magnification). A spore is considered germinated when the length of the hyphae produced by the spore is at least five times greater than the spore's diameter. Register the results of the assessment on the Excel spreadsheet provided by the project coordination.

NOTE 4: the germination assessment can be performed one day after the disassembly of the replicates, maximum. In that case, the "spore sandwiches" wrapped on moist paper towel must be stored in the refrigerator, at 4ºC, in the dark.

Validity criteria

- The mean number of recovered spores in all treatments is ≥ 25;
- The mean percentage of germinated spores in the control treatment is ≥ 75% at the end of the test.

Calculation and expression of results

The percentage of spore germination, per replicate, is calculated according to the following equation. adapted from Malfatti et al. (2021):

$$\%\ Germination = \frac{\mathrm{g\ x\ 100}}{n}$$

Where g is the number of germinated spores

n is the number of recovered spores

One-way analysis of variance, followed by the post hoc Dunnett test ($p \leq 0.05$) will be used to evaluate statistical significance of the differences between treatments and the control and estimate the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). Inhibition concentrations (ICx) causing 10 or 50% of inhibition in spore germination will be estimated using non-linear regressions when possible.

References

ISO, Soil quality — Effects of Pollutants on Mycorrhizal Fungi — Spore Germination Test. ISO 10832:2009. 2009.

Mallmann GC., Sousa JP., Sundh I., Pieper S., Arena M., da Cruz SP. et al. Placing Arbuscular Mycorrhizal Fungi on the Risk Assessment Test Battery of Plant Protection Products (PPPs). Ecotoxicology. 2018 Sep 1;27(7):809–18.

Gerdemann JW., Nicolson TH. Spores of Mycorrhizal Endogone Species Extracted from Soil by Wet Sieving and Decanting. Transactions of the British Mycological Society. 1963 Jun;46(2):235–44.

OECD, Test No. 232: Collembolan Reproduction Test in Soil. Paris; 2016.

ISO, Soil quality — Effects of pollutants on earthworms – Part 2: Determination of effects on reproduction of *Eisenia fetida/Eisenia andrei*. ISO 11268-2:2012. 2012.

Annex A

(informative)

Spore isolation with sucrose gradient

The recommended spore extraction method is based on a wet sieving technique, followed by a sucrose centrifugation after which spores are separated and counted (Gerdemann & Nicolson (1963).

Procedure

1) Prepare a 50 mL Falcon tube with a sucrose gradient: pour 15 ml of 20% sucrose solution in the tube. Then, dip a glass pipette in the 20% sucrose solution, reaching the bottom of the tube, and add 15 ml of 60% sucrose solution. The 60% sucrose solution must remain in the bottom of the Falcon tube, under the 20% sucrose solution;
2) In a 2 L beaker, vigorously mix a sample of 50 g of inoculant with 2 L of tap water for 30 seconds. Sieve the supernatant and the inoculant through stacked sieves of 250 µm (top) and 45 µm mesh (bottom). Repeat as many times as necessary to bring all the inoculant to the top sieve. With tap water, wash the inoculant retained on the top sieve so all the spores are dragged to the bottom sieve;
3) Wash the materials retained on the 45 µm sieve to a beaker. Add a maximum of 10 mL of the retained materials to the Falcon tube containing the sucrose gradient and centrifuge for 2 minutes at 2000 rpm;
4) After centrifugation, sieve the supernatant using the 45 µm sieve and wash the retained materials with distilled water to remove all sucrose residues;
5) Transfer the retained material (which includes the spores) to a watch-glass or Petri dish and select viable spores under the stereomicroscope at 50x amplification. Only viable spores, with intact walls, are collected. Old, empty or crushed spores are discarded;

6) Store the selected spores in microtubes with distilled water at 4ºC. Spores can be stored for one day before use in pre-symbiotic phase tests.

Annex B
(informative)

Determination of water holding capacity

The method described below for maximum water holding capacity (WHC) of the soil determination is based on Annex C of the ISO 11268-2 (Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction) and on the Annex 5 of the OECD 232 guideline (OECD guidelines for testing chemicals - Collembolan Reproduction Test in Soil).

Procedure

1) Prepare a tube with a filter paper in the bottom and weight (A);
2) Wet the piece of filter paper and weight (B);
3) Fill the tubes with a portion of the test soil (or with a mixture of test soil and inoculum. as applicable), without reaching the top of the tubes;
4) Place the tubes in a tray with tall borders;
5) Outside the tubes, put water until the soil is submerged (don't put water directly in the soil, the water must ascent by capillarity).
6) Let it rest, submerged, for three hours;
7) Drain the samples for two hours in a grid. Cover the tubes so water in the soil will not be lost by evaporation;
8) Weight the saturated tubes (C);
9) Dry at 105ºC for 14h;
10) Weight (D) and calculate the %WHC applying the equation below:

$$\%WHC = \frac{(C - B) - (D - A)}{D - A} * 100$$

Annex C
(informative)

Determination of soil water content

Soil water content should be measured following the steps below:

1) Weigh an empty vessel (A);
2) Without taring the scale, add about 10g of the fresh soil in the vessel and weight (B);
3) Dry the soil at 105ºC for at least 12h;
4) After letting settle down the temperature of the soil (5 to 10 min), weight the dried soil + vessel (C);
5) Calculate de soil water content, in percentage, by following the equation below:

$$\%\ Water\ content = \frac{(B - C)}{(C - A)} * 100$$

Annex D
(informative)

Determination of soil pH

The method described for determination of soil pH is based on the methodology described on the guideline ISO 10390: Soil Quality Determination of pH and on the OECD 232 guideline (OECD guidelines for testing chemicals - Collembolan Reproduction Test in Soil, Annex 6).

To measure the pH of a soil sample. follow the steps presented below:

1) Let the fresh soil air-dry at room temperature for at least 12h;
2) Once the soil is dry, weight 5 g of soil into a tall vessel;
3) Add 25 mL of KCl (1M) or $CaCl_2$ (0.01M);
4) Mix for 5 minutes and settle for at least 2h (but not more than 24h);
5) Measure the pH in the liquid phase using a previously calibrated pH-meter.

A.18 SOP Test 2: Assessing the effects of chemicals on the symbiotic phase of Arbuscular Mycorrhizal Fungi (AMF) (reproduction of the final version of the document, from January 2024).

Aim of this Standard Operating Procedure

Ensure an adequate evaluation of the effects of chemicals on arbuscular symbiotic root colonization, consolidation and functionality (formation of intra-radicular arbuscules and extra-radicular hyphae production) for the arbuscular mycorrhizal fungi (AMF) *Rhizophagus irregularis*.

Summary

This protocol describes a method to evaluate the potential effect of chemicals in the symbiotic phase of the AMF *Rhizophagus irregularis*. Seeds of the host plant (leek, *Allium ampeloprasum*) are sown in pots containing AMF mixed inoculum and test soil spiked with the test substance in different concentrations. After 8 weeks of incubation, effects on different AMF and plant related parameters are assessed (total and arbuscular intra-radicular colonization, extra-radicular mycelial production, plant shoot/root biomass).

NOTE 1: Mixed inoculum is considered a mixture with more than one source of AMF propagules (i.e., spores, hyphae, and colonized roots). Inoculant is a mixture of the mixed inoculum with a soil (usually a sandy soil) that is used as source of mixed inoculum in the experiment.

Equipment

- Growth chamber with controlled temperature and photoperiod (see Test conditions);
- Weighing scale;
- Oven;
- Autoclave.

Materials

- Inoculant (must be stored at 4ºC until its use for tests);
- Petri dish;
- Filter paper or paper towel;
- *Allium ampeloprasum* seeds;
- 250 mL plastic tubes or pots as test vessels;
- Vessels for soil moisture and pH assessment;
- Distilled water;
- Falcon tubes;
- Spoons;
- Long Asthon nutrient solution;
- Coarse sand or glass beads (particles > 2 mm);
- Modified standard artificial soil (see NOTE 2) or natural soil sieved at 2 mm.

NOTE 2: Modified artificial soil composition: 2.5% of peat (use mature peat, air dried and sieved at 2 mm - peat can be milled if necessary to make sieving easier), 20% of kaolin clay (with kaolinite content preferably above 30%) and 77.5% of air-dried industrial sand (with more than 50% of the particles between 50 and 200 microns). Soil pH should be adjusted to 6.0 ± 0.5 with the addition of $CaCO_3$.

Test conditions

The test is incubated for 56 days in a growth chamber with controlled temperature of 23 ± 2ºC, 16:8h light:dark photoperiod and light intensity of 1000 to 4000 lx.

When possible, a relative air moisture of 40% to 60% should be used (high air moisture reduces water losses in the replicates).

Procedure

A test timeline is presented in Annex F.

Before the test setup, some materials need previous preparation:

- Natural or artificial soils and coarse sand or glass beads must be autoclaved by 2 cycles at 121ºC ± 2ºC and 1.5 ± 0.1 atm for 60 ± 5 minutes. The soil and coverage

material must “rest” at least overnight between cycles. After autoclaving, the materials must be kept at room temperature for 5-7 days before being used in the tests;

- Test soils and mixtures soil + inoculum must be characterized before the start of the test. Test soil pH and maximum water holding capacity (WHC) should be measured after soil autoclavation as autoclaving process interferes in the soil pH and WHC (see Annex B and Annex D);
- Seeds must be presoaked before use in test. First, they are disinfected by immersion in a 1% sodium hypochlorite solution for 5 minutes and subsequently washed several times with distilled water. Then, they are placed in filter paper/paper towel moistened with distilled water in Petri dishes for 2 to 4 days. Turgid seeds must be used immediately before germination (note that AMF roots colonization may be reduced when seedlings with radicles length are used);
- In the day before the test setup, an aliquot of test soil must be dried as described below (see Test setup, 1st Step).

Test setup

Four treatment types should be considered in each experiment:

Sterilization control (CT_{ster})	Replicates with non-contaminated test soil, with host plants and without inoculant. Controls the persistence of active propagules in the soil after sterilization.
Solvent control (CT_{sol})	Replicates with test soil moistened with the organic solvent used in the stock solution, with inoculant and host plant but without test substance. This treatment is only applied in cases where a substance insoluble in water is used.
Negative control (C_0)	Replicates with the test soil without the test substance, with inoculant and the host plant.
Test treatments ($C_x = C_1. C_2.\ldots$)	Treatments with test soil spiked with the test substance in different concentrations, with inoculant and the host plant.

To setup the test use the provided spreadsheet (“Test setup”) and follow the steps described below:

1st Step – Aliquot contamination

1.1. Oven-dry one individual portion of fresh test soil per treatment at 70ºC overnight (“Soil aliquot – Fresh weight”);

1.2. Prepare the Stock Solution by diluting the contaminant (Azoxystrobin or Fluazinam) in acetone (“Stock solution preparation”);

1.3. Contaminate the soil portions (“Soil aliquot – Dry weight”) with a mixture of “Stock solution” and “Acetone” in a mortar (in alternative, glass or metallic vessels can be used). Mix very well and let the solvent volatilize totally before following for the 2nd step.

2nd Step – Contamination of soil by treatment

2.1. Once all solvent has volatilized, mix the “Soil aliquot” with the rest of the soil batch (“Fresh soil”). Add the volume of “Water” to reach 50% of the maximum WHC of the soil.

Each treatment must be prepared individually, from the lowest concentration to the highest.

Preferably, the five replicates of each treatment are filled after this step, following the steps described in the section “3rd Step – Replicate preparation and sampling”.

3rd Step – Replicate

3.1. Insert the soil for the bottom layer on the 5 treatment replicates, avoiding soil compaction. Prepare the top layer individually for each replicate by mixing soil,

inoculum and water. Lay the top layer on each of the 5 replicates. All replicates of the treatment must be ready before following to the next treatment;

3.2. For treatments CTsol, CTster and C1 to C5, collect three samples of 50 g of soil per treatment for chemical analysis; for C0 collect only one sample 50 g of soil for chemical analysis (use the Falcons tubes provided by the project coordination). These samples must be stored at -20ºC until analysis/shipment.
Collect one sample of 10 g of soil for determination of soil initial pH and one sample of 10 g of soil for determination of initial soil WC per treatment (see Annex C and D. Results must be registered in the spreadsheet provided by the project coordination);
3.3. Sow 3 presoaked seeds of leek in each replicate at approximately 0.5 cm depth. Cover with sterilized coarse sand or glass beads;
3.4. Weight each replicate individually and register the weight;
3.5. Randomly distribute the test containers in the growth chamber (positions must be changed once a week).

The test starts only when at least one seed has germinated in each replicate of the negative control (C_0). The time period for at least one seed germination cannot exceed two weeks. Despite three seeds being sown per replicate at the test setup, only the first emergent plant should remain in the replicate to the end of the test. For that, remove the later emergent plants by pulling them in order to also remove their root.

Soil water content should be adjusted twice a week on a weight basis: distilled water should be added to compensate water losses when the weight loss is higher than 2%.
At week 1, 3 and 5 after the beginning of the test (i.e., after germination in C0 replicates), 5 mL of Long Ashton nutrient solution (with 10% P (9.3 mg P kg^{-1} soil), see Annex A) is applied in each replicate.

Test final measurements

At the end of the 56 days, the replicates are individually disassembled by removing the coverage layer (sand or glass beads) and pouring the replicate content in a tray. Using the Falcon tubes provided by the project coordination, collect a soil sample (~50 g) from the bottom layer of three random replicates per treatment for chemical analysis (except for C0, where no replicates must be collected). These soil samples must be stored at -20ºC ± 4 ºC until analysis/shipment.

Also, a sample of 10 g of soil from a random replicate per treatment must be used to measure soil pH at the end of the test (also from the bottom layer). Register the measured pH in the spreadsheet provided by the project coordination.

Soil water content at the end of the test must be measured in each replicate as described in SOP 2.4 and registered in the spreadsheet provided by the project coordination.

Measurements of the endpoints

At the end of test period (56 days) the following parameters should be measured:

a. Intra-radicular total colonization (%) (see SOP 2.3)
b. Intra-radicular arbuscular colonization (%) (see SOP 2.3)
c. Extra-mycelial production (hyphal length) (see SOP 2.4)

Additionally, as complementary parameters, plant root fresh biomass and shoot fresh and dry biomass must be measured. To obtain shoot dry biomass, the plant structure is weighted fresh, oven-dried at 60ºC for at least 2 days and weighted again.

Validity criteria

At the end of the experiment (56 days), the following validiy criteria must be fulfilled:

- Total intra-radicular colonization of 0% in replicates of CT_{ster}.
- Total intra-radicular colonization higher than 30% on average in the replicates of C_0.

- Coefficient of variation lower than 50% in total intra-radicular colonization of C_0.
- Minimum length of roots of 10 cm should be observed in replicates of C_0.

Calculation and expression of results

Total and arbuscular root colonization are expressed in % of colonization per replicate, while extra-radicular mycelium length is expressed in meters of hyphae per gram of dry soil per replicate.

One-way analysis of variance, followed by the *post hoc* Dunnett test ($p \leq 0.05$) will be used to evaluate statistical significance of the differences between treatments and the control. Effect concentrations (EC_x) causing 10 or 50% of inhibition in the evaluated endpoints will be estimated using non-linear regressions.

References

ISO 11268-2. Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction

OECD 232 guideline. OECD guidelines for testing chemicals - Collembolan Reproduction Test in Soil

Annex A
(informative)

Composition of 1 L of a Long Ashton nutrient solution with low P (provided by the project coordination)

Component		Stock solution g L^{-1}	Volume of stock solutions to complete 1 L fertilizer solution
Macronutrients			**100 mL**
KNO_3	Potassium nitrate	4.04	
$Ca(NO_3)_2 \bullet 4H_2O$	Calcium nitrate tetrahydrate	9.44	
$NaH_2PO_4 \bullet 4H_2O$	Sodium Dihydrogen Phosphate Tetrahydrate	1.84	
$MgSO_4 \bullet 7H_2O$	Magnesium sulfate heptahydrate	3.68	
Micronutrients			**10 mL**
$MnSO_4 \bullet H_2O$	Manganese (II) sulfate monohydrate	1.68	
$CuSO_4 \bullet 5H_2O$	Copper sulphate pentahydrate	0.25	
$ZnSO_4 \bullet 7H_2O$	Zinc sulfate heptahydrate	0.29	
H_3BO_3	Boric acid	2.23	
Molybdenum			**1 mL**
$(NH_4)_6O_{24}Mo_7 \bullet 4H_2O$	Ammonium molybdate tetrahydrate	0.35	
Iron			**10 mL**
$FeSO_4 \bullet 7H_2O$	Iron (II) sulfate heptahydrate	3.05	
Ferric sodium EDTA	Ethylenediaminetetraacetic acid ferric sodium salt	4.09	
Distilled water			**879 mL**

Annex B
(informative)

Determination of water holding capacity

The method described below for maximum water holding capacity (WHC) of the soil determination is based on Annex C of the ISO 11268-2 (Soil Quality - Effects of pollutants on earthworms (*Eisenia fetida*). Part 2: Determination of effects on reproduction) and on the Annex 5 of the OECD 232 guideline (OECD guidelines for testing chemicals - Collembolan Reproduction Test in Soil).

Procedure

1. Prepare a tube with a filter paper in the bottom and weight (A);
2. Wet the piece of filter paper and weight (B);
3. Fill the tubes with a portion of the test soil (or with a mixture of test soil and inoculum. as applicable), without reaching the top of the tubes;
4. Place the tubes in a tray with tall borders;
5. Outside the tubes, put water until the soil is submerged (don't put water directly in the soil, the water must ascent by capillarity).
6. Let it rest, submerged, for three hours;
7. Drain the samples for two hours in a grid. Cover the tubes so water in the soil will not be lost by evaporation;
8. Weight the saturated tubes (C);
9. Dry at 105ºC for 14h;
10. Weight (D) and calculate the %WHC applying the equation below:

$$\%WHC = \frac{(C - B) - (D - A)}{D - A} * 100$$

Annex C
(informative)

Determination of soil water content

Soil water content should be measured following the steps below:

1. Weigh an empty vessel (A);
2. Without taring the scale, add about 10g of the fresh soil in the vessel and weight (B);
3. Dry the soil at 105ºC for at least 12h;
4. After letting settle down the temperature of the soil (5 to 10 min), weight the dried soil + vessel (C);
5. Calculate de soil water content, in percentage, by following the equation below:

$$\%\, Water\ content = \frac{(B - C)}{(C - A)} * 100$$

Annex D
(informative)

Determination of soil pH

The method described for determination of soil pH is based on the methodology described on the guideline ISO 10390: Soil Quality Determination of pH and on the OECD 232 guideline (OECD guidelines for testing chemicals - Collembolan Reproduction Test in Soil, Annex 6).

To measure the pH of a soil sample, follow the steps presented below:

1. Let the fresh soil air-dry at room temperature for at least 12h;
2. Once the soil is dry, weight 5 g of soil into a tall vessel;
3. Add 25 mL of KCl (1M) or $CaCl_2$ (0.01M);
4. Mix for 5 minutes and settle for at least 2h (but not more than 24h);
5. Measure the pH in the liquid phase using a previously calibrated pH-meter.

Annex E

(informative)

Test timeline

Time (days)	Action
-14 days before test setup	- Prepare artificial soil following the instructions of Note 2 (if applicable)
-7 to -5 days before test setup	- Autoclave soil and coarse sand/glass beads - Measure WHC_{soil} and $WHC_{soil+inoculum}$ (applicable only for artificial soil)
-4 or -2 days before test setup	- Sterilize leek seeds - Prepare test materials
Test setup	- Follow SOP Test 2 - Assess initial WC and pH - Sample for chemical analysis
≤ 2 weeks	Check for seeds germination (during this period, the replicates initial weight must be reestablished by addition of distilled water when weight loss >2% twice a week)

Day 0	- Observe the germination of at least one seed per replicate of C0 - Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 4	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 7	- Watering with 5 ml of Long Ashton nutrient solution per replicate - Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 11	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%

Time (days)	Action
Day 14	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 18	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 21	- Watering with 5 ml of Long Ashton nutrient solution per replicate - Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 25	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 28	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 32	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 35	- Watering with 5 ml of Long Ashton nutrient solution per replicate - Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 39	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 42	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 46	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 49	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 53	- Reestablish replicates initial weight by addition of distilled water when weight loss >2%
Day 56	- End of test - Assess final pH per treatment and WC per replicate - Evaluate test endpoints - Sample for chemical analysis

Time (days)	Action
After 56 days test	- Evaluate test endpoints

A.19 Raw data of the experiments from the 2nd phase of WP2.

Table A.19 – 1: Raw data of the experiments with boric acid (BA) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and soils OECD 2.5%, Bra8, Por3 and #Lufa 2.1. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

Table A.19 – 2: Raw data of the experiments with copper sulphate ($CuSO_4$) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and OECD 2.5% soil. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

Table A.19 – 3: Raw data of the experiments with Tebuconazole (TBC) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus clarus* and soils OECD 2.5% and Bra8. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

Table A.19 – 4: Raw data of the experiments with Terbuthylazine (TRZ) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus clarus* and soils OECD 2.5% and Bra8. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

Table A.19 – 5: Raw data of the experiments with Azoxystrobin (AZX) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and soils OECD 2.5%, #Lufa 2.1, Por3, Lufa 2.2, Est32 and Bra8. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

Table A.19 – 6: Raw data of the experiments with Fluazinam (FLZ) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and soils OECD 2.5%, Por3, Lufa 2.2, Est32 and Bra8. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

Table A.19 – 7: Raw data of the experiments with Pendimethalin (PDM) conducted during the second phase of WP2 using AMF inoculants of *Funneliformis mosseae* and *Rhizophagus irregularis* and soils OECD 2.5% and Est32. Only treatments with plant germination are presented.

Please refer to the "Raw data" supplementary Excel file.

A.20 Non-linear models for endpoint estimations for WP2.

Endpoint estimations (EC10 and EC50), based on nominal and initial measured concentrations, were performed for each test endpoint parameter: total colonization (TC), arbuscular colonization (AC) and hyphae length (EHL) using R Studio (version 2023.09.1), packages drc (v. 3.0-1), tidyverse (v. 2.0.0), readxl (v. 1.4.3) and PMCMRplus (v. 1.9.10).

Table A.20 – 1: Experiments with Boric acid (BA) (WP2, section 2.6.1.1).

Please refer to the "Supporting information" supplementary Excel file.

Table A.20 – 2: Experiments with copper sulphate (WP2, section 2.6.1.2).

Please refer to the "Supporting information" supplementary Excel file.

Table A.20 – 3: Experiments with Tebuconazole (WP2, section 2.6.1.3).

Please refer to the "Supporting information" supplementary Excel file.

Table A.20 – 4: Experiments with Terbuthylazine (WP2, section 2.6.1.4).

Please refer to the "Supporting information" supplementary Excel file.

Table A.20 – 5: Experiments with Azoxystrobin (WP2, section 2.6.1.5).

Please refer to the "Supporting information" supplementary Excel file.

Table A.20 – 6: Experiments with Fluazinam (WP2, section 2.6.1.6).

Please refer to the "Supporting information" supplementary Excel file.

Table A.20 – 7: Experiments with Pendimethalin (WP2, section 2.6.1.7).

Please refer to the "Supporting information" supplementary Excel file.

A.21 Data considered in the Generalized Linear Models to evaluate the influence of soil properties on AMF colonization.

Table A.21 – 1: Data considered in the Generalized Linear Models applied in Brodgar 2.7.5 software (www.brodgar.com) to evaluate the influence of soil properties on arbuscular mycorrhiza fungi (AMF) colonization.

Please refer to the "Supporting information" supplementary Excel file.

A.22 Data considered in the Generalized Linear Models to evaluate the influence of soil properties on AMF sensitivity to different test substances.

Table A.22-1: Data considered in the Generalized Linear Models applied in Brodgar 2.7.5 software (www.brodgar.com) to evaluate the influence of soil properties on sensitivity of *Rhizophagus irregularis* to Azoxystrobin and Fluazinam.

Please refer to the "Supporting information" supplementary Excel file.

A.23 Literature data on the contaminants tested on WP2.

Table A.23 – 1: Literature data on the effects of boric acid on soil non-target organisms.

Organism species	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Avena sativa	OECD 10%	Active ingredient	Boric acid	EC50 (shoot fresh weight)	182 (119-281)	mg a.i./kg of soil	Becker et al. 2011
Brassica napus	OECD 10%	Active ingredient	Boric acid	EC50 (shoot fresh weight)	175 (164-187)	mg a.i./kg of soil	Becker et al. 2011
Caenorhabditis elegans	OECD 10%	Active ingredient	Boric acid	EC50 (reprod)	747 (589-949)	mg a.i./kg of soil	Becker et al. 2011
Eisenia fetida	OECD 10%	Active ingredient	Boric acid	EC50 (reprod)	484 (465-504)	mg a.i./kg of soil	Becker et al. 2011
Eisenia fetida	OECD 10%	Active ingredient	Boric acid	NOEC (reprod)	316	mg a.i./kg of soil	Becker et al. 2011
Enchytraeus albidus	Lufa 2.2	Active ingredient	Boric acid	EC10 (reprod)	39.3 (33.1-44.9)	mg a.i./kg of soil	Amorim et al. 2012
Enchytraeus albidus	Lufa 2.2	Active ingredient	Boric acid	EC50 (reprod)	103.5 (96.6-110.9)	mg a.i./kg of soil	Amorim et al. 2012
Enchytraeus albidus	Lufa 2.2	Active ingredient	Boric acid	LC50	302.8 (233.4-391.5)	mg a.i./kg of soil	Amorim et al. 2012
Enchytraeus albidus	Lufa 2.2	Active ingredient	Boric acid	NOEC (reprod)	113	mg a.i./kg of soil	Amorim et al. 2012
Enchytraeus albidus	Lufa 2.2	Active ingredient	Boric acid	NOEC (surv)	226	mg a.i./kg of soil	Amorim et al. 2012

Organism species	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Enchytraeus crypticus	OECD 10%	Active ingredient	Boric acid	EC50 (reprod)	220 (280-233)	mg a.i./kg of soil	Becker et al. 2011
Enchytraeus luxuriosus	OECD 10%	Active ingredient	Boric acid	EC50 (reprod)	228 (201-259)	mg a.i./kg of soil	Becker et al. 2011
Folsomia candida	Lufa 2.2	Active ingredient	Boric acid	EC10 (reprod)	31.7 (24.9-36.5)	mg a.i./kg of soil	Amorim et al. 2012
Folsomia candida	Lufa 2.2	Active ingredient	Boric acid	EC50 (reprod)	54.5 (50.5-58.3)	mg a.i./kg of soil	Amorim et al. 2012
Folsomia candida	Lufa 2.2	Active ingredient	Boric acid	LC50	139.4 (125.7-153.2)	mg a.i./kg of soil	Amorim et al. 2012
Folsomia candida	Lufa 2.2	Active ingredient	Boric acid	NOEC (reprod)	25	mg a.i./kg of soil	Amorim et al. 2012
Folsomia candida	Lufa 2.2	Active ingredient	Boric acid	NOEC (surv)	110	mg a.i./kg of soil	Amorim et al. 2012
Folsomia candida	OECD 10%	Active ingredient	Boric acid	EC50 (avoid)	1441 (183-478293)	mg a.i./kg of soil	Becker et al. 2011
Hypoaspis aculeifer	OECD 5%	Active ingredient	Boric acid	EC50 (avoid)	1234 (749-2031)	mg a.i./kg of soil	Owojori et al. 2014
Hypoaspis aculeifer	OECD 5%	Active ingredient	Boric acid	EC50 (reprod)	332 (292-373)	mg a.i./kg of soil	Owojori et al. 2014
Hypoaspis aculeifer	OECD 5%	Active ingredient	Boric acid	LC50	668 (523-791)	mg a.i./kg of soil	Owojori et al. 2014

Organism species	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Soil microorganism	NA	Active ingredient	EC50 (nitrogen transformation)	> 240	mg a.i./kg of soil	Becker et al. 2011	EC50 (nitrogen transformation)

Table A.23 – 2: Literature data on the effects of copper sulphate on soil non-target organisms.

Organism species	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Eisenia andrei	Agricultural sandy loam	Active ingredient	Copper sulphate	EC50 (reprod)	124 (85-179)	µg/g of soil	Velicogna et al. 2021
Eisenia andrei	Agricultural sandy loam	Active ingredient	Copper sulphate	EC10 (reprod)	79 (34-185)	µg/g of soil	Velicogna et al. 2021
Folsomia candida	Agricultural sandy loam	Active ingredient	Copper sulphate	EC50 (reprod)	490 (380-646)	µg/g of soil	Velicogna et al. 2021
Folsomia candida	Agricultural sandy loam	Active ingredient	Copper sulphate	EC10 (reprod)	219 (123-398)	µg/g of soil	Velicogna et al. 2021
Folsomia candida	Natural soil	Active ingredient	Copper sulphate	EC10 (reprod)	50 (0.03-13095)	mg/kg of soil DW	Pedersen et al. 2000
Folsomia candida	Natural soil	Active ingredient	Copper sulphate	EC50 (reprod)	519 (21-13095)	mg/kg of soil DW	Pedersen et al. 2000
Folsomia fimetaria	Natural soil	Active ingredient	Copper sulphate	EC10 (reprod)	141 (20-1001)	mg/kg of soil DW	Pedersen et al. 2000
Folsomia fimetaria	Natural soil	Active ingredient	Copper sulphate	EC50 (reprod)	657 (293-1470)	mg/kg of soil DW	Pedersen et al. 2000

Table A.23 – 3: Literature data on the effects of Tebuconazole on soil non-target organisms.

Organism species	Soil	Product tested	Active ingredient(s)	Endpoint	Value	Units	Reference
Eisenia fetida	OECD	Active ingredient	Tebuconazole	LC50 (14 days)	1381	mg a.i./kg of soil DW	EFSA 2014
Eisenia fetida	OECD	Active ingredient	Tebuconazole	NOEC (chronic)	10	mg a.i./kg of soil DW	EFSA 2014
Eisenia fetida	OECD	Active ingredient	Tebuconazole	LC50 (7 days)	746.3	mg a.i./kg of soil DW	Chen et al. 2018
Eisenia fetida	OECD	Active ingredient	Tebuconazole	LC50 (14 days)	287.9	mg a.i./kg of soil DW	Chen et al. 2019
Eisenia fetida	OECD 10%	Folicur 25EW	Tebuconazole	LC50 (14 days)	180 (161–204)	mg a.i./kg of soil DW	Rico et al. 2016
Eisenia fetida	OECD 10%	Prosaro® 250 EC	Tebuconazole + Prothioconazole	EC10 (reprod)	133.3 (20.13–1776)	mg fung/kg of soil	Jorge-Escudero et al. 2022
Eisenia fetida	OECD 10%	Prosaro® 250 EC	Tebuconazole + Prothioconazole	EC20 (reprod)	233.3 (35.95–3269)	mg fung/kg of soil	Jorge-Escudero et al. 2023
Eisenia fetida	OECD 10%	Prosaro® 250 EC	Tebuconazole + Prothioconazole	LC50 (14 days)	>1000	mg fung/kg of soil	Jorge-Escudero et al. 2024
Enchytraeus crypticus	Lufa 2.2	Prosaro® 250 EC	Tebuconazole + Prothioconazole	LC10 (21 days)	429 (nd)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Prosaro® 250 EC	Tebuconazole + Prothioconazole	LC50 (21 days)	533 (nd)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Prosaro® 250 EC	Tebuconazole + Prothioconazole	EC10 (reprod)	139 (48-229)	mg a.i./kg of soil DW	Gomes et al. 2021

Organism species	Soil	Product tested	Active ingredient(s)	Endpoint	Value	Units	Reference
Enchytraeus crypticus	Lufa 2.2	Prosaro® 250 EC	Tebuconazole + Prothioconazole	EC50 (reprod)	350 (262-438)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Active ingredient	Tebuconazole	LC10 (21 days)	148 (145-150)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Active ingredient	Tebuconazole	LC50 (21 days)	164 (162-166)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Active ingredient	Tebuconazole	EC10 (reprod)	12 (-6-31)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Active ingredient	Tebuconazole	EC50 (reprod)	41 (16-66)	mg a.i./kg of soil DW	Gomes et al. 2021
Folsomia candida	OECD	Active ingredient	Tebuconazole	NOEC (chronic)	250	mg a.i./kg of soil DW	EFSA 2014
Folsomia candida	OECD 5%	Active ingredient	Tebuconazole	NOEC (chronic)	50	mg a.i./kg of soil DW	Guimarães 2018 (thesis)
Folsomia candida	OECD 5%	Active ingredient	Tebuconazole	LOEC (chronic)	111.8	mg a.i./kg of soil DW	Guimarães 2018 (thesis)
Folsomia candida	OECD 5%	Active ingredient	Tebuconazole	EC10 (reprod)	44.5 (6.2-82.8)	mg a.i./kg of soil DW	Guimarães 2018 (thesis)
Folsomia candida	OECD 5%	Active ingredient	Tebuconazole	LC50 (28 days)	> 1250	mg a.i./kg of soil DW	Guimarães 2018 (thesis)
Folsomia candida	OECD 5%	Active ingredient	Tebuconazole	EC50 48h (avoid)	14.2 (0-56.3)	mg a.i./kg of soil DW	Guimarães 2018 (thesis)

Organism species	Soil	Product tested	Active ingredient(s)	Endpoint	Value	Units	Reference
Glossoscolex rione	OECD 10%	Prosaro® 250 EC	Tebuconazole + Prothioconazole	LC50 (14 days)	285 (223-344)	mg fung/kg of soil	Jorge-Escudero et al. 2025
Hypoaspis aculeifer	OECD	Active ingredient	Tebuconazole	NOEC (chronic)	50	mg a.i./kg of soil DW	EFSA 2014
Lepidium sativum	OECD	Active ingredient	Tebuconazole	ER50 (vegetative vigour)	14	mg a.i./kg of soil DW	EFSA 2014
Lepidium sativum	OECD	Active ingredient	Tebuconazole	ER50 (seed emergence)	100	mg a.i./kg of soil DW	EFSA 2014
Soil microorganisms	NA	Active ingredient	Tebuconazole	Nitrogen mineralisation (28 days)	NS at 8.3	mg a.i./kg of soil DW	EFSA 2014
Soil microorganisms	NA	Active ingredient	Tebuconazole	Carbon mineralisation (28 days)	NS at 8.3	mg a.i./kg of soil DW	EFSA 2014

NA – information not available; NS – not significant

Table A.23 – 4: Literature data on the effects of Terbuthylazine on soil non-target organisms.

Organism species	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Collembola *(F. candida?)*	OECD	Gardoprim Plus Gold	Terbuthylazine	NOEC (28 days)	21.12	mg a.i/kg of soil DW	EFSA 2010
Eisenia fetida	OECD	Active ingredient	Terbuthylazine	LC50 (14 days)	> 141.7	mg a.i/kg of soil DW	EFSA 2010

Organism species	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Eisenia fetida	OECD	Gardoprim Plus Gold	Terbuthylazine	NOEC (56 days)	0.563	mg a.i/kg of soil DW	EFSA 2010
Eisenia fetida	OECD 10%	Terbis	Terbuthylazine	LC50 (14 days)	1.26 (2.89-5.50)	mg a.i/kg of soil DW	Jovana et al. 2014
Eisenia fetida	OECD 10%	Active ingredient	Terbuthylazine	LC50 (14 days)	258.52 (195.33–377.38)	mg a.i/kg of soil DW	Li et al. 2023
Lettuce	OECD	Gardoprim Plus Gold	Terbuthylazine	EC50 (pre-emerg)	321	mL c.f./ha	EFSA 2010
Lettuce	OECD	Gardoprim Plus Gold	Terbuthylazine	EC50 (post-emerg)	439	mL c.f./ha	EFSA 2010
Sugar beet	OECD	Terbuthylazine 500 g/L SC	Terbuthylazine	EC50 (pre-emerg)	16	mL c.f./ha	EFSA 2010
Tomato	OECD	Terbuthylazine 500 g/L SC	Terbuthylazine	EC50 (post-emerg)	164.61	mL c.f./ha	EFSA 2010
Soil microorganisms	NA	Terbuthylazine	Terbuthylazine	Nitrogen mineralisation	NS at 10.9	mg a.i/kg of soil DW	EFSA 2010
Soil microorganisms	NA	Terbuthylazine	Terbuthylazine	Carbon mineralisation	NS at 10.9	mg a.i/kg of soil DW	EFSA 2010

NA – information not available; NS – not significant

Table A.23 – 5: Literature data on the effects of Azoxystrobin on soil non-target organisms.

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Enchytraeus crypticus	Lufa 2.2	Amistar® XTRA	Azoxystrobin + Cyproconazole	LC10 (21 days)	172 (74-269)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Amistar® XTRA	Azoxystrobin + Cyproconazole	LC50 (21 days)	320 (286-355)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Amistar® XTRA	Azoxystrobin + Cyproconazole	EC10	38 (6-70)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	Amistar® XTRA	Azoxystrobin + Cyproconazole	EC50	161 (130-193)	mg a.i./kg of soil DW	Gomes et al. 2021
Enchytraeus crypticus	Lufa 2.2	a.i.	Azoxystrobin	LC10 (21 days)	21 (15-30)	mg a.i./kg of soil DW	Gomes et al. 2022
Enchytraeus crypticus	Lufa 2.2	a.i.	Azoxystrobin	LC50 (21 days)	39 (34-45	mg a.i./kg of soil DW	Gomes et al. 2023
Enchytraeus crypticus	Lufa 2.2	a.i.	Azoxystrobin	EC10	17 (2-33)	mg a.i./kg of soil DW	Gomes et al. 2024
Enchytraeus crypticus	Lufa 2.2	a.i.	Azoxystrobin	EC50	37 (25-49)	mg a.i./kg of soil DW	Gomes et al. 2025
Enchytraeus crypticus	Lufa 2.2	QUADRIS®	Azoxystrobin	LC50 (21 days)	≥150	mg a.i./kg of soil DW	Kovačević et al. 2021
Enchytraeus crypticus	Lufa 2.2	QUADRIS®	Azoxystrobin	EC50	93.10	mg a.i./kg of soil DW	Kovačević et al. 2022
Folsomia candida	Mediterranean	ORTIVA®	Azoxystrobin	EC50	92.0 (57.9–126.1)	mg a.i./kg of soil DW	Leitão et al. 2014

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Folsomia candida	Mediterranean	ORTIVA®	Azoxystrobin	EC20	54.9 (23.0–86.9)	mg a.i./kg of soil DW	Leitão et al. 2015
Eisenia andrei	Mediterranean	ORTIVA®	Azoxystrobin	EC50	42.0 (23.2–60.8)	mg a.i./kg of soil DW	Leitão et al. 2015
Eisenia andrei	Mediterranean	ORTIVA®	Azoxystrobin	EC20	12.2 (1.2–23.1)	mg a.i./kg of soil DW	Leitão et al. 2015
Enchytraeus crypticus	Mediterranean	ORTIVA®	Azoxystrobin	EC50	99.2 (73.3–125.7)	mg a.i./kg of soil DW	Leitão et al. 2015
Enchytraeus crypticus	Mediterranean	ORTIVA®	Azoxystrobin	EC20	42.6 (25.2–60.0)	mg a.i./kg of soil DW	Leitão et al. 2015
Eisenia fetida	Fluvo-aquic soil	a.i.	Azoxystrobin	LC50 (7 days)	53.8 (43.5–66.0)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	Fluvo-aquic soil	a.i.	Azoxystrobin	LC50 (14 days)	32.5 (18.6–44.8)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	Black soil	a.i.	Azoxystrobin	LC50 (7 days)	266.6 (235.8–297.3)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	Black soil	a.i.	Azoxystrobin	LC50 (14 days)	241.0 (185.2–244.1)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	Red clay soil	a.i.	Azoxystrobin	LC50 (7 days)	37.4 (33.7–42.2)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	Red clay soil	a.i.	Azoxystrobin	LC50 (14 days)	21.3 (19.8–28.7)	mg a.i./kg of soil DW	Xu et al. 2021

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Eisenia fetida	OECD 10%	a.i.	Azoxystrobin	LC50 (7 days)	607.2 (544.8–675.6)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	OECD 10%	a.i.	Azoxystrobin	LC50 (14 days)	528.7 (471.2–588.9)	mg a.i./kg of soil DW	Xu et al. 2021
Eisenia fetida	OECD	a.i.	Azoxystrobin	LC50 (14 days)	283	mg a.i./kg of soil DW	EFSA 2010
Lettuce	OECD	a.i.	Azoxystrobin	ER50 (seed emerg)	> 20	mg a.i./kg of soil DW	EFSA 2010
Radish	OECD	a.i.	Azoxystrobin	ER50 (seed emerg)	> 20	mg a.i./kg of soil DW	EFSA 2010
Wheat	OECD	a.i.	Azoxystrobin	ER50 (seed emerg)	> 20	mg a.i./kg of soil DW	EFSA 2010
Soil microorganisms	NA	R234886	Azoxystrobin	Nitrogen mineralisation (28 days)	NS at 10	mg a.i./kg of soil DW	EFSA 2010
Soil microorganisms	NA	R401553	Azoxystrobin	Nitrogen mineralisation (28 days)	NS at 2.643	mg a.i./kg of soil DW	EFSA 2010
Soil microorganisms	NA	R402173	Azoxystrobin	Nitrogen mineralisation (28 days)	NS at 4.131	mg a.i./kg of soil DW	EFSA 2010
Soil microorganisms	NA	R234886	Azoxystrobin	Carbon mineralisation (28 days)	NS at 10	mg a.i./kg of soil DW	EFSA 2010
Soil microorganisms	NA	R401553	Azoxystrobin	Carbon mineralisation (28 days)	NS at 2.643	mg a.i./kg of soil DW	EFSA 2010

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Soil microorganisms	NA	R402173	Azoxystrobin	Carbon mineralisation (28 days)	NS at 4.131	mg a.i./kg of soil DW	EFSA 2010

NA – information not available; NS – not significant

Table A.23 – 6: Literature data on the effects of Fluazinam on soil non-target organisms.

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Eisenia fetida	OECD	a.i.	Fluazinam	LC50 (14 days)	500	mg a.i./kg soil DW	EFSA 2008
Eisenia fetida	OECD	Fluazinam 500 SC	Fluazinam	LC50 (14 days)	> 341	mg a.i./kg soil DW	EFSA 2008
Eisenia andrei	OECD	Fluazinam 500 SC	Fluazinam	NOEC (chronic)	< 0.175	mg a.i./kg soil DW	EFSA 2008
Folsomia candida	OECD	Fluazinam 500 SC	Fluazinam	NOEC (chronic)	< 0.785	mg a.i./kg soil DW	EFSA 2008
Cucumber	OECD	a.i.	Fluazinam	ER50 (veg rigour)	> 1500	mg a.i./kg soil DW	EFSA 2008
Cucumber	OECD	a.i.	Fluazinam	ER50 (seed emerg)	> 1500	mg a.i./kg soil DW	EFSA 2008
Soil microorganisms	NA	Fluazinam 500 SC	Fluazinam	Nitrogen mineralisation (28 days)	NS at 2.27	mg a.i./kg soil DW	EFSA 2008
Soil microorganisms	NA	HYPA	Fluazinam	Nitrogen mineralisation (28 days)	NS at 0.38	mg a.i./kg soil DW	EFSA 2008
Soil microorganisms	NA	Fluazinam 500 SC	Fluazinam	Carbon mineralisation (28 days)	NS at 2.27	mg a.i./kg soil DW	EFSA 2008
Soil microorganisms	NA	HYPA	Fluazinam	Carbon mineralisation (28 days)	NS (increase) at 0.38	mg a.i./kg soil DW	EFSA 2008

NA – information not available; NS – not significant (< 25%)

Table A.23 – 7: Literature data on the effects of Pendimethalin on soil non-target organisms.

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Earthworm	OECD 10%	Active ingredient	Pendimethalin	NOEC	33.45	mg a.i./kg of soil DW	EFSA 2016
Earthworm	OECD 10%	Active ingredient	Pendimethalin	EC10 (reprod)	49	mg a.i./kg of soil DW	EFSA 2016
Folsomia candida	Natural soil (Field 55)	Active ingredient	Pendimethalin	NOEC	30	mg a.i./kg of soil DW	Belden et al. 2005
Folsomia candida	Natural soil (Field 55)	Active ingredient	Pendimethalin	LOEC	90	mg a.i./kg of soil DW	Belden et al. 2005
Folsomia candida	Natural soil (Field 55)	Active ingredient	Pendimethalin	EC50 (reprod)	47 (44-51)	mg a.i./kg of soil DW	Belden et al. 2005
Folsomia candida	OECD 5%	BAS455 48 H	Pendimethalin	NOEC	193	mg a.i./kg of soil DW	EFSA 2016
Folsomia candida	OECD 5%	BAS455 48 H	Pendimethalin	EC10 (reprod)	561	mg a.i./kg of soil DW	EFSA 2016
Hypoaspis aculeifer	OECD 5%	BAS455 48 H	Pendimethalin	NOEC	385	mg a.i./kg of soil DW	EFSA 2016
Hypoaspis aculeifer	OECD 5%	BAS455 48 H	Pendimethalin	EC10 (reprod)	257	mg a.i./kg of soil DW	EFSA 2016
Latuca sativa	Natural soil (Field 55)	Active ingredient	Pendimethalin	IC50 (seed growth 7 days)	7.74 (5.67-10.57)	mg a.i./kg of soil DW	Belden et al. 2005

Organism	Soil	Product tested	Active ingredient	Endpoint	Value	Units	Reference
Latuca sativa	Natural soil (Field 55)	Active ingredient	Pendimethalin	NOEC	1	mg a.i./kg of soil DW	Belden et al. 2005
Latuca sativa	Natural soil (Field 55)	Active ingredient	Pendimethalin	LOEC	4	mg a.i./kg of soil DW	Belden et al. 2005
Perionyx excavatus	Garden soil	Active ingredient	Pendimethalin	LC50	0.016 (0.01-0.04)	mg a.i./kg of soil DW	Sanyal et al. 2015
Perionyx excavatus	OECD 10%	Active ingredient	Pendimethalin	LC50	0.018 (0.01-0.08)	mg a.i./kg of soil DW	Sanyal et al. 2015
Ryegrass	OECD	BAS 455 48	Pendimethalin	ER50 (emergence)	543	g a.i./ha	EFSA 2016
Tomato	OECD	AG-P4-400-SC	Pendimethalin	ER50 (emergence)	402	g a.i./ha	EFSA 2016
Soil microorganisms	NA	BAS 455 48 H	Pendimethalin	Nitrogen mineralisation EC25 (28 days)	2.66	g a.i./ha	EFSA 2016
Soil microorganisms	NA	AG-P4-400-SC	Pendimethalin	Nitrogen mineralisation (28 days)	NS at 11.0	g a.i./ha	EFSA 2016
Soil microorganisms	NA	M455H001	Pendimethalin	Nitrogen mineralisation (28 days)	NS at 5.0	g a.i./ha	EFSA 2016
Soil microorganisms	NA	M455H033	Pendimethalin	Nitrogen mineralisation (28 days)	NS at 5.0	g a.i./ha	EFSA 2016

NA – information not available; NS – not significant (< 25%)

A.24 Survey for participation in the ring test.

Reproduction of the survey sent to the laboratories interested in participating in the ring test to infer which laboratories were able to perform the experiments required is presented below.

Survey for the ring testers:

This survey aims at collecting information from each ring test participant so we can better plan the work to be developed.

Contact information:

Institution/Company:	
Laboratory main contact:	**Name:**
	Email:
	Telephone Nº:
Ring Test Study Leader (if different from main contact):	**Name:**
	Email:
	Telephone Nº:
Full Mailing address (supplies will be shipped to this address)	**Attn:**

Please check if you have the following equipment available for the ring-test:

Type of Equipment:	**Do you have access to this equipment? (Yes/No)**	**Model (and capacity if applicable):**
1 temperature controlled room/chamber regulated for 23 ± 2 ºC, a photoperiod of 16:8 h light:dark and a relative air moisture of 60% or higher (consider that a minimum of 1 m^2 is needed for each experiment and a constant ventilation is required to avoid CO_2 accumulation)		
1 autoclave with capacity for autoclaving soil		
1 centrifuge ideally for ≥50 mL tubes *(needed only for the pre-symbiotic phase)*		
1 Oven set at 105 °C		

1 microscope with magnification up to 400x (10x ocular × 40x objective)		
1 Binocular microscope with ≥50x of magnification *(needed only for the pre-symbiotic phase)*		
Eye piece Whipple disc (i.e. net micrometer) - 10 x 10 grid (1 mm squares)		
1 water bath or any other system that allows continuous heating of water, e.g. pot on a stove (e.g. 4-14 L capacity)		
Blender (e.g. 2 L capacity)		
Vacuum filtration system/capability		
1 mm - mesh sieve (e.g. 20 cm diameter)		
0.30 mm - mesh sieve (e.g. 20 cm diameter). *(needed only for the pre-symbiotic phase)*		
0.25 mm - mesh sieve (e.g. 20 cm diameter)		
45 µm – mesh sieve (e.g. 20 cm diameter)		

To the following questions, please write freely in the right column:

Question:	**Answer:**
1. Do you have someone with experience in performing experiments and/or manipulating mycorrhiza in your laboratory?	
2. Do you have someone with experience in laboratory ecotoxicological tests following procedures from international standards?	
3. Would you require initial training to have the necessary skills to perform the experiments? For how many persons would it be needed? When would you be available to start the training?	
4. How many experiments would you be able to perform for the symbiotic phase, knowing that (i) 1 experiment encompasses 6 treatments + 1 control and (ii) each	

treatment and control consisting of 5 replicates (meaning a total of 35 replicates),(iii) each experiment takes 2 months (i.e. 56 days) to be performed; (iv) after these 2 months, the measurement of the endpoints take 2 additional months (1 person) but over these additional months, other tests can be performed in the meantime. ***NOTE:*** In an ideal situation we would expect that each laboratory would be able to perform 4 experiments in 6 months.	
5. Knowing that, for the experiments of symbiotic phase, each replicate is composed of a 500 mL/pot, how many replicates would you be able to handle simultaneously in your facilities (please consider manpower for management, and space available for the experiments).	
6. Are you able to obtain your own inoculum of *Funneliformis mosseae* and/or *Rhizophagus irregularis* for the ring-test experiments? We estimate that the total amount of inoculum needed for each laboratory is about 7kg for each of these two AMF species.	
7. Are you able to make OECD artificial soil yourself (i.e. kaolin clay, sand and *Sphagnum* sp. Peat)? We estimate that a total of 70kg of soil will be needed for each laboratory (4 experiments).	
8. Would you be available to perform experiments also for the pre-symbiotic phase? Please take into consideration that experiments for pre-symbiotic phase are performed in Petri dishes, over 14 days and a test design similar to that used for the symbiotic phase will be followed.	
9. We plan to ask to each laboratory a non-disclosure agreement and of intellectual property rights according to the requirements agreed with the sponsor of this	

project. If you have any internal incompatibility in signing this document, please let us know.	
10. Would you be able to receive the materials needed for the ring test, e.g. natural soils, inoculum, test substance, etc., in your facilities over the first 2 months of 2023 and to start the tests on March of 2023?	

Please send the completed form to Tiago Natal da Luz (tiagoluz@cloverstrategy.pt**) by November 11, 2022**

A.25 Example of an Excel spreadsheet for the symbiotic phase ring test (for instance for the test with Fluazinam in OECD 2.5% soil).

"Test setup" tab

ERAMYC test setup

Contaminant information	
Contaminant identification:	Fluazinam
99,77 % purity	

Soil information		
Soil identification:	OECD 2.5% soil	
WHC_{max} (soil)		%
$\%WHC_{max}$ (soil)	50	%
WC (soil)		%
WHC_{max} (soil+mixed inoc)		%

WHC - Water Holding Capacity; WC - Water Content

Test setup		
Soil weight/replicate:	180	g DW
Nº replicate/treatment or control	5	
Soil for pH measurement	10	g FW
Soil for WC measurement	10	g FW
Soil for chemical analyses	150	g FW

Test information	
Setup date	
Test start (germ in C0 replicates)	
1st nutrient addition	07/01/1900
2nd nutrient addition	21/01/1900
3rd nutrient addition	04/02/1900
Test end	25/02/1900

Please follow the date formate: dd/mm/yyyy

Partner information
Institution name:
Technician(s):

Please fill the green cells with your data.

For the experiment setup, please follow the measeurements marked by grey cells only (white cells are supporting calculations)

Page 1 of 4

ERAMYC test setup

1st Step - Aliquot contamination

1.1. Oven-dry (70ºC overnight) an aliquot of fresh soil

Treatments	Soil aliquot - Fresh weight (±0,1000) (g)
CTsol	10,6
C1	10,6
C2	10,6
C3	10,6
C4	10,6
C5	10,6

1.2. Prepare the Stock Solution (SS)

Treatments	[] Fluazinam (mg/kg)	Dry soil (g)	Fresh soil (g)	Fluazinam weight (mg)	Fluazinam weight (mg product)
C0solv	0	1070	1070,0	0	0,00
C1	0,5	1070	1070,0	0,535	0,54
C2	1,0	1070	1070,0	1,07	1,07
C3	2,0	1070	1070,0	2,14	2,14
C4	4,0	1070	1070,0	4,28	4,29
C5	8,0	1070	1070,0	9	8,58

Stock solution preparation:

Fluazinam (g)	0,0179
SS volume (mL)	50
Solvent	Acetone

1.3. Contaminate the oven-dried soil aliquote with SS+acetone. Let it dry completely before following for 2nd step

Treatments	Soil aliquot - dry weight (g)	Stock solution (mL)	Acetone (mL)
CTsol	10,6	0	24,0
C1	10,6	1,5	22,5
C2	10,6	3,0	21,0
C3	10,6	6,0	18,0
C4	10,6	12,0	12,0
C5	10,6	24,0	0

Page 2 of 4

ERAMYC test setup

2nd Step - Contamination of soil by treatment

2.1. Contaminate the soil of each treatment individually. Start from the lower concentration to the highest.

Treatments	Dry soil (g)	Fresh soil (g)	Soil aliquot (g)	Water (ml)	Total weight (g)
CTster	1170	1170,0	0	0,0	1170,0
C0	1070	1070,0	0	0,0	1070,0
CTsol	1059,3	1059,3	10,6	0,0	1069,9
C1	1059,3	1059,3	10,6	0,0	1069,9
C2	1059,3	1059,3	10,6	0,0	1069,9
C3	1059,3	1059,3	10,6	0,0	1069,9
C4	1059,3	1059,3	10,6	0,0	1069,9
C5	1059,3	1059,3	10,6	0,0	1069,9

3rd Step - Replicate preparation + sampling

3.1. Fill the 5 replicate vessels of the treatment. Start by filling the bottom layer of all 5 replicates and then follow for the top layer.

Treatments	Layer	Total dry soil/replicate (g)	Moistened soil/replicate (g)	Inoculum (g)	Water (ml)	Weight/ replicate (g)	Total weight/replicate (g)
CTster (no inoculum but with plant)	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	100	100,0	0	0,0	100,00	
CTsol	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	
C0	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	
C1	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	
C2	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	
C3	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	

ERAMYC test setup

Treatments	Layer	Total dry soil/replicate (g)	Moistened soil/replicate (g)	Inoculum (g)	Water (ml)	Weight/ replicate (g)	Total weight/replicate (g)
C4	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	
C5	Bottom	100	100,0	0	0,0	100,00	200,00
	Top	80	80,0	20	0,0	100,00	

3.2. Collect 3 samples of ~50 g of soil for chemical analysis + 1 sample of 10 g for soil initial WC + 1 sample for soil initial pH.
3.3. Sow 3 pre-sterilized leek seeds in the replicates. Cover each replicate with coarse sand/glass beads.
3.4. Weight each replicate individually and register the weight.

"Soil %WC and pH" tab

Measurements: Soil %Water Content and pH
Contaminant: Fluazinam
Soil: OECD 2.5% soil
Methodology description: SOP Test 2 - Symbiotic phase
Institution:
Technician:
Please fill the green cells with your data.

Soil initial %Water Content and pH								
Assessment date:								
Treatment	Vessel tare (g)	Tare + fresh soil (g)	Tare + dry soil (g)	Water weight (g)	Dry soil (g)	% Water Content	%WHC	pH
CTsol				0,00	0,00	#DIV/0!	#DIV/0!	
CTster				0,00	0,00	#DIV/0!	#DIV/0!	
C0				0,00	0,00	#DIV/0!	#DIV/0!	
C1				0,00	0,00	#DIV/0!	#DIV/0!	
C2				0,00	0,00	#DIV/0!	#DIV/0!	
C3				0,00	0,00	#DIV/0!	#DIV/0!	
C4				0,00	0,00	#DIV/0!	#DIV/0!	
C5				0,00	0,00	#DIV/0!	#DIV/0!	

Soil final %Water Content and pH								
Assessment date:								
Treatment	Vessel tare (g)	Tare + fresh soil (g)	Tare + dry soil (g)	Water weight (g)	Dry soil (g)	% Water Content	%WHC	pH
CTsol-R1				0,00	0,00	#DIV/0!	#DIV/0!	
CTsol-R2				0,00	0,00	#DIV/0!	#DIV/0!	
CTsol-R3				0,00	0,00	#DIV/0!	#DIV/0!	
CTsol-R4				0,00	0,00	#DIV/0!	#DIV/0!	
CTsol-R5				0,00	0,00	#DIV/0!	#DIV/0!	
CTster-R1				0,00	0,00	#DIV/0!	#DIV/0!	
CTster-R2				0,00	0,00	#DIV/0!	#DIV/0!	
CTster-R3				0,00	0,00	#DIV/0!	#DIV/0!	
CTster-R4				0,00	0,00	#DIV/0!	#DIV/0!	
CTster-R5				0,00	0,00	#DIV/0!	#DIV/0!	
C0-R1				0,00	0,00	#DIV/0!	#DIV/0!	
C0-R2				0,00	0,00	#DIV/0!	#DIV/0!	
C0-R3				0,00	0,00	#DIV/0!	#DIV/0!	
C0-R4				0,00	0,00	#DIV/0!	#DIV/0!	
C0-R5				0,00	0,00	#DIV/0!	#DIV/0!	
C1-R1				0,00	0,00	#DIV/0!	#DIV/0!	
C1-R2				0,00	0,00	#DIV/0!	#DIV/0!	
C1-R3				0,00	0,00	#DIV/0!	#DIV/0!	
C1-R4				0,00	0,00	#DIV/0!	#DIV/0!	
C1-R5				0,00	0,00	#DIV/0!	#DIV/0!	
C2-R1				0,00	0,00	#DIV/0!	#DIV/0!	
C2-R2				0,00	0,00	#DIV/0!	#DIV/0!	
C2-R3				0,00	0,00	#DIV/0!	#DIV/0!	
C2-R4				0,00	0,00	#DIV/0!	#DIV/0!	
C2-R5				0,00	0,00	#DIV/0!	#DIV/0!	
C3-R1				0,00	0,00	#DIV/0!	#DIV/0!	
C3-R2				0,00	0,00	#DIV/0!	#DIV/0!	
C3-R3				0,00	0,00	#DIV/0!	#DIV/0!	
C3-R4				0,00	0,00	#DIV/0!	#DIV/0!	
C3-R5				0,00	0,00	#DIV/0!	#DIV/0!	
C4-R1				0,00	0,00	#DIV/0!	#DIV/0!	
C4-R2				0,00	0,00	#DIV/0!	#DIV/0!	
C4-R3				0,00	0,00	#DIV/0!	#DIV/0!	
C4-R4				0,00	0,00	#DIV/0!	#DIV/0!	
C4-R5				0,00	0,00	#DIV/0!	#DIV/0!	
C5-R1				0,00	0,00	#DIV/0!	#DIV/0!	
C5-R2				0,00	0,00	#DIV/0!	#DIV/0!	
C5-R3				0,00	0,00	#DIV/0!	#DIV/0!	

C5-R4				0,00	0,00	#DIV/0!	#DIV/0!	
C5-R5				0,00	0,00	#DIV/0!	#DIV/0!	

Observations (please fill this space if you have any observation)

"Intraradicular colonization" tab

Measurements: Intraradicular colonization (% total and arbuscular colonization)

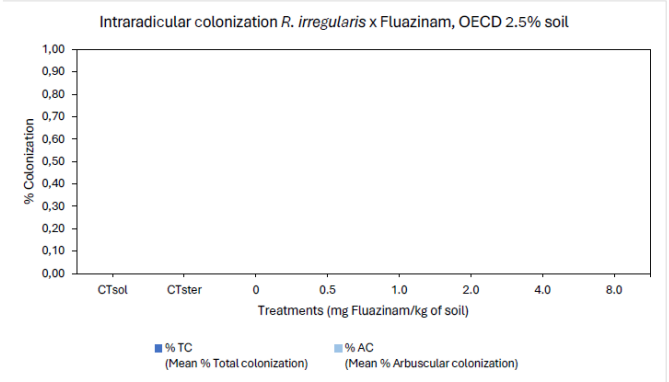
Contaminant: Fluazinam
Soil: OECD 2.5% soil
Methodology description: SOP 2.2 - Staining and SOP 2.3 - AMF structures
Institution:
Technician:
Sampling date: 15/04/2024
Please fill the green cells with your data.

Treatment-Replicate	Total colonization (%)	Arbuscular colonization (%)
CTsol-R1		
CTsol-R2		
CTsol-R3		
CTsol-R4		
CTsol-R5		
CTster-R1		
CTster-R2		
CTster-R3		
CTster-R4		
CTster-R5		
C0-R1		
C0-R2		
C0-R3		
C0-R4		
C0-R5		
C1-R1		
C1-R2		
C1-R3		
C1-R4		

C1-R5		
C2-R1		
C2-R2		
C2-R3		
C2-R4		
C2-R5		
C3-R1		
C3-R2		
C3-R3		
C3-R4		
C3-R5		
C4-R1		
C4-R2		
C4-R3		
C4-R4		
C4-R5		
C5-R1		
C5-R2		
C5-R3		
C5-R4		
C5-R5		

Observations (please fill this space if you have any observation)

Treatment (mg Fluaz/kg soil)	**% TC** (Mean % Total colonization)	**SD %TC** (Standard deviation %TC)	**% AC** (Mean % Arbuscular colonization)	**SD %AC** (Standard deviation %AC)
CTsol	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
CTster	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0.5	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
8.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!



"Extra-radicular hyphae lenght" tab

Measurements: Extraradicular hyphae lenght (EHL)
Contaminant: Fluazinam

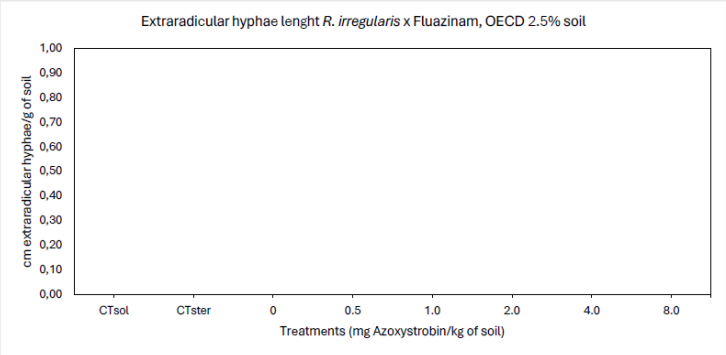
Soil: OECD 2.5% soil
Methodology description: SOP 2.4 - Total extraradicular mycellium lenght
Institution:
Technician:
Sampling date: 25/02/1900
Please fill the green cells with your data.

Equation correction factor (See SOP 2.4, NOTE)	0,037160233

Treatment-Replicate	Nº intersections	Water content in 10 g of soil (g)	m of extraradicular hyphae/g of soil
CTsol-R1		#DIV/0!	#DIV/0!
CTsol-R2		#DIV/0!	#DIV/0!
CTsol-R3		#DIV/0!	#DIV/0!
CTsol-R4		#DIV/0!	#DIV/0!
CTsol-R5		#DIV/0!	#DIV/0!
CTster-R1		#DIV/0!	#DIV/0!
CTster-R2		#DIV/0!	#DIV/0!
CTster-R3		#DIV/0!	#DIV/0!
CTster-R4		#DIV/0!	#DIV/0!
CTster-R5		#DIV/0!	#DIV/0!
C0-R1		#DIV/0!	#DIV/0!
C0-R2		#DIV/0!	#DIV/0!
C0-R3		#DIV/0!	#DIV/0!
C0-R4		#DIV/0!	#DIV/0!
C0-R5		#DIV/0!	#DIV/0!
C1-R1		#DIV/0!	#DIV/0!
C1-R2		#DIV/0!	#DIV/0!
C1-R3		#DIV/0!	#DIV/0!
C1-R4		#DIV/0!	#DIV/0!
C1-R5		#DIV/0!	#DIV/0!
C2-R1		#DIV/0!	#DIV/0!
C2-R2		#DIV/0!	#DIV/0!
C2-R3		#DIV/0!	#DIV/0!
C2-R4		#DIV/0!	#DIV/0!
C2-R5		#DIV/0!	#DIV/0!
C3-R1		#DIV/0!	#DIV/0!
C3-R2		#DIV/0!	#DIV/0!
C3-R3		#DIV/0!	#DIV/0!
C3-R4		#DIV/0!	#DIV/0!
C3-R5		#DIV/0!	#DIV/0!
C4-R1		#DIV/0!	#DIV/0!
C4-R2		#DIV/0!	#DIV/0!
C4-R3		#DIV/0!	#DIV/0!
C4-R4		#DIV/0!	#DIV/0!
C4-R5		#DIV/0!	#DIV/0!
C5-R1		#DIV/0!	#DIV/0!
C5-R2		#DIV/0!	#DIV/0!
C5-R3		#DIV/0!	#DIV/0!
C5-R4		#DIV/0!	#DIV/0!
C5-R5		#DIV/0!	#DIV/0!

Observations (please fill this space if you have any observation)

Treatment (mg Fluaz/kg soil)	EHL/g of soil	SD EHL/g of soil (Standard deviation EHL/g of soil)
CTsol	#DIV/0!	#DIV/0!
CTster	#DIV/0!	#DIV/0!
0	#DIV/0!	#DIV/0!
0.5	#DIV/0!	#DIV/0!
1.0	#DIV/0!	#DIV/0!
2.0	#DIV/0!	#DIV/0!
4.0	#DIV/0!	#DIV/0!
8.0	#DIV/0!	#DIV/0!



"Plant biomass" tab

Measurements: Shoot and root biomass

Contaminant: Fluazinam
Soil: OECD 2.5% soil
Methodology description: SOP Test 2 - Symbiotic phase
Institution:
Technician:
Sampling date: 25/02/1900
Please fill the green cells with your data.

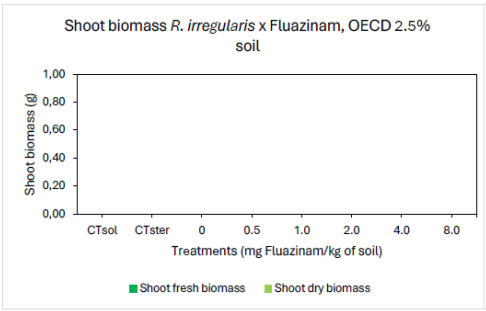
Treatment-Replicate	Shoot fresh biomass (g)	Shoot dry biomass (g)	Root fresh biomass (g)
CTsol-R1			
CTsol-R2			
CTsol-R3			
CTsol-R4			
CTsol-R5			
CTster-R1			
CTster-R2			
CTster-R3			
CTster-R4			
CTster-R5			
C0-R1			
C0-R2			
C0-R3			
C0-R4			
C0-R5			
C1-R1			
C1-R2			
C1-R3			

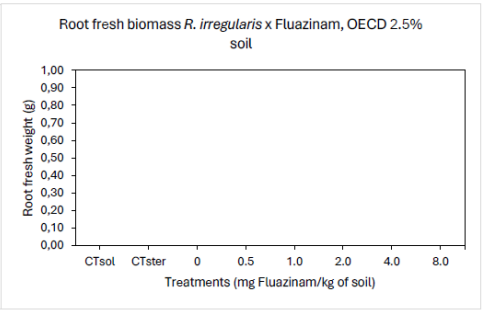
C1-R4			
C1-R5			
C2-R1			
C2-R2			
C2-R3			
C2-R4			
C2-R5			
C3-R1			
C3-R2			
C3-R3			
C3-R4			
C3-R5			
C4-R1			
C4-R2			
C4-R3			
C4-R4			
C4-R5			
C5-R1			
C5-R2			
C5-R3			
C5-R4			
C5-R5			

Observations (please fill this space if you have any observation)

Treatment (mg Fluaz/kg soil)	Shoot fresh biomass	SD SFB (Standard deviation SFB)	Shoot dry biomass	SD SDB (Standard deviation SDB)
CTsol	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
CTster	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
0.5	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
8.0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Treatment (mg Fluaz/kg soil)	Root fresh biomass	SD RFB (Standard deviation RFB)
CTsol	#DIV/0!	#DIV/0!
CTster	#DIV/0!	#DIV/0!
0	#DIV/0!	#DIV/0!
0.5	#DIV/0!	#DIV/0!
1.0	#DIV/0!	#DIV/0!
2.0	#DIV/0!	#DIV/0!
4.0	#DIV/0!	#DIV/0!
8.0	#DIV/0!	#DIV/0!





A.26 Raw data of the symbiotic phase ring test experiments with Azoxystrobin.

Table A.26 – 1: Raw data from the experiments conducted during the symbiotic phase ring test using inoculant of *Rhizophagus irregularis*, the test soil OECD 2.5% soil and the test substance Azoxystrobin (AZX).

Please refer to the "Raw data" supplementary Excel file.

A.27 Raw data of the symbiotic phase ring test experiments with Fluazinam.

Table A.27 – 1: Raw data from the experiments conducted during the symbiotic phase ring test using inoculant of *Rhizophagus irregularis*, the test soil OECD 2.5% soil and the test substance Fluazinam (FLZ).

Please refer to the "Raw data" supplementary Excel file.

A.28 Example of an Excel spreadsheet for the pre-symbiotic phase ring test (for instance for the test with Azoxystrobin in OECD 2.5% soil).

"Test setup" tab

ERAMYC pre-symbiotic phase test setup

Contaminant information	
Contaminant identification:	Azoxystrobin
99,7	% purity

Test information	
Setup date	
Test end	14/01/1900

Please follow the date formate: dd/mm/yyyy

Soil information		
Soil identification:	OECD 2.5% soil	
WHC_{max} (soil)		%
$\%WHC_{max}$ (soil)	50	%
WC (soil)		%

WHC - Water Holding Capacity; WC - Water Content

Partner information
Institution name:
Technician(s):

Test setup		
Soil weight/replicate:	80	g DW
N° replicate/treatment or control	6	
Soil for pH measurement	10	g FW
Soil for WC measurement	10	g FW
Soil for chemical analyses	150	g FW

Please fill the green cells with your data.
For the experiment setup, please follow the measeurements marked by grey cells only (white cells are supporting calculations)

ERAMYC pre-symbiotic phase test setup

1st Step - Aliquot contamination

1.1. Oven-dry (70°C overnight) an aliquot of fresh soil

Treatments	Soil aliquot - Fresh weight (±0,1000) (g)
CTsol	6,4
C1	6,4
C2	6,4
C3	6,4
C4	6,4
C5	6,4
C6	6,4

1.2. Prepare the Stock Solution (SS)

Treatments	[] Azoxystrobin (mg/kg)	Dry soil (g)	Fresh soil (g)	Azoxystrobin weight (mg)	Azoxystrobin weight (mg product)
Ctsol	0	650	650,0	0	0,00
C1	1,25	650	650,0	0,81	0,81
C2	3,125	650	650,0	2,03	2,04
C3	7,8	650	650,0	5,07	5,09
C4	19,5	650	650,0	12,68	12,71
C5	39,0	650	650,0	25,35	25,43
C6	78,0	650	650,0	50,70	50,85

Stock solution preparation:	
Azoxystrobin (g)	0,1589
SS volume (mL)	50
Solvent	Acetone

1.3. Contaminate the oven-dried soil aliquote with SS+acetone. Let it dry completely before following for 2nd step

Treatments	Soil aliquot - dry weight (g)	Stock solution (mL)	Acetone (mL)
CTsol	6,4	0	16,0
C1	6,4	0,3	15,7
C2	6,4	0,6	15,4
C3	6,4	1,6	14,4

ERAMYC pre-symbiotic phase test setup

C4	6,4	4,0	12,0
C5	6,4	8,0	8,0
C6	6,4	16,0	0,0

1.4. Prepare the "spore sandwiches"

2nd Step - Contamination of soil by treatment

2.1. Contaminate the soil of each treatment individually. Start from the lower concentration to the highest.

Treatments	Dry soil (g)	Fresh soil (g)	Soil aliquot (g)	Water (ml)	Total weight (g)
C0	650	650,0	0	0,0	650,0
CTsol	643,5	643,5	6,4	0,0	649,9
C1	643,5	643,5	6,4	0,0	649,9
C2	643,5	643,5	6,4	0,0	649,9
C3	643,5	643,5	6,4	0,0	649,9
C4	643,5	643,5	6,4	0,0	649,9
C5	643,5	643,5	6,4	0,0	649,9
C6	643,5	643,5	6,4	0,0	649,9

3rd Step - Replicate preparation and sampling

3.1. Fill the 6 replicate vessels of the treatment. Start by filling the bottom of the Petri dish of all 6 replicates, put the spore sandwich and then pour the top layer.

Treatments	Layer	Total dry soil/replicate (g)	Moistened soil/replicate (g)	Weight/ replicate (g)	Total weight/replicate (g)
CTsol	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	
C0	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	
C1	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	
C2	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	

ERAMYC pre-symbiotic phase test setup

C3	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	
C4	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	
C5	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	
C6	Bottom	40	40,0	40,00	80,00
	Top	40	40,0	40,00	

3.2. Collect 3 samples of ~50 g of soil for chemical analysis of treatments C1 to C6 + 1 sample of ~50 g of soil for C0 + 1 sample of 10 g for soil initial WC + 1 sample for soil initial pH.

"Soil %WC and pH" tab

Measurements: Soil %Water Content and pH
Contaminant: Azoxystrobin
Soil: OECD 2.5% soil
Methodology description: SOP Test 1 - Pre symbiotic phase
Institution:
Technician:
Please fill the green cells with your data.

Soil initial %Water Content and pH

Assessment date:

Treatment	Vessel tare (g)	Tare + fresh soil (g)	Tare + dry soil (g)	Water weight (g)	Dry soil (g)	% Water Content	% WHC	pH
CTsol				0,00	0,00	#DIV/0!	#DIV/0!	
C0				0,00	0,00	#DIV/0!	#DIV/0!	
C1				0,00	0,00	#DIV/0!	#DIV/0!	
C2				0,00	0,00	#DIV/0!	#DIV/0!	
C3				0,00	0,00	#DIV/0!	#DIV/0!	
C4				0,00	0,00	#DIV/0!	#DIV/0!	
C5				0,00	0,00	#DIV/0!	#DIV/0!	
C6				0,00	0,00	#DIV/0!	#DIV/0!	

Soil final %Water Content and pH

Assessment date:

Treatment	Vessel tare (g)	Tare + fresh soil (g)	Tare + dry soil (g)	Water weight (g)	Dry soil (g)	% Water Content	% WHC	pH
CTsol				0,00	0,00	#DIV/0!	#DIV/0!	
C0				0,00	0,00	#DIV/0!	#DIV/0!	
C1				0,00	0,00	#DIV/0!	#DIV/0!	
C2				0,00	0,00	#DIV/0!	#DIV/0!	
C3				0,00	0,00	#DIV/0!	#DIV/0!	
C4				0,00	0,00	#DIV/0!	#DIV/0!	
C5				0,00	0,00	#DIV/0!	#DIV/0!	
C6				0,00	0,00	#DIV/0!	#DIV/0!	

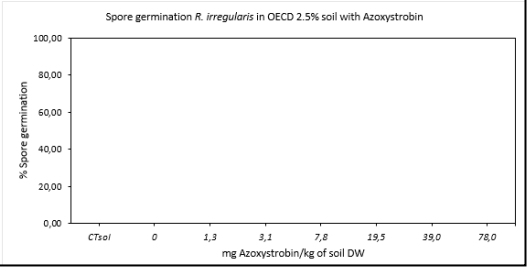
Observations (please fill this space if you have any observation)

"%Spore germination" tab

Measurements: % Spore germination
Contaminant: Azoxystrobin
Soil: OECD 2.5% soil
Methodology description: SOP Test 1 - Pre symbiotic phase
Institution:
Technician:
Sampling date: 14/01/1900
Please fill the green cells with your data.

Treatment-Replicate	Nº of germinated spores	Nº of recovered spores	% of Spore Germination
CTsol-R1			#DIV/0!
CTsol-R2			#DIV/0!
CTsol-R3			#DIV/0!
CTsol-R4			#DIV/0!
CTsol-R5			#DIV/0!
CTsol-R6			#DIV/0!
C0-R1			#DIV/0!
C0-R2			#DIV/0!
C0-R3			#DIV/0!
C0-R4			#DIV/0!
C0-R5			#DIV/0!
C0-R6			#DIV/0!
C1-R1			#DIV/0!
C1-R2			#DIV/0!
C1-R3			#DIV/0!
C1-R4			#DIV/0!
C1-R5			#DIV/0!
C1-R6			#DIV/0!
C2-R1			#DIV/0!
C2-R2			#DIV/0!
C2-R3			#DIV/0!
C2-R4			#DIV/0!
C2-R5			#DIV/0!
C2-R6			#DIV/0!

Treatment (mg Azoxi/kg soil)	Nº of recovered spores	% Spore germination (SG)	SD %SG (Standard deviation %SG)
CTsol	#DIV/0!	#DIV/0!	#DIV/0!
0	#DIV/0!	#DIV/0!	#DIV/0!
1,3	#DIV/0!	#DIV/0!	#DIV/0!
3,1	#DIV/0!	#DIV/0!	#DIV/0!
7,8	#DIV/0!	#DIV/0!	#DIV/0!
19,5	#DIV/0!	#DIV/0!	#DIV/0!
39,0	#DIV/0!	#DIV/0!	#DIV/0!
78,0	#DIV/0!	#DIV/0!	#DIV/0!



C3-R1			#DIV/0!
C3-R2			#DIV/0!
C3-R3			#DIV/0!
C3-R4			#DIV/0!
C3-R5			#DIV/0!
C3-R6			#DIV/0!
C4-R1			#DIV/0!
C4-R2			#DIV/0!
C4-R3			#DIV/0!
C4-R4			#DIV/0!
C4-R5			#DIV/0!
C4-R6			#DIV/0!
C5-R1			#DIV/0!
C5-R2			#DIV/0!
C5-R3			#DIV/0!
C5-R4			#DIV/0!
C5-R5			#DIV/0!
C5-R6			#DIV/0!
C6-R1			#DIV/0!
C6-R2			#DIV/0!
C6-R3			#DIV/0!
C6-R4			#DIV/0!
C6-R5			#DIV/0!
C6-R6			#DIV/0!

Observations (please fill this space if you have any observation)

A.29 Raw data from the pre-symbiotic phase ring test with Azoxystrobin.

Table A.29 – 1: Raw data from the experiments conducted during the pre-symbiotic phase ring test using spores of *Rhizophagus irregularis*, the test soil OECD 2.5% soil and the test substance Azoxystrobin (AZX).

Please refer to the "Raw data" supplementary Excel file.

A.30 Raw data from the pre-symbiotic phase ring test with Fluazinam.

Table A.30 – 1: Raw data from the experiments conducted during the pre-symbiotic phase ring test using spores of *Rhizophagus irregularis*, the test soil OECD 2.5% soil and the test substance Fluazinam (FLZ).

Please refer to the "Raw data" supplementary Excel file.

A.31 Chemical analysis conducted for the pre-symbiotic and symbiotic phase ring test experiments.

Table A.31 – 1: **Mean (± standard deviation, *n=3*) measured initial and final concentrations of Azoxystrobin on soil samples from valid experiments of the symbiotic phase ring test. Percentages of nominal concentration (initial and final) were estimated based on raw data and nominal concentrations.**

Please refer to the "Raw data" supplementary Excel file.

Table A.31 – 2: **Mean (± standard deviation, *n=3*) measured initial and final concentrations of Fluazinam on soil samples from valid experiments of the symbiotic phase ring test. Percentages of nominal concentration (initial and final) were estimated based on raw data and nominal concentrations.**

Please refer to the "Raw data" supplementary Excel file.

Table A.31 – 3: **Mean (± standard deviation, *n=3*) measured initial and final concentrations of Azoxystrobin on soil samples from valid experiment of the pre-symbiotic phase ring test. Percentages of nominal concentration (initial and final) were estimated based on raw data and nominal concentrations.**

Please refer to the "Raw data" supplementary Excel file.

Table A.31 – 4: **Mean (± standard deviation, *n=3*) measured initial and final concentrations of Fluazinam on soil samples from valid experiment of the symbiotic phase ring test. Percentages of nominal concentration (initial and final) were estimated based on raw data and nominal concentrations.**

Please refer to the "Raw data" supplementary Excel file.

A.32 Non-linear models for endpoint estimations for WP3.

Table A.32 – 1: Non-linear models (and respective coefficient of regression, r^2) used on the estimation of the endpoints based on nominal and measured concentrations for the symbiotic phase experiments of the ring test with Azoxystrobin.

Please refer to the "Supporting information" supplementary Excel file.

Table A.32 – 2: Non-linear models (and respective coefficient of regression, r^2) used on the estimation of the endpoints based on nominal and measured concentrations for the symbiotic phase experiments of the ring test with Fluazinam.

Please refer to the "Supporting information" supplementary Excel file.

Table A.32 – 3: Non-linear models (and respective coefficient of regression, r^2) used on the estimation of the endpoints based on nominal and measured concentrations for the pre-symbiotic phase experiments of the ring test with Azoxystrobin.

Please refer to the "Supporting information" supplementary Excel file.

Table A.32 – 4: Non-linear models (and respective coefficient of regression, r^2) used on the estimation of the endpoints based on nominal and measured concentrations for the pre-symbiotic phase experiments of the ring test with Fluazinam.

Please refer to the "Supporting information" supplementary Excel file.

A.33 Draft guideline for the AMF pre-symbiotic phase tests.

Reproduction of the document prepared following the OECD formatting guidelines.

INTRODUCTION

1. This document is designed for assessing the effects of contaminants in soil on the pre-symbiotic phase (spore germination) of the development of Arbuscular Mycorrhizal Fungi (AMF). This test guideline has been ring-tested.

2. AMF are mutualist organisms living in symbiotic association with most vascular plant species. Besides the benefits provided to the host plant during the symbiosis (e.g. enhanced tolerance to environmental stress and nutrient acquisition), AMF also contribute with a large spectrum of ecosystem services (e.g. supporting and regulating services) (1,2).

3. Appropriate spore germination is essential for the subsequent establishment of the symbiosis between the AMF and the host plant. Spores released outside the host-plant roots are particularly vulnerable to the presence of contaminants in the soil, as they are in direct contact with them. The acknowledged sensitivity of AMF to contaminants led the European Food Safety Authority (EFSA) to list these organisms as potentially important for the risk assessment of Plant Protection Products (PPP) (2).

4. The standardized guideline from the International Organization for Standardization (ISO), guideline 10832:2009 (3), and the studies published by Mallmann et al. (2018), Malfatti et al. (2021) and Malfatti et al. (2023) provided essential information for the development of the present document.

PRINCIPLE OF THE TEST

5. The test assesses the effects of test chemicals present in the soil on the percentage of germination of AMF mature spores, inside a membrane filter “spore sandwich”, after 14 days of exposure under controlled laboratory conditions.

6. The percentage of spore germination on the treatments is compared to the response on the negative control to determine the no-observed effect concentration (NOEC), the lowest observed effect concentration (LOEC) and, when possible, the inhibition concentrations (IC_x; e.g. IC_{10}, IC_{50}) may be estimated through non-linear regression models.

INFORMATION ON THE TEST CHEMICAL

7. Information on the test chemical such as common name. chemical name (preferably IUPAC name), structural formula, CAS registry number, purity, safety precautions, proper storage conditions and analytical methods should be obtained before the beginning of the study. In addition, the following information should be known:
   - Solubility in water and/or in organic solvents;
   - Octanol-water partition coefficient, K_{ow};
   - Soil-water partition coefficient, expressed as K_{oc};
   - Vapour pressure;

- Degradability (e.g. in soil, water);
- Known metabolites.

REFERENCE SUBSTANCE

8. The recommended reference substance on ISO 10832:2009 (3) is cadmium nitrate ($CdNO_3 \cdot 4H_2O$). This substance caused a 50% inhibition on *Glomus (=Funneliformis) mosseae* spore germination at concentrations between 0.15 and 1.7 mg kg^{-1}.

9. An alternative reference substance, boric acid, is recommended by Mallmann et al. (2018). Boric acid caused a 50% inhibition of spore germination on different AMF species on tropical artificial soil (TAS, (7)) between 359.84 and 684.27 mg kg^{-1}.

VALIDITY CRITERIA

10. For the test to be valid, the following criteria are applied:
    - The mean number of recovered spores in all treatments is ≥ 25;
    - The mean percentage of germinated spores in the control treatment is ≥ 75% at the end of the test.

DESCRIPTION OF THE TEST

Test apparatus

11. Usual laboratory equipment and the following should be used:

a. Sterilized plastic Petri dishes (Ø 9 cm), or other recipient with lid, with enough capacity for 80 g of soil dry weight (DW) can be used for replicates;

b. Distilled water with pH ≥ 5.5;

c. Nitrocellulose membrane filters (47 mm diameter, 0.45 µm porosity, white, 3 mm grid lined);

d. Watch-glass;

e. Ultra-fine tweezers;

f. Blue or black ink;

g. Sucrose (or regular white sugar);

h. Centrifuge, capable of 2000 rpm;

i. Stereomicroscope with 50x magnification.

Biological materials

12. AMF species suitable for the performance of these tests are *Glomus (=Funneliformis) mosseae* (3), *Rhizophagus irregularis* (8), *Rhizophagus clarus* and *Gigaspora albida*

(4–6). Other AMF species may be adapted to the method after evaluation of germination responses and test validation criteria.

13. Sporocarps or isolated spores can be purchased commercially. Alternatively, sporocarps or spores can be extracted from monoxenic cultures by wet sieving (9) and using a sucrose gradient solution followed by centrifugation (Annex B).

14. Sporocarps containing spores can be placed on moisturized filter paper and carefully opened with ultra-fine tweezer under the stereo microscope to extract the spores. Spores can be transferred to a microtube with distilled water and stored at 4ºC until use in tests, which should be within one day.

Test soil

15. Test soils (either natural or artificial soil) should not have negative effects on the development of AMF spores. Soils with physical or chemical characteristics that can inhibit the development of AMF should be avoided (e.g. high content of phosphorous, aluminum or manganese) (10–15).

16. Test soils should be characterized before their use in tests, with, at least, the following information:
    - Collection site (for natural soils);
    - Origin of the components used in preparation of artificial soil;
    - Organic carbon content;
    - Particle size distribution (% sand, clay and silt);
    - Maximum water holding capacity (% WHC_{max}) (Annex C);
    - Water content (% WC) (Annex D);
    - pH (Annex E);
    - Aluminum, manganese and phosphorus content.

17. Modified artificial soil, with reduced organic matter content, can be prepared based on the information presented in the guideline OECD 232:2016 (16):

Soil component	Content expressed on % dry mass basis
- *Sphagnum* peat (with no visible plant remains, use mature peat, air dried and sieved at 2 mm)	2.5
- Kaolin clay (kaolinite content preferably above 30 %)	20
- Industrial quartz sand (air dried, with more than 50 % of the particles between 50 and 200 microns)	77.5
- Calcium carbonate ($CaCO_3$, pulverized, analytical grade) to obtain an initial pH of 6.0 ± 0.5	< 1

Note 1: The amount of $CaCO_3$ required depends on its constituents and should be determined by measuring the pH of pre-incubated moist soil sub-samples before the test.

Note 2: Tropical artificial soil (TAS) (7) can be applied in this methodology by replacing the *Sphagnum* peat by coconut fiber on the preparation of the artificial soil.

18. Natural soils should be collected from unpolluted sites and be free of PPP, manure from treated animals and organic fertilizers for at least one year prior to use. Sieving at 2 mm is recommended to homogenize the soil and remove coarser particles. Natural soils can be stored at room temperature. for the shorter time possible before use in tests.

Preparation of test concentrations

19. Test soils are contaminated by homogenizing a solution of test chemical directly in the test soil.

Contaminating soil with water soluble test chemicals

20. A stock solution is prepared by dissolving the test chemical in distilled water. A portion of test soil enough for the test treatment is homogeneously contaminated with an amount of stock solution and distilled water to adjust the soil moisture to 50% WHC_{max} before preparing the replicates.

Contaminating soil with water insoluble chemicals

21. Contamination of soil with water insoluble chemicals requires the previous coating of an aliquot of test soil. A portion of test soil is contaminated with a solution of the test chemical dissolved in an appropriate organic solvent (e.g. acetone). After complete evaporation of the solvent, the portion of contaminated soil is mixed with an amount of test soil enough for all treatment and the soil moisture should be adjusted to 50% WHC_{max} with distilled water.

22. In this case, the preparation of a solvent control is recommended (paragraph 24).

TEST PROCEDURES

Test treatments

23. A negative control, composed by the test soil without the test chemical is prepared for all tests.

24. A solvent control is recommended in test with water insoluble chemicals. In this case, the soil is spiked with the solvent, but no test chemical is added.

Range finding test

25. A range-finding test could be necessary to help select appropriate test concentrations for the definitive test. Three replicates can be used per treatment and the percentage of spore germination should be assessed after 14 days of exposure.

26. For the range-finding test. the test concentrations should be widely spaced (e.g. 0.1, 1.0, 10, 100 and 1000 mg/kg dry soil) or others if data exist to support the selection of different concentrations.

Definitive test

27. Test concentrations can be based on the results observed on the range-finding test or on data from literature, which should be reported in any case. At least five treatments are recommended.

28. Ideally, the selected concentrations should cover concentrations with no effect and concentrations which totally inhibited spore germination on the range finding test.

Test design

29. Each test treatment is composed of, at least, six replicates with 30 spores each.

30. Previously isolated spores are selected under the stereo microscope (50x amplification). Only spores with intact and clean walls are selected.

31. Viable spores are transferred to wet nitrocellulose membranes and placed inside the grid squares. The membrane is covered with another wet nitrocellulose membrane, forming a "spore sandwich".

32. The "spore sandwich" is transferred to a Petri dish with 40 g DW of test soil (uncontaminated for control or contaminated for test treatments other than control) and covered with more 40 g DW of the same soil.

33. The Petri dish is sealed with parafilm and incubated under controlled conditions for 14 days.

Test conditions

34. Replicates are incubated under controlled conditions: 26 ± 2ºC in the dark (alternatively Petri dishes can be covered with aluminum foil). Other temperatures can be used according to the AMF species.

Test measurements

35. After 14 days of exposure, the "spore sandwich" is removed from each replicate. Excess soil is removed from the surface of the "sandwich" with a brush or with distilled water. The spore sandwich should always be kept horizontally to avoid loss of spores.

36. The "sandwiches" are soaked in a 1:1 solution of a 5% blue or black aqueous pen ink solution and a 5% acetic acid solution for 15 min to make hyphal germination visible. After this period, the "sandwiches" are carefully dried with absorbent paper and opened.

37. The number of recovered and germinated spores is determined using a stereo microscope (50x magnification). A spore is considered germinated when the hyphae length is at least five times greater than the spore diameter.

DATA AND REPORTING

Treatment of results

38. The percentage of germination and recovery of spores is calculated according to the following equation (1) adapted from Malfatti et al. (2021):

$$(1)\ \ \%(Germ/Rec) = \frac{g\ x\ 100}{n}$$

Where ***g*** is the number of germinated spores

n is the number of recovered spores

39. The mean percentage of spore germination and its standard deviation should be reported and compared to the control treatment. NOEC/LOEC and IC_x should be calculated if possible.

NOEC/LOEC

40. The no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) are estimated through the evaluation of significant differences of treatments when compared to the controls, considering $p < 0.05$.

41. Data normal distribution and homogeneity of data variances must be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Bartlett test, respectively, before statistical analysis. One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) can be used to calculate if there are significant differences ($p \leq 0.05$) between the controls and the treatments. Non-parametric methods (e.g. Bonferroni-U-test) could also be applied for NOEC/LOEC estimation.

ICx

42. Effect concentration for x% of inhibition of the spore germination when compared to the respective control (and its 95% confidence intervals) is estimated through non-linear regression models (e.g. logistic model, exponential model, Hormesis model).

Test report

43. The test report must contain the following information:

Test chemical

- Data from previous works, if available;
- Physical and chemical characterization of the test chemical;
- Details on soil contamination and verification of the tested concentrations.

Inoculant and host plant

- Scientific name, strain/variety, source.

Test conditions

- Description of the test design and procedures;
- Description of environmental conditions during the exposure period (photoperiod, % relative humidity, etc.);
- Nominal test concentrations;
- Characterization of the test soil.

Results

- Percentage of spore germination;
- Methods for statistical analysis;
- Compliance with the validity criteria;
- Further observations.

REFERENCES

1. Chen M., Arato M., Borghi L., Nouri E., Reinhardt D., Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application. Vol. 9. Frontiers in Plant Science. Frontiers Media S.A.; 2018.

2. EFSA. Scientific Opinion Addressing the State of the Science on Risk Assessment of Plant Protection Products for In-soil Organisms. EFSA Journal. 2017 Feb 1;15(2).

3. ISO. Soil quality — Effects of Pollutants on Mycorrhizal Fungi — Spore Germination Test. ISO 10832:2009. 2009.

4. Mallmann GC., Sousa JP., Sundh I., Pieper S., Arena M., da Cruz SP., et al., Placing Arbuscular Mycorrhizal Fungi on the Risk Assessment Test Battery of Plant Protection Products (PPPs). Ecotoxicology. 2018 Sep 1; 27(7):809-18.

5. Malfatti A de LR., Oliveira Filho LCI., Carniel LSC., Mallmann GC., da Cruz SP., Klauberg-Filho O. Risk Assessment Tests of Neonicotinoids on Spore Germination of Arbuscular Mycorrhizal Fungi Gigaspora albida and Rhizophagus clarus. J Soils Sediments. 2023 Mar 5;23(3):1295–303.

6. Malfatti A de LR., Mallmann GC., Oliveira Filho LCI., Carniel LSC., Cruz SP., Klauberg-Filho O. Ecotoxicological Test to Assess Effects of Herbicides on Spore Germination of Rhizophagus clarus and Gigaspora albida. Ecotoxicol Environ Saf. 2021 Jan 1;207.

7. Garcia M. Effects of Pesticides on Soil Fauna: Development of Ecotoxicological Test Methods for Tropical Regions. Cuvillier; 2004. 281 p.

8. Buysens C., Dupré de Boulois H., Declerck S. Do Fungicides Used to Control Rhizoctonia solani Impact the Non-target Arbuscular Mycorrhizal Fungus Rhizophagus irregularis? Mycorrhiza. 2015 May 1;25(4):277–88.

9. Gerdemann JW., Nicolson TH. Spores of Mycorrhizal Endogone Species Extracted from Soil by Wet Sieving and Decanting. Transactions of the British Mycological Society. 1963 Jun;46(2):235–44.

10. Alotaibi MO., Saleh AM., Sobrinho RL., Sheteiwy MS., El-sawah AM., Mohammed AE., et al. Arbuscular Mycorrhizae Mitigate Aluminum Toxicity and Regulate Proline Metabolism in Plants Grown in Acidic Soil. Journal of Fungi. 2021 Jul 1;7(7).

11. Liu W., Zhang Y., Jiang S., Deng Y., Christie P., Murray PJ., et al. Arbuscular Mycorrhizal Fungi in Soil and Roots Respond Differently to Phosphorus Inputs in an Intensively Managed Calcareous Agricultural Soil. Sci Rep. 2016 Apr 22;6.

12. Miao F., Wang S., Yuan Y., Chen Y., Guo E., Li Y. The Addition of a High Concentration of Phosphorus Reduces the Diversity of Arbuscular Mycorrhizal Fungi in Temperate Agroecosystems. Diversity (Basel). 2023 Oct 1;15(10).

13. Nogueira MA., Magalhães GC., Cardoso EJBN. Manganese Toxicity in Mycorrhizal and Phosphorus-Fertilized Soybean Plants. J Plant Nutr. 2004;27(1):141–56.

14. Nogueira MA., Cardoso EJBN. Eficiência Micorrízica e Toxidez de Manganês em Soja em Função do Tipo de Solo e da Espécie do Endófito. Sci Agric. 2003 Apr 1;60(2):329–35.

15. Rouphael Y., Franken P., Schneider C., Schwarz D., Giovannetti M., Agnolucci M., et al. Arbuscular Mycorrhizal Fungi act as Biostimulants in Horticultural Crops. Vol. 196. Scientia Horticulturae. Elsevier; 2015. p. 91–108.

16. OECD. Test No. 232: Collembolan Reproduction Test in Soil. Paris; 2016.

ANNEX A

Definitions

Hyphae: AMF filamentous structures ramifying from the spore after its germination.

ICx (concentration for x% inhibition effect): the concentration that causes x% inhibition on the germination of spores when compared to the response on the control.

LOEC (lowest observed effect concentration): the lowest test treatment for which there is an effect on the measured endpoint parameter. Statistically significant differences are observed when comparing the treatment to the control ($p < 0.05$).

NOEC (no observed effect concentration): the highest test treatment for which there is no effect on the measured endpoint parameter, since no statistically significant differences are observed when comparing the treatment to the control ($p < 0.05$).

Spore: AMF spherical structures which store nutrients but can also be dormant structures or propagules for germination and colonization.

Sporocarp: capsules containing clusters of spores.

ANNEX B

Spore extraction

The recommended spore extraction method is based on a wet sieving technique, followed by a sucrose centrifugation after which spores are separated and counted, as described by (Gerdemann & Nicolson (1963):

1. Prepare a Falcon tube with a sucrose gradient: pour 15 ml of 20% sucrose solution in a Falcon tube. Then, dip a glass pipette in the 20% sucrose solution, reaching the bottom of the tube, and add 15 ml of 60% sucrose solution. The 60% sucrose solution must remain in the bottom of the Falcon tube, under the 20% sucrose solution;

2. Vigorously mix a sample of 50 g of inoculant with 2L of tap water in a beaker for 30 seconds. After 2 minutes of sedimentation, sieve the supernatant through stacked sieves of 300 µm (top) and 45 µm mesh (bottom). This step must be repeated twice with the sedimented inoculant;

3. Wash the materials retained on the 45 µm sieve into the Falcon tube containing the sucrose gradient. Centrifuge for 2 minutes at 2000 rpm;

4. Sieve the supernatant using a 45 µm sieve and wash the retained materials with distilled water to remove the sucrose residues;

5. Transfer the retained material (which includes spores) to a watch-glass or Petri dish and select viable spores under the stereo microscope at 50x amplification. Viable spores, with intact walls, are collected and empty or crushed spores are eliminated;

6. Selected spores can be stored, at 4ºC, for two days in microtubes with distilled water until use in pre-symbiotic phase tests.

ANNEX C

Determination of water holding capacity

The method described below for maximum water holding capacity (WHC_{max}) of the soil is based the Annex 5 of the OECD 232 guideline (16).

1. Prepare a tube with a filter paper in the bottom and weight (A);
2. Wet the piece of filter paper and weight (B);
3. Fill the tubes with a soil, without reaching the top of the tubes;
4. Place the tubes in a tray with tall borders;
5. Outside the tubes, put water until the soil is submerged (don't put water directly in the soil, the water must ascent by capillarity).
6. Let it rest, submerged, for three hours;
7. Drain the samples for two hours in a grid. Cover the tubes to avoid water evaporation;
8. Weight the saturated tubes (C);
9. Dry at 105ºC for 14h;
10. Weight (D) and calculate the $\%WHC_{max}$ applying the equation below:

$$\%WHC_{max} = \frac{(C - B) - (D - A)}{D - A} * 100$$

ANNEX D

Determination of soil water content

Soil water content should be measured following the steps below:

1. Weigh an empty vessel (A);
2. Without taring the scale, add approximately 10 g of fresh soil into the vessel and weight (B);
3. Dry the soil at 105ºC for at least 12h;
4. After letting settle down the temperature of the soil (5 to 10 min), weight the dried soil + vessel (C);
5. Calculate de soil water content, in percentage, by following the equation below:

$$\%\, Water\ content = \frac{(B - C)}{(C - A)} * 100$$

ANNEX E

Determination of soil pH

The method described for determination of soil pH is based on the methodology described on the OECD 232 guideline (16):

1. Let the fresh soil air-dry at room temperature for at least 12h;
2. Once the soil is dry, weight 5 g of soil into a container;
3. Add 25 mL of KCl (1M) or $CaCl_2$ (0.01M);
4. Mix for 5 minutes and settle for at least 2h (but not more than 24h);
5. Measure the pH in the liquid phase using a previously calibrated pH-meter.

A.34 Draft guideline for the AMF symbiotic phase tests.

Reproduction of the document prepared following the OECD formatting guidelines.

INTRODUCTION

1. This Test document is designed for assessing the effects of chemicals in soil on the symbiotic phase (intra-radicular colonization and extra-radicular mycelium length) of the development of Arbuscular Mycorrhizal Fungi (AMF). This test guideline has been ring-tested.

2. AMF are organisms that live in obligate symbiosis with most plant species. They have an important role to plants and soil ecology by increasing plant nutrient acquisition and tolerance to several environmental stressors (e.g., metals, pathogens, drought, salinity, and others) and by increasing soil carbon sequestration and soil aggregation and stabilization (1,2).

3. Due to their recognized ecological relevance and their sensitivity to chemicals, AMF have been indicated by the European Food Safety Authority (EFSA) as a potential group of non-target organisms for ecotoxicological testing with Plant Protection Products (PPPs) (2).

4. Despite the lack of published national and international guidelines. the documentation fascicule from the Association Française de Normalisation (AFNOR) for the effects of pollutants on AMF (3) provides useful information for the performance of the present methodology.

PRINCIPLE OF THE TEST

5. The test assesses the effects of the test chemical present in the test soil on the establishment of the symbiosis between the AMF and the host plant through the evaluation of the intra-radicular colonization and the evaluation of the production of extra-radicular mycelium after 56 days of exposure under controlled laboratory conditions.

6. Intra-radicular colonization (i.e. percentage of total and arbuscular colonization) is quantified through observation, at the microscope, of AMF colonizing structures on previously stained root fragments collected from the replicates after the exposure period. The length of extra-radicular hyphae produced during the exposure period is evaluated through a grid line intersection method after observation, on the microscope, on filtered soil samples collected from the replicates.

7. The intra-radicular colonization and extra-radicular mycelium production observed on the treatments is compared to the response on the negative control to determine the no-observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). When possible, effective concentrations (ECx; e.g. EC_{10}, EC_{50}) may be estimated through the fitting in non-linear regression models.

INFORMATION ON THE TEST CHEMICAL

8. Information on the test chemical such as common name, chemical name (preferably IUPAC name), structural formula, CAS registry number, purity, safety precautions, proper storage conditions and analytical methods should be obtained before the beginning of the study. In addition, the following information should be known:
   - Solubility in water and/or in organic solvents;
   - Octanol-water partition coefficient, K_{ow};
   - Soil-water partition coefficient, expressed as K_{oc};
   - Vapour pressure;
   - Degradability (e.g. in soil, water);
   - Known metabolites.

REFERENCE SUBSTANCE

9. The recommended reference substance on AFNOR (2014) (3) is cadmium nitrate ($CdNO_3 \cdot 4H_2O$). This substance caused a 20% effect on *Glomus (=Funneliformis) mosseae* mycorrhization frequency at concentrations between 1.6 and 4.1 mg kg^{-1}.

VALIDITY OF THE TEST

10. For the test to be valid, the following criteria apply:
    - Percentage of total intra-radicular colonization higher than 30%, on average, in the replicates of the negative control;
    - Percentage of total intra-radicular colonization of 0% in replicates of sterilization control;
    - Coefficient of variation in total intra-radicular colonization lower than 50% in replicates of the negative control;
    - A minimum length of roots to prepare at least one preparation with 10 fragments of root (of approximately 1 cm) must be found in at least 80% of the replicates of the negative control.

DESCRIPTION OF THE TEST

Test vessels

11. Cylindrical vessels made of non-toxic plastic with capacity for 200 g of soil dry weight (dw) can be used for the replicates. Vessel size should allow good development of the root. The vessels should not be transparent to avoid biofilm production during the test.

Host plant

12. The selected host plant should be known for its capacity to form symbiosis with AMF and for its ability to thrive under laboratory conditions. Some common host plants are *Allium ampeloprasum/porrum*, *Zea mays* and *Glycine max*. A list of other possible host plant species is presented in Annex B.

13. Seeds of the host plant should be from reliable seed sources and produce consistent and regular germination. and uniform plant growth. It is recommended to use uncoated

    seeds (i.e. free of any plant protection product [PPP]), unless this is in the scope of the test.

AMF species and inoculant selection

14. The selected AMF species should have known capacity to colonize the selected host plant. Common selected species are *Rhizophagus irregularis*, *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Rhizophagus intraradices*; other possible AMF species are recommended in Annex C.

15. AMF inoculant can be obtained from pure laboratory cultures (Annex D) or purchased from certified AMF germplasm banks. An appropriate inoculant should contain, at least, 1 spore g^{-1} of inoculant.

16. Before the definitive test, the performance of an inoculum potential test (Annex E) is recommended to assess the suitability of the inoculant to validate the test according to the validity criteria defined in paragraph 10.

Test soil

17. Test soils (either natural or artificial soil) should allow the good development of the host plant and the establishment of the symbiosis with the AMF. Soils with high contents of phosphorus, aluminum, and manganese are not recommended, as these nutrients are known to limit the symbiosis plant-AMF (Alotaibi et al., 2021; Liu et al., 2016; Miao et al., 2023; Nogueira et al., 2004; Nogueira & Cardoso, 2003; Rouphael et al., 2015).

18. Test soils should be characterized prior to their use in the test with, at least, the following information: collection site (for natural soils), origin of the constituents (for artificial soil), pH, organic carbon content, particle size distribution (% sand, silt, and clay), maximum water holding capacity ($WHC_{max\ soil}$, Annex F), % water content (WC, Annex G) and phosphorous, aluminum and manganese content.

19. Natural soils from unpolluted sites can be used as test soil. Agricultural soils should be free of PPPs, manure from treated animals and organic fertilizers for at least one year prior to use in tests. Natural soils must be sieved at 2 mm to homogenize the soil and remove coarser particles. Natural soils can be stored at room temperature, for the shorter time possible before use in tests.

20. Modified artificial soil, with reduced organic matter content, can be prepared based on the information presented in the guideline OECD 232 (10):

Soil component	Content expressed on % dry mass basis
- *Sphagnum* peat (with no visible plant remains, use mature peat, air dried and sieved at 2 mm)	2.5
- Kaolin clay (kaolinite content preferably above 30%)	20
- Industrial quartz sand (air dried, with more than 50% of the particles between 50 and 200 microns)	77.5
- Calcium carbonate ($CaCO_3$. pulverized, analytical grade) to obtain an initial pH of 6.0 ± 0.5	< 1

Note 1: The amount of $CaCO_3$ required depends on its constituents and should be determined by measuring the pH of pre-incubated moist soil sub-samples before the test.

Note 2: Tropical artificial soil (TAS) (11) can be applied in this methodology by replacing the *Sphagnum* peat with coconut fiber on the preparation of the artificial soil.

Preparation of test concentrations

21. Soil contamination methods depend on the characteristics of the test chemical and on the aim of the study. Generally, the test chemical is homogenized into the soil but other modes of application (e.g. spraying) can be performed if required.

Contaminating soil with water soluble test chemicals

22. A stock solution is prepared by completely dissolving the test chemical in deionized water. The stock solution is mixed with an amount of deionized water to adjust soil moisture to 40% to 60% of the $WHC_{max\ soil}$. The mixture of stock solution and deionized water is homogenized with the portion of test soil needed for each treatment, before distributing the soil by the replicates.

Contaminating soil with water insoluble test chemicals

23. Water insoluble chemicals require the coating of an aliquot of test soil: an oven-dried (70ºC, overnight) portion of soil is coated with a solution of the test chemical dissolved in an appropriate organic solvent (e.g. acetone) and left to evaporate completely before being mixed with the remaining test soil. Deionized water is added to adjust the final moisture of the soil to be between 40% and 60% of the $WHC_{max\ soil}$.

24. When an organic solvent is used, the preparation of a solvent control is recommended.

Application of test chemical on the soil surface

25. The test chemical may be applied directly on the surface of the replicates, which are previously prepared according to paragraphs 39 and 40. The test chemical is applied to

the surface of the soil as evenly as possible using a suitable laboratory-scale spraying device to simulate spray application in the field.

TEST PROCEDURES

Preparation of test components

26. A recommended test timeline is presented in Annex I.

27. Before the test setup, test soil and covering material (coarse sand or glass beads with size > 2 mm) are autoclaved twice, with at least one night of interval between cycles, at 121ºC and 1.5 atm for 60 minutes. The soil and covering materials are aerated at room temperature for 5 to 7 days before test setup. Alternatively, gamma arrays can be used to sterilize the test soil and covering material.

28. Host plant seeds are disinfected with a 1% sodium hypochlorite solution, washed with deionized water and soaked in a paper filter with deionized water 2 to 4 days before the test setup. Turgid seeds must be used immediately before germination (AMF root colonization may be reduced when seedlings with radicles are used). At least 3 seeds should be sown in each test replicate.

29. The maximum water holding capacity of the mixture soil + inoculant ($WHC_{max\ soil+inoculum}$) should be assessed prior to the test setup, using the proportion inoculum:soil 2:8 (*w*:*w*) used in the upper soil layer of the test replicates (paragraph 39).

Test treatments

30. A negative control consisting of replicates with the test soil without the test chemical. with inoculant and host plant seeds are prepared for all tests.

31. A sterilization control should be prepared to confirm that no active propagules remain in the soil after sterilization. The replicates are composed of non-contaminated test soil and host plant seeds. Note that no inoculant must be included.

32. A solvent control is recommended in tests with test chemicals that are water insoluble. The replicates are composed of test soil moistened with the organic solvent used in soil spiking (see paragraph 23) without test chemical. These replicates also include the inoculant and host plant seeds.

33. Soil water content (Annex G) and soil pH (Annex H) should be determined at the beginning and at the end of the test in a sample of control soil and a sample of each test concentration. Soil samples for chemical determination can be collected if needed according to the objective of the study.

Range-finding test

34. A range-finding test could be necessary to help select appropriate test concentrations for the definitive test. Three replicates can be used per treatment and the intra-radicular

colonization is assessed after 8 weeks after the emergence of 70% of the control replicates.

35. For the range-finding test, the test concentrations should be widely spaced (e.g. 0.1, 1.0, 10, 100 and 1000 mg/kg dry soil). Other concentrations may be used if supported by existing data.

Definitive test concentrations

36. Test concentrations can be based on the results observed on the range-finding test or on data from literature, which should be reported in any case.

37. The concentrations should not affect significantly the development of the host plant as this may indirectly affect the development of the symbiosis AMF-plant.

Test procedure

38. The symbiosis AMF-host plant is exposed to the test chemical during 56 days after the emergence of, at least, 70% of the negative control replicates.

39. At least 6 replicates are prepared per treatment and control. The replicates are composed of two layers: the bottom layer with 100 g dw of soil (uncontaminated for control or contaminated for test treatments other than control) and the top layer composed by 100 g dw of mixture of AMF inoculant + soil (uncontaminated or contaminated) in a proportion of 2:8 (*w*:*w*).

40. At least 3 pre-soaked turgid seeds (paragraph 28), with no evidence of germination, are sown on the top layer of each replicate at 0.5 cm depth. The first emerged plant must remain in the replicate until the end of the test. Other emerged plants are removed by pulling them to remove the root and avoid resprouting (if more than one plant emerged at the same time, the plant with a development state more similar to the plants of the other replicates of the same treatment is selected).

41. The replicates are covered with autoclaved covering material (coarse sand or glass beads with size > 2 mm) to reduce water evaporation during the test.

Watering and nutrient supplementation

42. Soil water content is adjusted twice a week on a weight basis: deionized water is added to compensate water losses when the weight loss is higher than 2% (weight resulting from plant growth is neglected).

43. At weeks 1, 3 and 5 after germination of at least 70% of negative control replicates, 5 mL of Long Ashton nutrient solution (Annex J) are applied in each replicate.

Test conditions

44. Tests are incubated under a controlled temperature of 23ºC ± 2ºC, 16h:8h light:dark photoperiod and a light intensity of 1000 to 4000 lx. Air relative moisture should be maintained between 40% and 60% (higher air moisture reduces water loss in the replicates but can also promote biofilm production).

45. Replicates position should be randomized at least once a week to reduce variability in plant development due to differences in the test conditions inside the incubation space.

Test measurements

46. At the end of the exposure period (56 days), replicates are individually disassembled by removing the coverage material and pouring the replicate content in a tray.

47. Samples for final pH determination (and, if necessary, for chemical determination) should be collected from the bottom layer in each treatment. Samples for final WC determination should be collected from each replicate after homogenizing the two soil layers.

48. Host plant roots are collected and washed in a fine sieve (≤ 1mm) using deionized water. If necessary, roots can be dried with paper towel and weighted to obtain fresh root biomass. Roots can be stored in deionized water for a maximum of 5 days before being stained.

49. Shoots can also be collected, and fresh and/or dry biomass can be assessed (to obtain dry biomass the shoots must be oven-dried at 60ºC for 48h). Signs of phytotoxicity observed during the test must be recorded.

50. A sample of at least 10 g of homogenized soil (fresh weight, fw) is collected for evaluation of extra-radicular hyphae length. This sample can be stored at -20ºC for a maximum of 6 months until assessment of this endpoint parameter.

Root staining and intra-radicular colonization assessment

51. The assessment of intra-radicular colonization requires the previous staining of the AMF inside the roots collected at the end of the test. The staining process includes (i) root clearing in KOH; (ii) root acidification and (iii) staining of AMF structures with aqueous pen ink. Detailed steps for the recommended staining process are presented in Annex K.

52. The assessment of intra-radicular colonization is based on the presence/absence of mycorrhizal structures in root fragments observed under a microscope (Annex K). The percentage of total colonization is determined by the presence/absence of any AMF structures (i.e. hyphae, vesicles, spores and/or arbuscules) in fragments of root from the replicate. The percentage of arbuscular colonization is determined by the presence/absence of arbuscules in the same root fragment.

Extra-radicular mycelium production assessment

53. The extraction of the extra-radicular mycelium is made through successive washing and filtering steps of the soil collected in paragraph 50. Materials retained in a sieve are

filtered in a vacuum pump through a cellulose nitrate membrane with a grid. The number of intersections of the extra-radicular hyphae with a gridded Eye piece Whipple disc in the microscope are counted in random sampling points of the membrane. Detailed steps for the recommended methodology and the equation to estimate the length of produced mycelium are presented on Annex L.

DATA AND REPORTING

Data treatment

NOEC/LOEC

54. The no-effect observed concentration (NOEC) and the lowest observed effect concentration (LOEC) are estimated through the evaluation of significant differences of treatments when compared to the controls. considering $p < 0.05$.

55. Data normal distribution and homogeneity must be tested using an appropriate statistical test, e.g. the Shapiro-Wilk test and Bartlett test, respectively, before statistical analysis. One-way ANOVA and subsequent multi-comparison tests can be performed. Multiple comparisons (e.g. Dunnett's test) can be used to calculate if there are significant differences ($p \leq 0.05$) between the controls and the treatments. Non-parametric methods (e.g. Bonferroni-U-test) could also be applied for NOEC/LOEC estimation.

ECx

56. Effect concentration for x% of reduction on a specific parameter when compared to the respective control (and its 95% confidence intervals) is estimated through non-linear regression models (e.g. logistic model, exponential model, Hormesis model).

Test report

57. The test report must contain the following information:

Test chemical

- Data from previous works, if available;
- Physical and chemical characterization of the test chemical;
- Details on soil contamination and verification of the tested concentrations, if needed.

Inoculant and host plant

- Scientific name, strain/variety, source.

Test conditions

- Description of the test design and procedures;
- Description of environmental conditions during the exposure period (temperature, photoperiod, % relative humidity, etc.);
- Nominal test concentrations;
- Characterization of the test soil.

Results

- Percentage of total and arbuscular colonization;
- Extra-radicular hyphae length;
- Additional parameters *(optional)*;
- Methods for statistical analysis;
- Compliance with the validity criteria;
- Further observations.

LITERATURE

1. Chen M., Arato M., Borghi L., Nouri E., Reinhardt D. Beneficial Services of Arbuscular Mycorrhizal Fungi – From Ecology to Application. Vol. 9. Frontiers in Plant Science. Frontiers Media S.A.; 2018.

2. EFSA, Scientific Opinion Addressing the State of the Science on Risk Assessment of Plant Protection Products for In-soil Organisms. EFSA Journal, 2017 Feb 1;15(2).

3. AFNOR, Qualité du sol - Effets des Polluants Vis-à-vis des Champignons Mycorhizogènes - Partie 2 : Essai de Mycorhization Racinaire. Normes nationales et documents normatifs nationaux. Association Française de Normalisation; 2014.

4. Liu W., Zhang Y., Jiang S., Deng Y., Christie P., Murray PJ., et al. Arbuscular Mycorrhizal Fungi in Soil and Roots Respond Differently to Phosphorus Inputs in an Intensively Managed Calcareous Agricultural Soil. Sci Rep, 2016 Apr 22;6.

5. Miao F., Wang S., Yuan Y., Chen Y., Guo E., Li Y. The Addition of a High Concentration of Phosphorus Reduces the Diversity of Arbuscular Mycorrhizal Fungi in Temperate Agroecosystems. Diversity (Basel), 2023 Oct 1;15(10).

6. Nogueira MA., Cardoso EJBN. Eficiência Micorrízica e Toxidez de Manganês em Soja em Função do Tipo de Solo e da Espécie do Endófito. Sci Agric, 2003 Apr 1;60(2):329–35.

7. Nogueira MA., Magalhães GC., Cardoso EJBN. Manganese Toxicity in Mycorrhizal and Phosphorus-Fertilized Soybean Plants. J Plant Nutr, 2004;27(1):141–56.

8. Alotaibi MO., Saleh AM., Sobrinho RL., Sheteiwy MS., El-sawah AM., Mohammed AE., et al. Arbuscular Mycorrhizae Mitigate Aluminum Toxicity and Regulate Proline Metabolism in Plants Grown in Acidic Soil. Journal of Fungi, 2021 Jul 1;7(7).

9. Rouphael Y., Franken P., Schneider C., Schwarz D., Giovannetti M., Agnolucci M., et al. Arbuscular Mycorrhizal Fungi Act as Biostimulants in Horticultural Crops. Vol. 196. Scientia Horticulturae, Elsevier; 2015, p. 91–108.

10. OECD, Test No. 232: Collembolan Reproduction Test in Soil. Paris; 2016.

11. Garcia M, Effects of Pesticides on Soil Fauna: Development of Ecotoxicological Test Methods for Tropical Regions. Cuvillier; 2004. 281 p.

12. Brundrett M., Bougher N., Dell B., Grove T., Malajczuk N. Working with Mycorrhizas in Forestry and Agriculture. Australian Centre for International Agricultural Research, 1996.

13. Kormanik P., McGraw A. Quantification of Vesicular-Arbuscular Mycorrhizal in Plant Roots. Schenck. NC. Ed. Methods and Principles of Mycorrhizal Research, American Phytopathological Society, St Paul, 1982;37–45.

14. Vierheilig H., Coughlan AP., Wyss U., Piché Y. Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi [Internet]. Vol. 64. APPLIED AND ENVIRONMENTAL MICROBIOLOGY, 1998. Available from: https://journals.asm.org/journal/aem

15. Phillips JM., Hayman DS. Improved Procedures for Clearing Roots and Staining Parasitic and Vesicular-arbuscular Mycorrhizal Fungi for Rapid Assessment of Infection. Transactions of the British Mycological Society [Internet], 1970 Aug; 55(1):158-IN18. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0007153670801103

16. Gerdemann JW. Relation of a Large Soil-Borne Spore to Phycomycetous Mycorrhizal Infections. Mycologia, 1955 Sep;47(5):619–32.

17. McGonigle TP., Miller MH., Evans DG., Fairchild GL., Swan JA. A New Method Which Gives an Objective Measure of Colonization of Roots by Vesicular-arbuscular Mycorrhizal Fungi. New Phytol, 1990.

18. Melloni RA. Quantificação do Micélio Extrarradicular de Fungos Micorrízicos Arbusculares em Plantas Cítricas. [Piracicaba]: Escola superior de Agricultura “Luiz de Queiroz”, Universidade de São Paulo; 1996.

19. Melloni R., Cardoso EJBN. Quantificação de Micélio Extrarradicular de Fungos Micorrízicos Arbusculares em Plantas Cítricas e Endófitos: I. Método empregado. Rev Bras Cienc Solo, 1999 Mar;23(1):53–8.

20. Berbara RLL., Souza FA., Fonseca HMAC. Fungos Micorrízicos Arbusculares: Muito Além da Nutrição. FERNANDES. M S (Ed) Nutrição mineral de plantas Viçosa: SBCS, 2006 p 53-88. 2006.

21. Cardoso-Filho JA. Quantificação do Micélio Extramatricial de Glomus etunicatum e da Sua Atividade, em Simbiose com o Milho. [Piracicaba]: Escola superior de Agricultura “Luiz de Queiroz”. Universidade de São Paulo; 1994.

22. Nogueira AN. Colonização Radicular de Produção de Micélio Externos por Duas Espécies de Fungos Micorrízicos Arbusculares em Soja Aubmetida a Doses de Fósforo. [Piracicaba]: Escola superior de Agricultura “Luiz de Queiroz”. Universidade de São Paulo; 1997.

ANNEX A

Definitions

Arbuscules: tree-shaped structures growing inside the root cells. Arbuscules are the AMF structure where nutrient exchange between the AMF and host plant occurs.

ECx (effect concentration for x% effect): the concentration that causes x% effect on the assessed endpoint parameter when compared to the response on the control.

Hyphae: AMF filamentous structures ramifying through the root cortex (intra-radicular hyphae) or though the soil (extra-radicular hyphae). Their main functions are nutrient acquisition and propagation of the colonization.

Inoculant: mixture of the mixed inoculum and the culture medium. The inoculant is used as source of mixed inoculum for an experiment.

Inoculum potential: capacity of an inoculant to promote symbiosis establishment and, therefore, root colonization.

LOEC (lowest observed effect concentration): the lowest test treatment for which there is an effect on the measured endpoint parameter. Statistically significant differences are observed when comparing the treatment to the control ($p < 0.05$).

Mixed inoculum: a mixture with more than one source of AMF propagules.

NOEC (no-observed effect concentration): the highest test treatment for which there is no effect on the measured endpoint parameter, since no statistically significant differences are observed when comparing the treatment to the control ($p < 0.05$).

Propagules: AMF spores, arbuscules, hyphae and vesicles.

Spores: AMF spherical structures forming from hyphae in the root or in the soil. Spores store nutrients but can also be used as dormant structures or propagules for further germination and colonization.

Vesicles: AMF structures formed from hyphae where the nutrients acquired by the symbiosis are stored.

ANNEX B

Host plant species used on ecotoxicological studies with AMFs according to published literature

Allium cepa
Allium ampeloprasum/porrum
Anthylis vulneraria sub sp. Sampaiana (transformed roots)
Arachis hipogea
Avena sativa
Bromus tectorum
Capsicum annum
Cichorium intybus
Cichorium intybus (transformed roots)
Citrus aurantium
Cucumis sativus
Daucus carota
Daucus carota (transformed roots)
Glycine max
Lactuca sativa
Leucaena leucocephala
Lolium multiflorum
Lotus tenuis
Medicago truncatula
Paspalum notatum
Phaseolus vulgaris
Pisum sativum
Schizolobium parahyba var amazonicum
Sesamum indicum
Sitanion hystrix
Solanum lycopersicum
Trifolium repens
Trifolium subterraneum

Triticum aestivum
Xanthium strumarium
Zea mays

ANNEX C

Arbuscular mycorrhiza fungi species used on ecotoxicological studies according to published literature

Acaulospora sp.
Acaulospora laevis
Claroideoglomus spp.
Claroideoglomus claroideum
Dentiscutata nigra
Funneliformis caledonium
Glomus caledonoim = F. caledonium
Funneliformis monosporum
Funneliformis mosseae
Glomus mosseae = F. mosseae
Gigaspora spp.
Gigaspora albida
Gigaspora decipiens
Gigaspora margarita
Gigaspora rosea
Glomus sp.
Glomus aggregatum
Glomus etunicatum = Claroideoglomus etunicatum

Glomus fascicularum
Glomus geosporum
Glomus leptotichum
Glomus monosporum
Paraglomus brasilianum
Paraglomus occultum
Glomus occultum = P. occultum
Glomus clarum = Rhizophagus clarus
Rhizoglomus clarum = Rhizophagus clarus
Rhizophagus intraradices
Glomus intraradices = R. intraradices
Rhizophagus irregularis
Glomus irregularis = R. irregularis
Scutellospora heterogama
Gigaspora calospora = S. heterogama
Native AMF species from cultivated fields, pastures, and grassland

ANNEX D

Production of AMF inoculant under laboratory conditions

AMF inoculant for the performance of symbiotic phase tests can be produced though multiplication of the selected AMF in plant pots under laboratory conditions. It is recommended to, at least initially, acquire mixed inoculum from an entity that ensures the purity of the AMF species and the viability of propagules. The production of AMF mixed inoculum is established for 120 days.

1. A substrate composed by a mixture of a suitable natural soil and fine sand (1:1; pH adjusted to 6.0 ± 0.5) is autoclaved at 121ºC, 1.5 atm for 60 minutes. After 5-7 days of aeration, the substrate is moistened to 60-70% WHC_{max} and transferred into plant pots with capacity for 3 kg of soil;

2. 30 mL of mixed inoculum containing viable propagules are deposited above the substrate and 10 mL of seeds of a host plant, previously disinfected in 1% sodium hypochlorite for 5 minutes, are placed on top of the substrate. Suggested host plants include C4 grasses, legumes and perennial or long-lived annuals. The number of seeds may vary depending on the host plant; however, competition between plants on the culture pot should be avoided;

3. Over the mixed inoculum and the seeds, a thin layer of the substrate should be placed, and above this, a thin layer of sterile covering material (~1 cm of coarse sand or glass beads, with size > 2 mm);

4. Culture vessels are incubated at 23ºC ± 2ºC, 16h:8h light:dark photoperiod and a light intensity of 1000 to 4000 lx. Air relative moisture should be maintained between 40% and 60%. Other culture conditions may be used according to the optimal conditions for the development of the selected host plant;

5. After 90-100 days, approximately 50 g of culture substrate can be sampled to monitor the AMF multiplication and production of new spores. If new spores' production is observed, watering is suspended until complete senescence of the host plants;

6. On day 120, the culture substrate and the roots of the host plants are collected. A new sample of culture substrate is monitored for quantification of spores and verification of the presence of contamination (i.e. presence of spores other than the AMF species of interest). Intra-radicular colonization can also be assessed. Contaminated substrate must be discarded. Uncontaminated substrate should be stored in plastic bags with approximately 20% moisture, identified with the species name and number of spores per g of soil, and stored in a refrigerator at 4ºC until use.

Each new batch of inoculant should be characterized before use in tests. Only inoculants with at least 1 spore g^{-1} of inoculant are suitable for testing.

ANNEX E

Assessing the inoculum potential of an inoculant

Inoculum potential is evaluated through the assessment of intra-radicular colonization after 28 days of incubation of different proportions inoculant:soil in containers with seeds of the host plant. The performance of an inoculum potential test is essential to confirm if the inoculant has sufficient propagules to be used in the tests or if it must be improved or multiplied according to the procedures described in Annex D before being used in the tests.

1. Test soil (artificial or natural soil) and covering materials (coarse sand or glass beads with size > 2 mm) are sterilized by two cycles of 60 minutes at 121ºC and 1.5 atm (resting overnight between cycles). The autoclaved soils and covering materials are aerated at room temperature for 5-7 days before being used. Alternatively, soils and covering materials can be sterilized through gamma arrays.

2. Host plant seeds are disinfected by immersion in a 1% sodium hypochlorite solution for 5 minutes and afterwards washed with deionized water. Disinfected seeds are placed in filter paper moistened with deionized water in Petri dishes for 2 to 4 days before the beginning of the test (only turgid seeds with no evidence of germination should be sown in the test);

3. Five replicates should be prepared for each inoculant:soil mixture. In each test container, add 100 g dw of soil with the moisture adjusted to 50% $WHC_{max\ soil}$ in the bottom and, at the top, a mixture of 100 g of soil + inoculant in different proportions. Table 1 presents a suggested gradient of mixtures;

4. Disinfected seeds are buried at 0.5 cm depth. Then, the replicates' surface is covered with autoclaved covering material.

The test has a duration of 4 weeks (12) starting after at least one plant has germinated in no less than 70% of the replicates. Only one plant must remain per replicate to the end of the test (later emerged seeds are extracted by pulling them to remove the root and avoid resprouting).
Replicates' moisture is corrected twice a week, on a weight basis, by adding deionized water. 5 mL of Long Ashton solution (Annex J) can be added 5 to 7 days after the test beginning.
After 28 days, the percentage of total intra-radicular colonization is evaluated in each replicate following the procedures described in Annex K.

Table 1. Suggested proportions of test soil and inoculant for the performance of a potential of inoculum test.

Bottom layer	Top layer	
Soil (g dw)	Soil (g dw)	Soil/substrate inoculant (g dw)
100	100	0
100	95	5
100	90	10
100	80	20
100	70	30
100	60	40
100	50	50

ANNEX F

Determination of water holding capacity

The method described below for maximum water holding capacity (WHC_{max}) of the soil (or mixture inoculant + soil) determination is based the Annex 5 of the OECD 232 guideline (10).

1. Prepare a tube with filter paper in the bottom and weight (A);
2. Wet the piece of filter paper and weight (B);
3. Fill the tubes with soil (or with a mixture of soil and inoculum, as applicable), without reaching the top of the tubes;
4. Place the tubes in a tray with tall borders;
5. Outside the tubes, put water until the soil is submerged (do not put water directly in the soil, the water must ascent by capillarity);
6. Let it rest, submerged, for three hours;
7. Drain the samples for two hours in a grid. Cover the tubes to avoid water evaporation;
8. Weight the saturated tubes (C);
9. Dry at 105ºC for 14h;
10. Weight (D) and calculate the $\%WHC_{max}$ applying the equation below:

$$\%WHC_{max} = \frac{(C - B) - (D - A)}{D - A} * 100$$

ANNEX G

Determination of soil water content

Soil water content should be measured following the steps below:

1. Weigh an empty vessel (A);
2. Without taring the scale, add approximately 10 g of fresh soil into the vessel and weight (B);
3. Dry the soil at 105ºC for at least 12h;
4. After letting settle down the temperature of the soil (5 to 10 min), weight the dried soil + vessel (C);
5. Calculate de soil water content, in percentage, by following the equation below:

$$\%\ Water\ content = \frac{(B - C)}{(C - A)} * 100$$

ANNEX H

Determination of soil pH

The method described for determination of soil pH is based on the methodology described on the OECD 232 guideline (10):

1. Let the fresh soil air-dry at room temperature for at least 12h;
2. Once the soil is dry, weight 5 g of soil into a container;
3. Add 25 mL of KCl (1M) or $CaCl_2$ (0.01M);
4. Mix for 5 minutes and settle for at least 2h (but not more than 24h);
5. Measure the pH in the liquid phase using a previously calibrated pH-meter.

ANNEX I

Suggested timeline for AMF symbiotic phase testing

Time (days)	Action
-14 days before test setup	- Prepare artificial soil (if applicable)
-7 to -5 days before test setup	- Autoclave test soil and covering material - Measure $WHC_{max\ soil}$ and $WHC_{max\ soil+inoculum}$
-4 or -2 days before test setup	- Sterilize host plant seeds - Prepare test materials
-1 day before test setup	- Oven-dry, at 70ºC, soil aliquots for contamination (if applicable)
Test setup	- Follow *Test Procedures* - Assess initial WC and pH - Sample for chemical analysis (if applicable)
Day 0	- Observe the germination of at least one seed on 70% of the negative control replicates - Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 4	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 7	- Watering with 5 ml of Long Ashton nutrient solution per replicate - Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 11	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 14	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 18	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 21	- Watering with 5 ml of Long Ashton nutrient solution per replicate - Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 25	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 28	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 32	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%

Time (days)	Action
Day 35	- Watering with 5 ml of Long Ashton nutrient solution per replicate - Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 39	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 42	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 46	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 49	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 53	- Reestablish replicates initial weight by addition of deionized water when weight loss >2%
Day 56	- End of test (collect roots, shoots and soil samples, if applicable) - Assess final pH per treatment and WC per replicate - Sample for chemical analysis (if applicable)
After 56 days test	- Evaluate test endpoints

ANNEX J

Composition of 1L of a Long Ashton nutrient solution with low P (10% P (9.3 mg P kg^{-1} soil)

The Long Ashton nutrient solution is prepared based on the mixture of 4 stock solutions (macronutrients, micronutrients, molybdenum, and iron solutions) and deionized water. The Long Ashton nutrient solution can be stored at 4ºC in the dark and used within 9 months.

Component		Stock solution (g L^{-1})	Volume of stock solutions for 1 L of fertilizer solution
Macronutrients			**100 mL**
KNO_3	Potassium nitrate	4.04	
$Ca(NO_3)_2•4H_2O$	Calcium nitrate tetrahydrate	9.44	
$NaH_2PO_4•4H_2O$	Sodium Dihydrogen Phosphate Tetrahydrate	1.84	
$MgSO_4•7H_2O$	Magnesium sulfate heptahydrate	3.68	
Micronutrients			**10 mL**
$MnSO_4•H_2O$	Manganese (II) sulfate monohydrate	1.68	
$CuSO_4•5H_2O$	Copper sulfate pentahydrate	0.25	
$ZnSO_4•7H_2O$	Zinc sulfate heptahydrate	0.29	
H_3BO_3	Boric acid	2.23	
Molybdenum			**1 mL**
$(NH_4)_6O_{24}Mo_7•4H_2O$	Ammonium molybdate tetrahydrate	0.35	
Iron			**10 mL**
$FeSO_4•7H_2O$	Iron (II) sulfate heptahydrate	3.05	
Ferric sodium EDTA	Ethylenediaminetetraacetic acid ferric sodium salt	4.09	
Deionized water			**879 mL**

ANNEX K

Root staining and assessment of AMF intra-radicular colonization

Root staining

Host plant roots collected at the end of the test should be stained to allow visualization of AMF intra-radicular structures. The recommended staining methods are adapted from (13–16).
Thiner roots should be selected, and old lignified roots rejected. Staining procedures work better with small amounts of roots (< 2 g). The staining of AMF structures inside the roots comprises 3 steps: root clearing, root acidification and staining of AMF structures, which must be followed sequentially.

Root clearing

1. Wash the roots collected from the replicate with tap water to remove soil and organic residues;

2. Coarsely cut the roots and place the fragments in an Erlenmeyer flask or other type of vessel;

3. Fully immerse the roots in 10% KOH overnight (~14 hours) at room temperature. For thicker roots, the 10% KOH solution may be changed at least once over this period;

4. Place the flasks in a water-bath at 90°C and incubate for 5-7 minutes. This step removes the cytoplasmic content from the cells. At the end of this period, the solution is expected to present a brownish color. Note that if roots are left in hot KOH for too long, the cortex will disintegrate;

5. Remove the flasks from the water bath and pour the roots and KOH in a fine sieve (mesh ≤ 1 mm). Wash the roots with tap water to remove KOH residue and place the roots back into the empty flasks.

Alternatively, roots can be cleared in a 2.5% KOH solution by placing cassettes with roots in a beaker inside a water bath at 90ºC in a fume cupboard. Periodically, cassettes must be agitated so that the KOH is refreshed inside the cassettes. After 15 minutes the cassettes are drained and washed thoroughly under tap water.

Root acidification

6. Immerse the cleared roots in a 5% acetic acid solution for 50 minutes at room temperature. Acetic acid opens cortex cell membranes, improving the staining process of AMF structures. Alternatively, 4% hydrochloric acid may be used for 60 minutes;

7. Remove the acid solution. There is no need of washing the roots before following to the next steps.

Staining of AMF structures

8. Immerse the roots, inside the flask, in a 1:1 solution of 5% black ink solution (e.g. Parker ink) and 5% acetic acid;

9. Place the flasks in a water bath at 90°C and incubate for 5-7 minutes;

10. Remove the flasks from the water bath;

11. Pour the roots and ink-acetic acid solution in a fine sieve (mesh ≤ 1 mm) so that the roots are retained while the ink-acetic acid solution is removed by runoff;

12. Wash the roots with tap water to remove ink excess

Alternatively, cleared roots inside the cassettes, may be immersed in 0.05% trypan blue at 90ºC in a fume cupboard. Periodically, cassettes must be agitated so that the dye is refreshed inside the cassettes. After 15 minutes, roots must be drained and washed thoroughly under tap water and immersed with acid glycerol (50 mL 1% HCl + 450 mL deionized water + 500 mL glycerol) for 10-20 minutes in a water bath at 90ºC or overnight at room temperature to remove excessive coloration from empty root cells.

Stained roots can be stored, at room temperature, in deionized water for a period not longer than 6 weeks or in acidified glycerol (50 mL 1% HCl + 450 mL deionized water + 500 mL glycerol or 70 mL 5% acetic acid + 230 mL deionized water + 700 mL glycerol, depending on the acid used in the acidification step of the staining process).
It is recommended to let the root destain at least overnight before proceeding for intra-radicular colonization assessment.

Intra-radicular colonization assessment

Evaluation of intra-radicular colonization is based on the observation of the presence/absence of AMF structures in previously stained root fragments from each replicate under microscope.
Total and arbuscular root colonization are determined following the method described by (17). An observation point is considered colonized when there is the presence of at least one type of the four fungal structures: vesicles, spores, arbuscles and/or hyphae (Figure 1).

13. Cut the stained roots collected from the replicate into fragments of approximately 1 cm;

14. Place the fragments on a microscope slide. One slide with 10 root fragments must be prepared for each replicate. When less than 10 root fragments are available, a microscope slide composed by a lower number of fragments is acceptable;

15. Cover the root fragments with a coverslip and gently smash the roots to avoid root cell superposition;

16. Place the microscope preparation under a microscope at 400x magnification (10x ocular and 40x objective);

17. Observe a randomly selected observation point in a root fragment;

18. Observe and record the presence or absence (not the number) of any of the four AMF structures (total colonization) and individually record the presence of arbuscules (arbuscular colonization);

19. Assess 9 more randomly selected observation points in the same root fragment. A total of 10 observation points is assessed per root fragment. Observation points must not overlap;

20. Repeat for all root fragments present in the microscope slide.

Calculation and expression of results

The percentage of total colonization and arbuscular colonization is calculated considering the number of observation points where at least one AMF structure and one arbuscule is observed, respectively. The percentage of colonization (total or arbuscular) on each replicate is calculated through equation (1):

Eq. (1) $$\%\ Colonization\ (total\ or\ arbuscular) = \frac{Nº\ of\ colonized\ observation\ points\ x\ 100}{Nº\ of\ observation\ points}$$

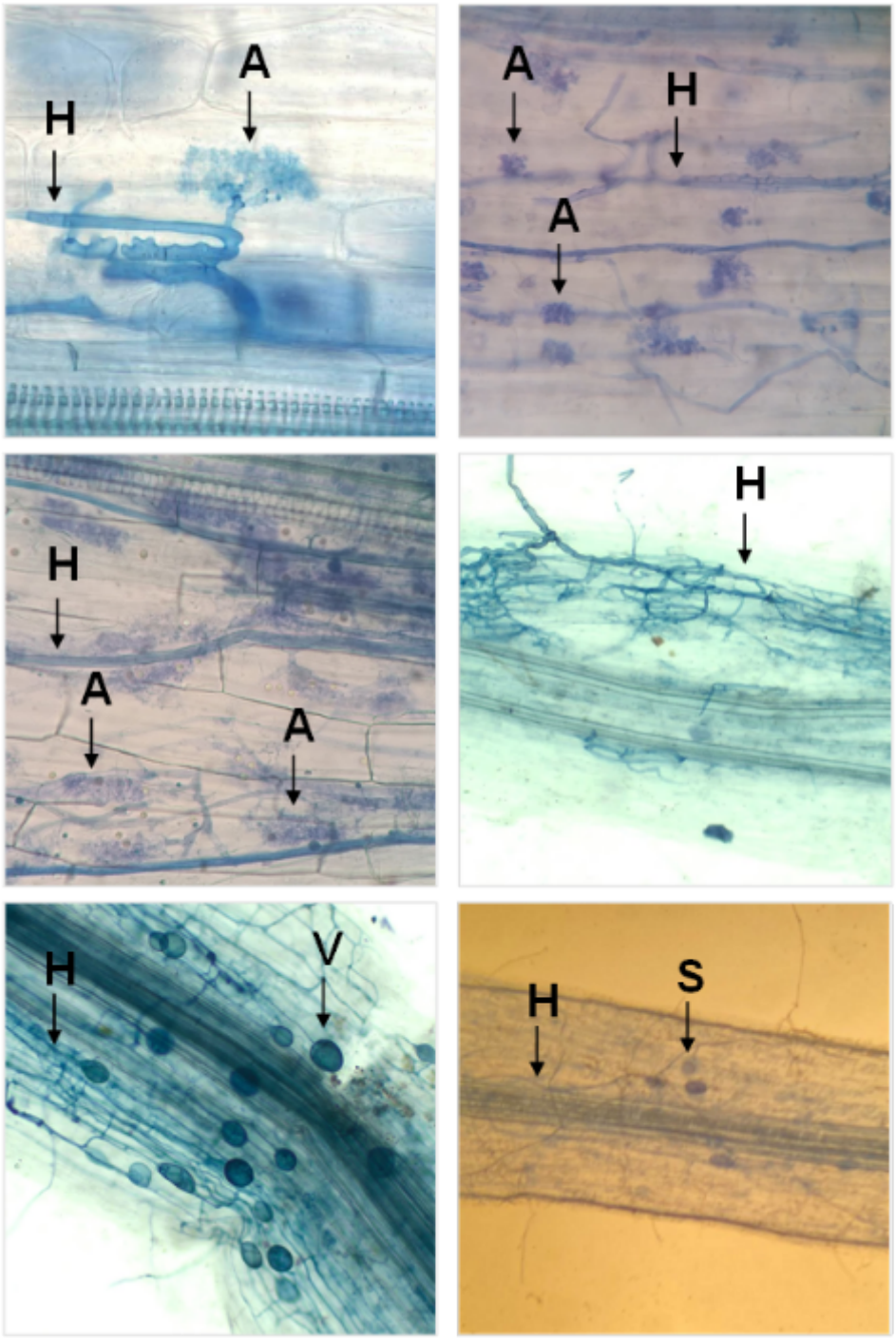


Figure 1. Examples of AMF colonizing structures: (H) hyphae, (V) vesicles, (A) arbuscules and (S) spores. Note that arbuscules, vesicles and immature spores are always connected to hyphae. Mature spores can be separated from hyphae. Credits: Julia dos Santos Ganen, Patrícia Ferreira and Tanel Vahter.

ANNEX L

Extra-radicular mycelium length determination

Extra-radicular hyphae are extracted from soil samples collected from the replicates. These are retained in a cellulose nitrate membrane and measured through a grid line intersect method. The recommended procedures are based on literature (18,19).

Sample preparation

1. Place the soil sample collected at the end of the test (10 g of soil fw, paragraph 50) in a 1 L beaker with 500 ml of tap water;

2. Stir vigorously with a glass rod for 30 seconds and leave to settle down for 1 minute;

3. Stack a 1 mm mesh sieve (top) and a 0.25 mm sieve (bottom) above a 2 L beaker;

4. Sieve the supernatant and collect it in the beaker placed under the sieves;

5. Repeat steps 2 and 4 twice to reach a total sieved supernatant volume of 1.5 L;

6. Stir the 1.5 L of supernatant for 30 seconds in a blender at the lowest speed. Let settle down for 2 minutes;

7. In a cellulose nitrate membrane (4.7 cm diameter, 0.45 µm porosity, 3 mm grid) mark, with a pen, a square of 8 x 8 grid. Place the membrane in the filtration compartment of the vacuum pump;

8. Sieve 500 ml of the supernatant in a 45 µm sieve;

9. With a wash bottle. wash the materials retained on the 45 µm sieve to the filtration compartment of the vacuum pump;

10. Filter the retained materials until there is no water in the compartment with the membrane;

11. The materials retained in the membrane (composed of soil/substrate residue, mycelium, and other particles) are submitted to evaluation under microscope.

Evaluation of extra-radicular hyphae length

12. Air dry the membrane with the filtered material and place it on a glass slide, lubricated with a drop of almond oil to improve visualization at the microscope;

13. Place the preparation under a microscope. Observation with a 400x magnification (10x ocular and 40x objective) is recommended;

14. Place an Eye piece Whipple disc (10 x 10 grid, squares with 1 mm^2) inside the microscope's ocular;

15. Observe and register the number of intersections of the hyphae (see Note 3) with horizontal and vertical lines of the Eye piece Whipple disc grid. Note that when the point of intersections of the hyphae with the grid is the intersection of a vertical and a horizontal line, it only counts as one intersection (Figure 2);

16. Assess one sampling site inside each one of the 64 fields (8 x 8 grid) of the cellulose nitrate membrane;

17. Register the number of intersections observed for each replicate (Figure 3).

Note 3: Hyphae are generally colorless and have a diameter of around 2 µm. Roots are generally thicker (root hairs: Ø 10-20 µm; lateral roots: 100-500 µm) and can be colored (20).

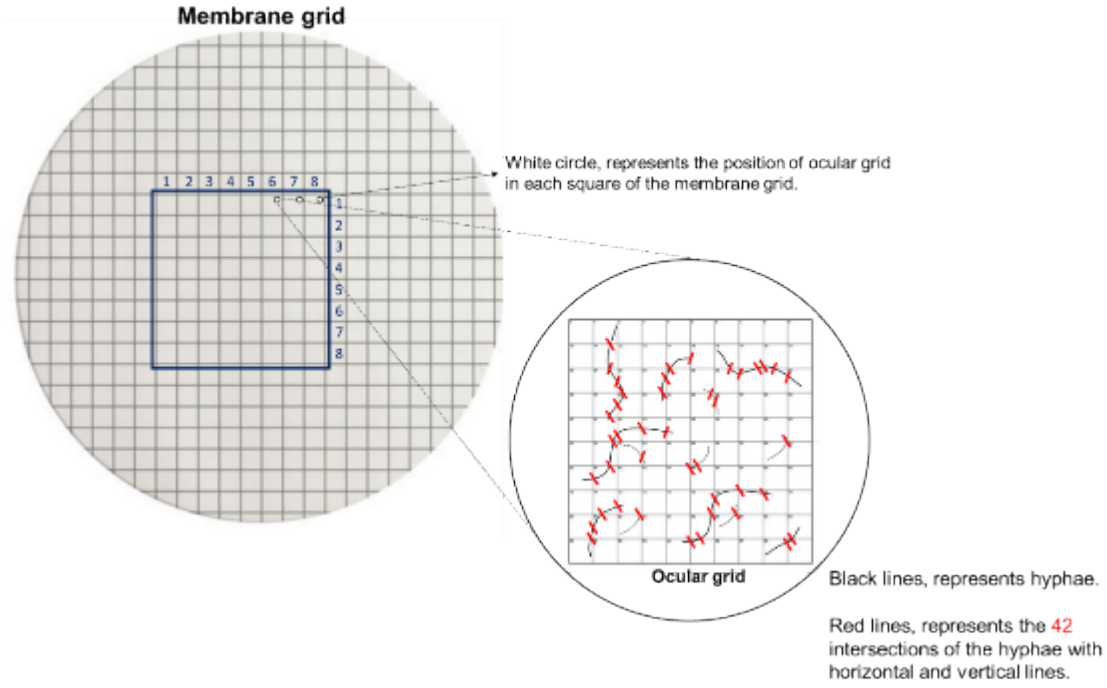


Figure 2. Procedure for quantification of the number of intersections of extra-radicular hyphae observed though the Eye piece Whipple disc grid. Credits: Luís Carlos Iuñes Oliveira Filho.

Calculation and expression of results

Extra-radicular hyphae length (EHL, m of hyphae g^{-1} of soil) is determined by extrapolation of the number of intersections of the hyphae with horizontal and vertical lines of the Eye piece Whipple disc grid using equation (2), adapted from root measurements (18,21,22).

$$\textbf{Eq. (2)} \quad EHL = \frac{0.037160233 \times n}{10 - \theta}$$

Where:
EHL is the length of the extra-radicular mycelium present in the replicate (in m g^{-1} of soil) evaluated in the 64 membrane fields
n is the number of hyphae intersections in the horizontal and vertical lines of the Eye piece Whipple disc grid
θ is the water content in 10 g of fresh soil (measured in paragraph 47)

This formula is derived from the simplification of equation (3), considering the following factors:
Af is the useful filtering area in the cellulose nitrate membrane (π x r^2 = π x (47 mm /2)^2 = 1734.89 mm^2)
Sq is the number of squares on one line of the Eye piece Whipple disc (10 squares/line)
L is the number of vertical and horizontal lines on the Eye piece Whipple disc (22 lines)

Eq. (3) $$EHL = \frac{\frac{\pi \times Af}{2 \times Sq \times L}}{10-\theta} \times 3n \times 10^{-3}$$

Note: different characteristics of the Eye piece Whipple disc and different useful filtering area are admitted and can be replaced on equation 3.

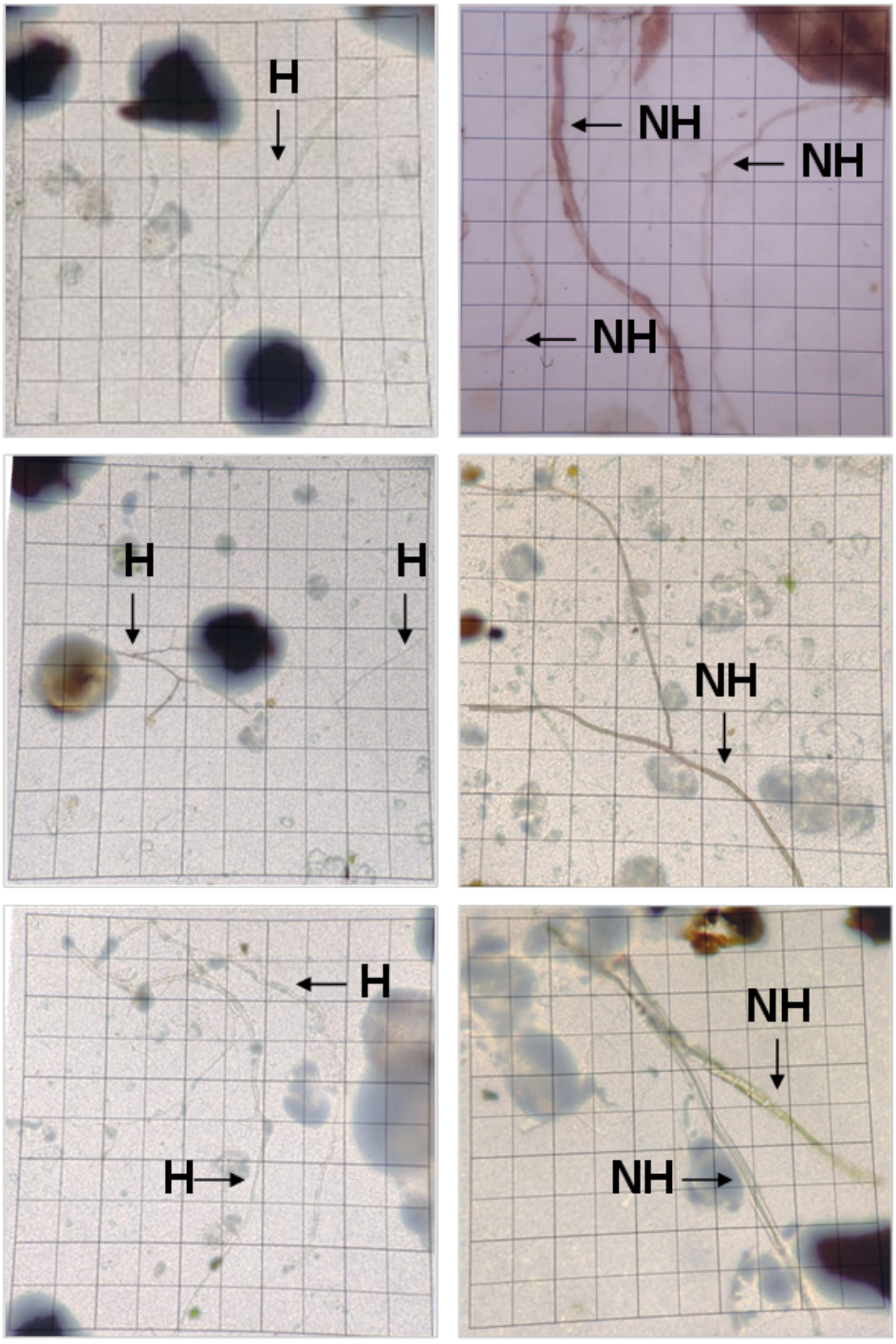


Figure 3. Examples of extra-radicular hyphae (H) and structures commonly mistaken by hyphae (NH; probably root hairs, organic matter or soil particles). Credits: Patrícia Ferreira, Isadora Pinheiro Ávila and Julia Merkle Ramos.

A.35 Extended abstract of the ERAMYC project submitted to the SETAC Europe 32th Annual Meeting in Copenhagen, Denmark (reproduction of the original document).

ERAMYC - The need of a test system with Arbuscular Mycorrhizal Fungi including pre-symbiotic and symbiotic phases for environmental risk assessment

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1. Introduction

Arbuscular Mycorrhizal Fungi (AMF) are recognized as ecologically relevant in supporting key soil functions, providing important ecosystem services (e.g. increasing resistance of host plants to several stressors, acquiring nutrients from soil for host plants, stabilizing soil structure and increasing soil carbon stocks). Due to their recognized ecological relevance and their sensitivity to chemicals, AMF have been indicated by the European Food Safety Authority (EFSA PPR [1]) as a potential group of non-target test organisms for ecotoxicological tests, used in the risk assessment of Plant Protection Products (PPPs).

The existing ISO protocol 10832, which aims to evaluate the effect of pollutants on AMF, considers only the pre-symbiotic phase. However, spore germination, germ tube formation, and asymbiotic hyphal growth are important phases of the fungal life cycle and are essential for establishing symbiosis. Some other processes like root colonization could be affected by pollutants in the soil and are not investigated when following existing test protocols. A literature search indicates that some studies have additionally analysed how stressors can influence AMF colonization, but at the moment there is no standardized test protocol availabe which includes effects of soil contaminants on AMF species during the symbiotic phase.

ERAMYC is a project which integrates a consortium of experts and aims to i) improve the existing protocol for the pre-symbiotic phase by including additional AMF species, test conditions and procedures embracing the symbiotic phase of AMF species; ii) standardize the developed advanced test protocol through a ring-test with different chemicals; iii) develop a draft OECD Test Guideline and evaluate experimental results in reference to the existing framework for the risk assessment of soil organisms exposed to chemicals.

2. Materials and methods

To comply with the objectives defined, the ERAMYC project is structured along four work packages (WPs).

WP1 comprises a literature review where a comparative assessment of studies with AMF exposed to chemicals is performed to collect data regarding test species sensitivity, test conditions and measured endpoints. Based on the data collected, a general test protocol was developed to be used in the WP2.

WP2 is divided in two phases or two clusters of experiments. In the first phase (without chemicals), the aim is to evaluate the performance/suitability of the selected AMF species in different soils under specific test conditions like environmental conditions and selected host plants. The most relevant parameters will be assessed to evaluate the operational aspects of their measurement, the intra-specific variability and also to define adequate validity criteria for combinations of AMF species/measured parameters. The second phase will include the use of test chemicals and the aim is to assess how the different AMF species and parameters measured react to the selected chemicals.

WP3 will comprise a ring test using the developed test protocol (involving a minimum of 6 laboratories) for the performance of tests comprising both the pre-symbiotic and symbiotic phases of AMF to evaluate its reproducibility.

WP4 will comprise the proposal of a draft OECD Test Guideline and validation report for testing AMF exposed to chemical substances.

3. Results and discussion

3.1. Literature review

The literature review provided an overview of AMF ecological relevance and their life cycle and delivered information about experiments conducted with the target organic chemicals of the project (i.e., PPPs, biocides and veterinary phamaceuticals) to test their effects on AMF species and on the symbiosis with selected host plants. Aspects dealing with tested species, test conditions and parameters measured (and their sensitivity) were also discussed.

Regading global occurrence, a total of 113,601 AMF records were found, distributed among 4 taxa and 1 unknown order (Figure 1).

In terms of geographical distribution, *Gigaspora margarita*, *Rhizophagus irregularis*, *Rhizophagus intraradices*, *Rhizophagus clarus* and *Funneliformis mosseae* were identified as the AMF species with the highest frequency of occurrence and/or global distribution.

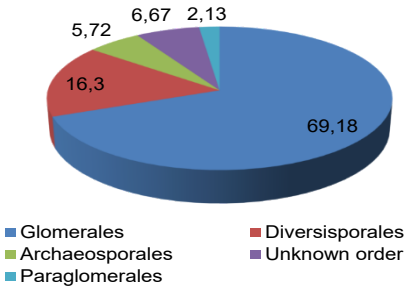

Figure 1: Percentage of AMF species distributed in Glomerales, Archaeosporales, Paraglomerales, Diversisporales or unknown order. Data obtained from PlutoF Database, April 2021 [3]

Concerning the test parameters, according to the data from literature, spore germination is the parameter mostly used, but not always the most sensitive when compared to mycelium growth. Fungicides are usually more toxic to AMF (15 out of 21 showed toxicity) than herbicides (6 out of 13 showed toxicity). Most of the biocides tested induced reduction in spore germination and no information is available on the effects of veterinary pharmaceuticals.

3.2. Ongoing WPs

The first phase of the WP2 is ongoing and some preliminary results of the experiments to evaluate the performance/suitability of the selected AMF species in the selected soils under specific test conditions (environmental conditions and selected host plants) will be presented.

4. Conclusions

The literature review evidenced that the sensitivity of test parameters can be species and substance dependent. When developing a standard protocol for the symbiotic phase, it is important to take these aspects into account. Additionally, a trade-off between measuring some of the parameters and the duration of the test might become apparent (i.e., to observe effects on spore germination, usually longer test periods are necessary).

5. References

[1] European Food Safety Authority, PPR Panel. 2017. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. In: EFSA Journal, 15(2), 4690

[2] International Organization for Standardization. 2009. ISO – 10832: Soil quality – Effects of pollutants on mycorrhizal fungi – Spore germination test. Geneve, Switzerland.

[3] Abarenkov K, et al. 2010. PlutoF - a Web Based Workbench for Ecological and Taxonomic Research, with an Online Implementation for Fungal ITS Sequences. In: Evolutionary Bioinformatics, 6, p. 189–196.

Acknowledgement - The authors thank the German Federal Environment Agency for funding this project (FKZ 3720 64 407 0).

A.36 Extended abstract of the ERAMYC project submitted to the SETAC Europe 33rd Annual Meeting in Dublin, Ireland (reproduction of the original document).

ERAMYC – Assessing the sensitivity of Arbuscular Mycorrhizal Fungi to chemicals in soil

Tiago Natal-da-Luz[1,2], Luís CI Oliveira Filho[3], Patrícia Ferreira[1], Tanel Vahter[4], Pia Kotschik[5], Silvia Pieper[5], Susanne Walter-Rohde[5], Sonia Purin da Cruz[6], Susana Rodríguez-Echeverría[2], Matthias C Rillig[7], Mari Moora[4], José P Sousa[2], Osmar Klauberg-Filho[3]

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1 Introduction

Arbuscular Mycorrhizal Fungi (AMF) are organisms that live in symbiosis with most plant species, being responsible for supporting key soil functions (e.g. increasing resistance of host plants to several stressors, acquiring nutrients from soil for host plants, stabilizing soil structure and increasing soil carbon stocks) and providing important ecosystem services. Due to their ecological relevance and sensitivity to chemicals, AMF have been indicated by the European Food Safety Authority (EFSA PPR [1]) as a potential group of non-target test organisms to be used in the risk assessment of Plant Protection Products (PPPs).

The existing ISO protocol 10832 [2] describes a method to evaluate the effect of chemicals on AMF, but considers only spore germination (pre-symbiotic phase). Germ tube formation and asymbiotic hyphal growth are also important phases of the fungal life cycle and are essential for establishing symbiosis. However, other processes like root colonization could be affected by chemicals in the soil and are not investigated when following the existing test protocol.

ERAMYC is a project which integrates a consortium of experts with the aim to i) improve the existing protocol for the pre-symbiotic phase by including additional AMF species, test conditions and procedures embracing the symbiotic phase of AMF species; ii) standardize the developed test protocol through a ring-test with different chemicals; iii) develop a draft OECD Test Guideline and evaluate experimental results in reference to the existing framework for the risk assessment of soil organisms exposed to chemicals.

To support the preparation of the ring-test, ERAMYC integrates a Work Package (WP) that, beyond the optimisation of the method and definintion of endpoints to be measured, also intends to evaluate the toxicity of several chemicals in four soils to different AMF species. The present oral platform aims

to provide the current status of the ERAMYC project and to present the toxicity data of chemicals to different AMF species in both pre-symbiotic and symbiotic phases in reference soils.

2 Materials and methods

To comply with the objective of the ERAMYC project of improving the existing method for the pre-symbiotic phase and of standardizing the procedures embracing the symbiotic phase of AMF species before the ring-test, two phases of preliminary experiments were performed. In the first phase, experiments were conducted without chemicals to evaluate the performance and suitability of the selected AMF species in different test soils under specific environmental conditions and selected host plants. The most relevant parameters were assessed to evaluate the operational aspects of their measurement, the inter-specific variability and also to define adequate validity criteria for combinations of AMF species and measured parameters. The second phase included the use of test chemicals aiming to assess how the different AMF species and respective parameters react to the selected chemicals.

3 Results and discussion

3.1 First phase

Laboratory experiments showed that a higher colonization rate is reached by the use of mixed inoculum of *Funneliformis mosseae*, *Rhizophagus irregularis* and *Rhizophagus clarus* per replicate compared to the use of extracted spores of the same AMF species. The amount of mixed inoculum per replicate depends on the inoculum potential, a parameter that must be measured previously for each inoculum batch (when aquired) and host plant used or routinely when multiplied in the lab. Regarding the performance of host plants, the use of tomato (*Solanum licopersicum*) and leek (*Allium ampeloprasum*) seeds previously soaked in water, at a temperature of 23 ± 2 ºC, in replicates of 200 to 500 g of soil (+inoculum) over a time period of 28 to 56 days is the most suitable setup for laboratory testing. In some cases, a fertilizer solution may be needed to ensure a good development of the host plants which will promote higher percentages of total AMF colonization.

3.2 Second phase

Laboratory experiments will be conducted with different chemicals with potential to be used as reference substance in positive controls of AMF symbiotic phase laboratory tests and/or suitable to be used in the planned ring-test. One of this substances could be the fungicide Chlorothalonil.

Results of previous studies [3] showed that a chlorothalonil-based fungicide significantly inhibited spore germination of *Gigaspora albida* (IC50 = 12.14 mg kg^{-1}) and *Rhizophagus clarus* (IC50 = 36.20 mg kg^{-1}) in the pre-symbiotic phase in tropical artificial soil (TAS). Effects at the symbiotic phase were evaluated using isolates of the same AMF species in combination with the host plants soybean (*Glycine max*) and brachiaria (*Braquiaria brizantha*) in TAS [4]. Fifty percent of effects (compared to control) were found at concentrations greater than 17 mg kg^{-1} depending on the AMF-host plant combination and the endpoint measured (Table 1).

Table 1. Inhibition concentration values [IC_{50} (CI95%) - mg kg^{-1}] for the percentage of total and arbuscular mycorrhizal root colonization and total extra-radicular mycelium length (TEML) in tropical artificial soil (TAS) spiked with a chlorothalonil-based fungicide [4]

Test battery	Total colonization (%)	Arbuscular colonization (%)	TEML (cm hyphae g^{-1} dry soil)
G. albida + *G. max*	> 72 [not calculated]	> 72 [not calculated]	**44.0** [39.8 – 48.1]
G. albida + *B. brizantha*	**69.7** [42.8 – 97.0]	**17.8** [11.4 – 24.2]	**69.3** [40.2 – 98.3]
R. clarus + *G. max*	**17.5** [15.0 – 21.0]	**20.9** [13.1 – 28.8]	**55.1** [43.9 – 66.3]
R. clarus + *B. brizantha*	**56.5** [35.9 – 77.2]	**23.8** [16.4 – 31.2]	**53.5** [43.0 – 63.9]

IC_{50} = concentration originating 50% inhibition of the germination; CI - confidence intervals.

4 Conclusions

The use of seeds pre-soaked in water and a total AMF colonization higher than 40% in controls have been required to validate the experiments. Chlorothalonil had effects on both the pre-symbiotic and symbiotic phases in both tested AMF species. In the symbiotic phase, arbuscular colonization was, in most cases, more sensitive when compared to total colonization and TEML. However, this patern can be different when testing an herbicide or/and a fungicide with a different mode of action. Percentage of colonization (total and arbuscular) and TEML seem to be adequate endpoints in the evaluation of the symbiosis between AMF-host plant.

5 References

[1] European Food Safety Authority, PPR Panel. 2017. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. In: EFSA Journal, 15(2), 4690

[2] International Organization for Standardization, 2009. ISO – 10832: Soil quality – Effects of pollutants on mycorrhizal fungi – Spore germination test. Geneve, Switzerland.

[3] Mallmann. et al, 2018. Placing arbuscular mycorrhizal fungi on the risk assessment test battery of plant protection products (PPPs). Ecotoxicology, 27:809-818.

[4] Mallmann. G., 2020. Establishment of ecotoxicological protocols to evaluate the effect of chemicals on arbuscular mycorrhizal fungi. PhD Thesis in Soil Science, UDESC, Lages, Brazil, 2020, 167 pp.

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A.37 Extended abstract of the ERAMYC project submitted to the SETAC Europe 35th Annual Meeting in Viena, Austria (reproduction of the original document).

ERAMYC: Results of a Ring Test for Standardization of Arbuscular Mycorrhizal Fungi Pre-Symbiotic and Symbiotic Phases Tests for Environmental Risk Assessment Schemes

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1. Introduction

Arbuscular mycorrhizal fungi (AMF) are mutualistic organisms living in obligate symbiosis with a wide range of plants. The presence of AMF in ecosystems is essential because of the benefits these organisms provide to host plants (e.g., enhanced tolerance to environmental stressors and increased nutrient and water uptake) and because of their major contributions to key ecosystem services (e.g., carbon sequestration, and soil aggregation and stabilization).

The ecological importance and sensitivity of AMF to contaminants led the European Food Safety Authority (EFSA) to identify this group of microorganisms as a potential group of non-target in-soil organisms to be included in risk assessment of plant protection products (PPPs) [1]. To date, there are no standardized OECD Test Guidelines for the performance of tests with AMF, but two standardized methods are available, one from ISO (ISO/TS 10832:2009) for pre-symbiotic testing [2] and one from Association Française de Normalisation (AFNOR, FD X 31-205-2) for symbiotic testing [3].

The ERAMYC project aims to fill some of the gaps identified by EFSA by i) improving the existing ISO document [2] for AMF pre-symbiotic phase testing by including new AMF species, improved test conditions and procedures and ii) developing and standardizing a laboratory method to assess the effects of chemicals on the AMF symbiotic phase. A ring test was conducted to assess the feasibility and reproducibility of the methods developed in the project with the final objective of developing an OECD Test Guideline for AMF symbiotic and pre-symbiotic phase tests.

2. Materials and Methods

2.1. Symbiotic phase ring test

The methodology for the performance of AMF symbiotic phase tests was developed and refined in two phases: i) definition of test procedures, assessment of the suitability of AMF and host plant and of the environmental conditions and definition of relevant parameters and respective validity criteria and ii) evaluation of the AMF response towards different test substances to be used in the interlaboratory test.

The protocol resulting from this work was ring-tested by 9 laboratories (3 from the consortium + 7 independent laboratories) using one AMF species (*Rhizophagus irregularis*), one host plant (*Allium ampeloprasum*), one test soil (OECD 2.5%) and two test substances (azoxystrobin and fluazinam). After 56 days of incubation, the total and arbuscular intra-radicular colonization and the extra-radicular hyphae length were assessed to evaluate the effect of the test substances on the establishment of the AMF-plant symbiosis.

2.2. Pre-symbiotic phase ring test

The methodology described in the ISO/TS 10832:2009 [2] was adapted and refined by including new methodological steps and considering different test conditions. The updated protocol was distributed to 7 laboratories (3 from the consortium + 4 independent laboratories) to be ring-tested using the AMF species *R. irregularis*, the test soil OECD 2.5% and the same test substances used for the symbiotic phase ring test, azoxystrobin and fluazinam. After 14 days of incubation, the number of recovered spores and the percentage of germinated spores were assessed to evaluate the effect of the test substances on the AMF pre-symbiotic development.

3. Results and Discussion

3.1. Symbiotic phase ring test

Most tests performed by the laboratories of the ring test fulfilled the validity criteria defined in the first phase of the project (i.e., total colonization ≥ 30%, coefficient of variation < 50% and root length ≥ 10 cm in control replicates). Measurements of total colonization generally provided more robust data compared to arbuscular colonization and sensitivity of both parameters did not differ considerably, independently of the test substance. Extra-radicular hyphae length revealed to be the least sensitive parameter measured. Data showed some variability within and between laboratories and so far it was not possible to find a suitable reference substance. Despite the data variability, results suggest that toxicity of the tested substances to AMF is similar to higher than the toxicity data reported in the literature for standard in-soil organisms.

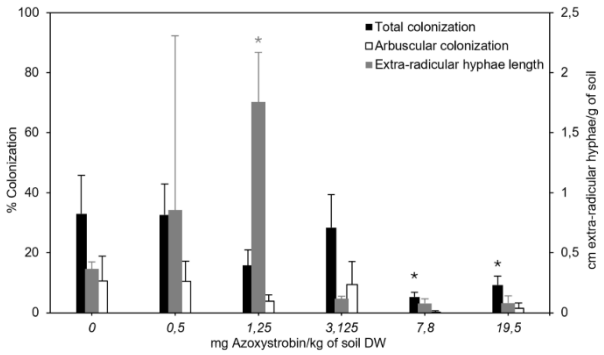

Figure 1: Outcome of an AMF symbiotic phase test of a laboratory of the ring test exposing* R. irregularis *in OECD 2.5% spiked with increasing concentrations of azoxystrobin.

3.2. Pre-symbiotic phase ring test

Most tests performed by the laboratories of the ring test showed that the new suggested species and environmental conditions continue to guarantee the compliance of the experiments with the validity criteria considered in the ISO document (i.e., nº recovered spores in all treatments ≥ 25, germinated spores in control ≥ 75%). Toxicity observed in pre-symbiotic phase tests tended to be lower than that observed in the symbiotic phase tests.

4. Conclusions

The variability of data obtained especially in the new method for AMF symbiotic phase testing and the difficulty in finding a suitable reference substance may hamper the standardization of the method. On the other hand, the fact that the toxic effects of the test substances to AMF observed in the ring test tended to be in the same order of magnitude or higher than the toxicity data reported in the literature for non-target standard in-soil species, suggests that the AMF communities may, in some cases, be insufficiently covered by the standard species currently considered in the ERA of PPPs in European Union. However, further tests are needed with a larger set of test substances to substantiate this assumption.

5. References

[1] EFSA, PPR Panel. 2017. Scientific Opinion addressing the state of the science on risk assessment of plant protection products for in-soil organisms. In: EFSA Journal, 15(2), 4690
[2] ISO. 2009. ISO/TS 10832:2009: Soil quality — Effects of pollutants on mycorrhizal fungi — Spore germination test. Geneve, Switzerland.
[3] AFNOR. 2014. FD X 31-205-2: Qualité du sol - Effets des polluants vis-à-vis des champignons mycorhizogènes - Partie 2 : essai de mycorhization racinaire. Normes nationales et documents normatifs nationaux. Association Française de Normalisation.

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A.38 Extended abstract of the ERAMYC project submitted to the SETAC Latin America 16th Biennial Meeting in Lima, Peru (reproduction of the original document).

Are the current Environmental Risk Assessment Schemes adequately protecting Arbuscular Mycorrhizal Fungi? Developing a method to assess AMF sensitivity

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Arbuscular Mycorrhizal Fungi (AMF) form symbiotic relationships with the roots of most land plants aiding in nutrient uptake and enhancing host plant resistance to diseases and stressors. AMF also increase soil carbon stocks and contribute to the formation of soil aggregates improving soil structure and moisture retention.

Ecological relevance and sensitivity of AMF to contaminants led the European Food Safety Authority (EFSA) to identify AMF as a potential group of non-target in-soil organisms to be included in environmental risk assessment (ERA) of plant protection products (PPPs). However, the existing ISO/TS 10832:2009 protocol that evaluates the effect of pollutants on AMF only considers spore survival and germination. Germ tube formation and asymbiotic hyphal growth, essential for establishing the symbiosis, could be also affected by pollutants in the soil, but they are not addressed by the current standards. Given this reality, German Environment Agency (UBA) funded the ERAMYC project that aimed to improve the existing ISO document for pre-symbiotic testing and develop and standardize a laboratory method for including the symbiotic phase. Laboratory tests were carried out with different AMF species (*Funneliformis mosseae*, *Rhizophagus irregularis and Rhizophagus clarus*), test soils (OECD 2.5% and 4 natural soils) and chemicals (5 fungicides and 2 herbicides) to assess the sensitivity of AMF and to develop and optimize the method. Lastly, a ring test was conducted using the AMF *R. irregularis,* the host plant *Allium ampeloprasum*, OECD 2.5% soil and the test substances Azoxystrobin and Fluazinam, to assess the precision and reproducibility of these methodologies with the final objective of developing an OECD international standard for AMF pre-symbiotic and symbiotic testing.

Symbiotic phase ring test results showed high variability and it was not possible to find a suitable reference substance, which may hamper the standardization of the method. The pre-symbiotic phase ring test did not confirm the reproducibility of the method due to a high number of invalid tests. Laboratory tests showed that Boric acid, Tebuconazole, Azoxystrobin and Pendimethalin are 1.85 to 333 times more toxic to AMF when compared to the lowest endpoints reported in the literature for the

non-target standard in-soil species considered in the current ERA of PPPs in European Union. This highlights the urgency of including AMF in ERA schemes.

Key-words: Arbuscular mychorrizal fungi; Ecological risk assessment; Non-target species; Ring test

Proposed session: “Soil: Ecotoxicity and Risk Assessment”

Preference: Oral platform