Deliverable 4.1: Seed inoculation of cover crops with native AMF inocula (version 1)

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Classification: Public
Associated Work Package(s)

WP1	WP2	WP3	WP4	WP5	WP6	WP7	WP8
☐	☐	☐	☑	☐	☐	☐	☐

Version History

Version number	Implemented by	Notes
1.0	UNIPI	

Table of contents

Action number: Grant agreement ID: 101083589

Action acronym: GOOD
Action title: AGrOecOlogy for weeDs

Date: 30/04/2024

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1. Introduction

The main ambition of GOOD is to foster the agroecological transition for weed management across Europe. To this aim GOOD is proposing innovative and sustainable solutions to accelerate the rate of adoption of proper agroecological weed managements by farmers.

Cover cropping is an important part of integrated crop and weed management systems, applying pressure to weeds and assisting the reduction of herbicides. In the GOOD project, different Agroecological Weed Management (AWM) strategies are tested to suppress weeds. Among the AWM strategies combined with cover crops, seed inoculation with beneficial microorganisms, such as arbuscular mycorrhizal fungi (AMF), represents one of the possible key novel interventions to accelerate the transition to sustainable, safe, and healthy food systems, reducing herbicide use and risk.

AMF are soil symbionts fulfilling a key function in the complex networks of belowground/aboveground biotic interactions as they live in association with the roots of most (80%) land plant families and influence not only soil fertility, but also plant nutrition, diversity and productivity, increasing plant resistance to biotic and abiotic stresses (Smith and Read, 2008). In addition, AMF may reduce aggressive agricultural weeds growth, while enhancing the yield of agricultural crops (Rinaudo et al., 2010). AMF may give advantages to cover crops to the detriment of weeds, favouring their rapid soil coverage and growth, smothering the weeds, through competition for light and being important also for the subsequent main crops or for the perennial crops growing in the systems. AMF, providing multifunctional ecosystem services, are classified as plant biostimulants according to the new Regulation (EU) 2019/1009. Their use as inoculum represents one of the most promising tools for sustainable management of agricultural soils, being fundamental for organic food production, reducing agrochemicals and decreasing environmental damage.

In the GOOD project, AMF seed inoculation will be tested in 14 experimental Living labs (LLs) from 7 European countries (the Netherlands, Serbia, Italy, Greece, Portugal, Spain & Cyprus), ranging from the Atlantic area to Continental areas through Mediterranean areas to ensure a balanced representation of different production systems.

AMF inoculation of the cover crops selected by WP2 and WP3 on the basis of climate, water availability and synchronization with the target crops (annuals/perennials) will be performed during the second and third year of the project. AMF are naturally present in the soils, but their abundance or taxonomic diversity may differ depending on a multitude of factors (pedological, climatic, agricultural practices intensity, etc.). For this reason, in the GOOD project native AMF will be massively reproduced from the original LLs soils and used as inoculants in the same LL soil.

Work Package (WP) 4 focuses on the examination of native soil microbiota and the coating of seeds with AMF to be performed in the activities of WP2 and WP3 to increase the crop and cover crop competitive ability against weeds, in order to reduce the dependence on herbicides and restore lost ecosystem services. Furthermore, it will examine the effects of the diverse AWM strategies implemented on WPs 2-4 on crop productivity, weed diversity and soil health.
The objectives of this WP are:
O4.1 To assess the effect of various AWM strategies on composition, diversity, and abundance of weed communities and the impact on yield components in the previously tested AWM initiatives.
O4.2 To assess weed management issues in the transition phase in relation to soil health.
O4.3 To assess any positive effects (i.e., nitrogen fluxes from legume cover crops) and drawbacks of weed management strategies on soil health and biological fertility.
O4.4 To research and innovate seed inoculation with biostimulant native AMF symbionts, adapted to the different ecosystem characteristics, to guarantee cover crop establishment, edaphic biodiversity enhancement and sustainable weed management.

The description of Task 4.1, as written in the Grant Agreement (GA) on page 83, is the following:

"Task 4.1: Use and assessment of native AMF inocula for the improvement of cover crops competitive ability against weeds.
Each LL in Portugal, Italy, Greece, Serbia, Cyprus, Spain & the Netherlands will send pooled soil samples from the experimental sites to UNIPI, where native AMF will be morphologically identified and

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*reproduced in pot cultures given their status of obligate biotrophic symbionts, using highly mycothrophic host plants. A second cycle of massive reproduction will be carried out, to obtain the necessary quantity of native AMF for seed inoculation. The obtained native AMF inocula will be sent to the relevant LLs for pre-sowing seed-coating of selected cover crop species to be performed in the second year. During the second year the roots of cover crops will be analyzed by UNIPI to assess mycorrhizal colonization of inoculated and non-inoculated plants and to evaluate the symbiotic competence of native AMF, providing protocols and guidelines for successful seed inoculation of cover crops (**D4.1,** D4.6)".*

Task 4.1 is led by UNIPI (Alessandra Turrini).
The following partners are participating in Task 4.1: UC, LSSV, AUA, CNR, MRIZP, CUT, USC, CICYTEX, DELPHY.
The Deliverable D4.1 "Seed inoculation of cover crops with native AMF inocula (version 1)" is due in Month 12 of the project (i.e., 30 April 2024).

Issues of the Deliverable D4.1

- List of native AMF taxa from 7 European countries with different edaphoclimatic conditions.
- Production of native AMF inocula for seed coating in the relevant LLs.
- Protocols and guidelines for successful seed inoculation of cover crops.

2. List of native AMF taxa from 7 European countries with different edaphoclimatic conditions

2.1 Methodology

The pooled soil samples of 14 experimental LLs from 7 European countries (the Netherlands, Serbia, Italy, Greece, Portugal, Spain & Cyprus), received in the period July-December 2023, were analysed for the morphological identification of the autochthonous AMF.

Table 1. Living Labs (LLs) of Organic and Mixed systems.

Annual crops			Permanent crops		
CROP	**Country/Partner**	LL code number	**CROP**	**Country/Partner**	LL code number
ONION	**Netherlands/DELPHY**	NL_onion/12	**OLIVES**	**Portugal/LSSV**	PT_olives/22
SOYBEAN	**Serbia/MRIZP**	RS_soybean/13	**CITRUS**	**Italy/AIAB**	IT_citrus/23
MAIZE	**Serbia/MRIZP**	RS_maize/14	**GRAPES**	**Italy/AIAB**	IT_grapes/24
TRITICALE	**Italy/CNR**	IT_triticale/15	**GRAPES**	**Greece/AUA**	GR_grapes/25
WHEAT	**Greece/AUA**	GR_wheat/16	**OLIVES**	**Cyprus/CUT**	CY_olives/26
COWPEA	**Portugal/LSSV**	PT_cowpea/17	**CHERRY**	**Spain/CICYTEX**	ES_cherry/27
RICE	**Spain/CICYTEX**	ES_rice/18	**APPLE/GRAPES**	**Spain/USC**	ES_apple-grapes/28-29

Soil samples from conventional Portugal LLs (PT_cowpea/17 and PT_olives/22) were sent as pooled soil samples, while soils from organic PT_cowpea/17 and organic PT_olives/22 were sent as separate samples. Soils from LLs RS_soybean/13 and RS_maize/14 were sent as pooled samples.

The diversity of AMF in the different experimental sites included in the GOOD project, has been assessed, by evaluating the number of spores and the variety of morphological types, occurring in the field soil used to reproduce the autochthonous communities.
Spores contained in aliquots of 50 g (dry weight) of each pooled soil were separated by the wet sieving and decanting procedure (Gerdemann and Nicolson 1963), followed by a sucrose density gradient centrifugation. Soil was suspended in water, stirred and poured through stacked sieves with 300, 100 and 50 µm mesh size. Materials retained on the 300 µm sieve were transferred to Petri dishes and observed under a dissecting microscope (Leica M205 C) for sporocarps and spores attached to root fragments.

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Materials from the other sieves were mixed, and transferred to 50 mL centrifuge tubes in water, and centrifuged at 3000 rpm for 3 min. After discarding the supernatant, the pellet was resuspended in the sucrose solution (45 mL of 45% sucrose) and centrifuged at 2000 rpm for 2 min. The supernatant was collected and washed under tap water to remove excess of sucrose, over a 50 µm sieve. The trapped material, largely containing spores and spore clusters, was flushed into 9 cm diameter Petri dishes and inspected under a dissecting microscope at magnifications up to ×160, with illumination by incident light from two fibre-optic quartz- halogen light sources. The spores were manually isolated using capillary pipettes, and mounted on slides in polyvinyl-lactic acid-glycerine (PLVG) (Koske & Tessier, 1983).

The slides were systematically examined under a Reichert-Jung (Vienna, Austria) Polyvar compound microscope at up to ×400 magnification to count all AMF spores and separate the morphologically distinct types. Detection of morphotypes was based on colour, shape and presence and mode of spore formation - acaulosporoid, glomoid, or gigasporoid.

The measure of spore diameter and capture of images were performed using the Leica DFC 450C color video camera and software package (LAS X 5.2.0261303 Life Science Microscope Software Platform, Leica Microsystems srl, Italy) connected to the microscope.

Very old and decaying spores, with missing features for identification, were counted but not included in any morphotype.

The spores were tentatively identified, when possible, using original species descriptions, updated taxonomic studies on the described taxa, available identification manuals and keys (Błaszkowski 2012; Oehl et al. 2011a, b). Other information was retrieved in the sites: https://glomeromycota.wixsite.com/lbmicorrizas and http://www.amf-phylogeny.com/. Spore abundance and richness (the number of AMF morphospecies in each soil sample) are expressed per 50 g of dry wright soil.

2.2 Results

The total number of AMF spore analysed was about 1,500. The mean number of AMF spores found in the different LLs soils was 109.1±71.6 (ST.D.) and ranged from 31 to 256 per 50 g soil (Fig. 1). The highest numbers were found in LLs IT_triticale/15 (IT15: 256 spores), GR_grapes/25 (GR25: 225 spores) and NL_onion/12 (NL12: 217 spores). On the opposite side, the lowest numbers were found in CY_olives/26 (CY26: 31 spores) and PT_cowpea/17-PT_olives/22 (PT17-22: 37 spores) (Fig. 1, Table 2).

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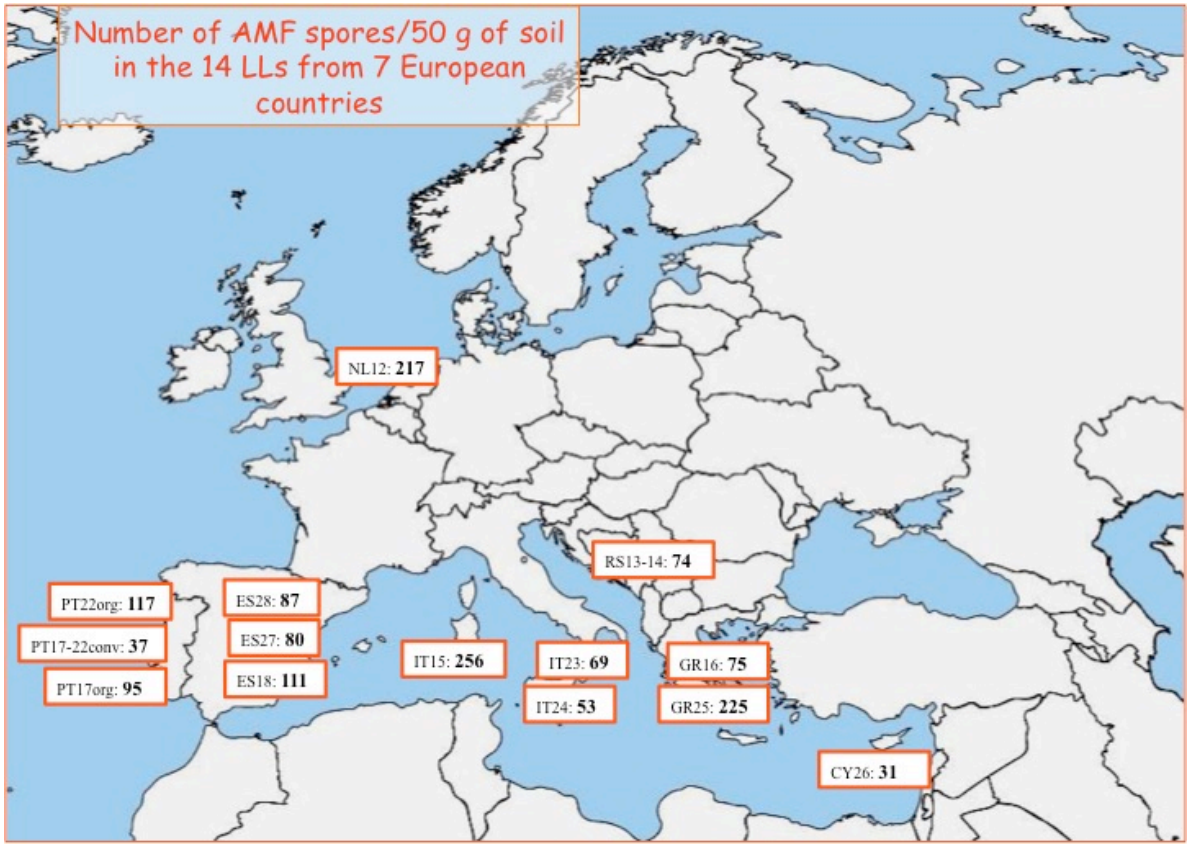

Figure 1. Number of AMF spores per 50 g of the original soil of the 14 LLs from 7 European countries, including unidentified spores.

Table 2. Number of AMF spores and morphotypes, separated by mode of spore formation, per 50 g of the original soil of the 14 LLs.

Spore types	Glomoid		Acaulosporoid		Gigasporoid		Total morphotypes
Living Labs Code numbers	No. morphotypes	No. spores	No. morphotypes	No. spores	No. morphotypes	No. spores	
NL12	10	193	1	5	1	5	12
RS13-14	6	59	3	9	-	-	9
PT22org[a]	12	90	-	-	1	8	13
PT17-22conv[b]	3	10	2	5	2	13	7
PT17org	9	73	1	10	-	-	10
ES28	11	61	2	8	4	14	17
ES27	11	57	3	6	3	12	17
ES18	13	87	2	21	-	-	15
IT23	9	51	2	5	1	5	12
IT24	4	49	-	-	-	-	4
IT15	6	229	3	11	1	3	10
GR25	10	194	1	5	-	-	11
GR16	6	55	2	6	-	-	8
CY26	5	27	-	-	-	-	5

[a]org=organic soil; [b]conv=conventional soil

The glomoid spore type was the most frequent, with about 80% of the total spores. Only small fractions of spores were represented by acaulosporoid/entrophosporoid (6%) and gigasporoid (4%) types.
Glomoid morphotypes represented the large majority in all LLs except for PT17-22.
Morphotypes richness differed among the different LLs and ranged from 4 to 17.
The highest species richness was found in ES27 and ES28 with 17 morphotypes, while in IT24 and CY26 only four and five morphotypes were detected (Fig. 2, Table 2).

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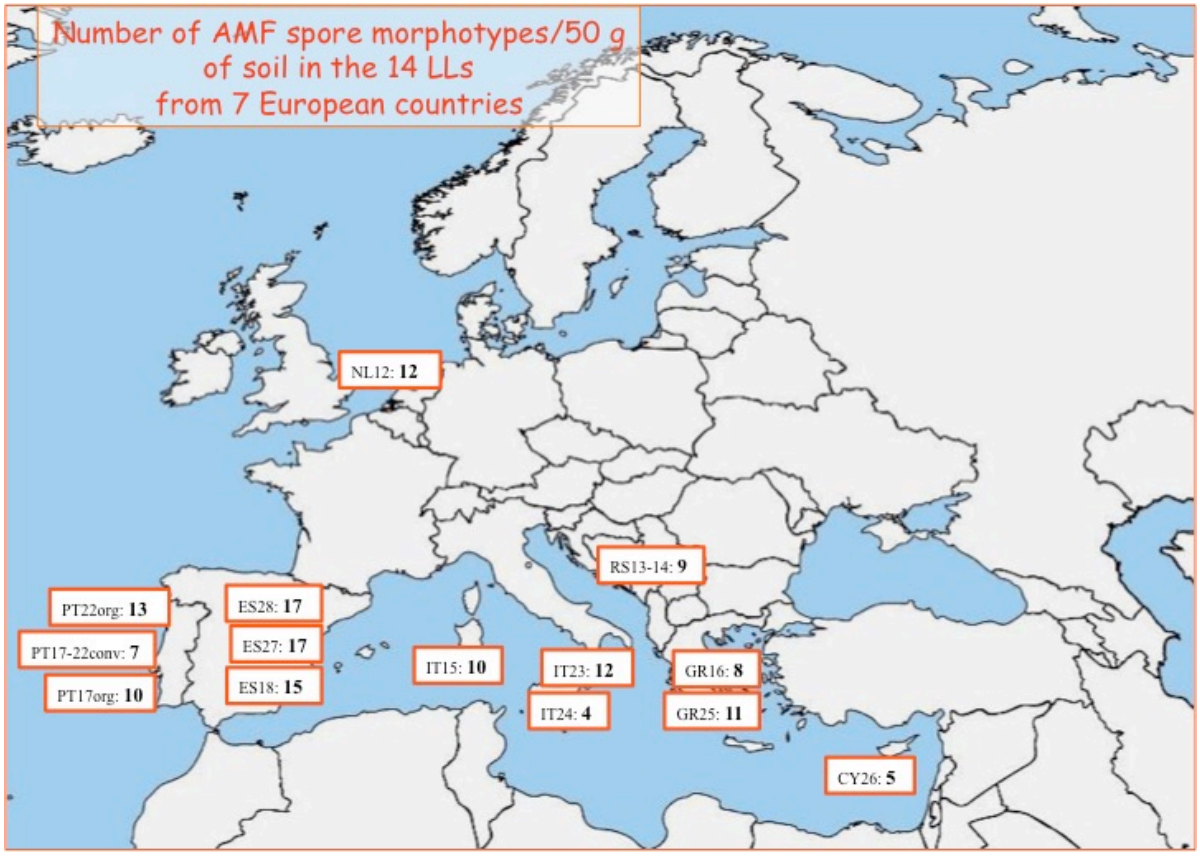

Figure 2. Number of AMF spore morphotypes per 50 g of the original soil of the 14 LLs from 7 European countries.

Some morphotypes were retrieved from different LLs. For example, morphotypes identified as *Entrophospora etunicata* (Figs 5C, 10A, 13C, 14A, 16A) *Pacispora* sp. (Figs 11B, 14B and 15E) and *Glomus badium* (Figs 3A, 5E, 9B, 13F), which occurred in five, three and four LLs, respectively.
Morphotypes resembling species belonging to *Rhizoglomus* genus occurred in at least five LLs (Figs 3B, 5A-B, 6A-C, 10B and 11E).
Spores belonging to Ambisporaceae, Archaeosporaceae (Figs 4B, 5F, 8B, 9C, 10G), Acaulosporaceae (4A, 13B, 15C) and Scutellosporacerae (Figs 3C-D, 6D, 12A, 14C) were also found, usually at very low density, in many LLs.

Representative spore morphotypes retrieved in the 14 pooled soils from the different LLs are reported in the following pages.

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1) **NL_onion/12**

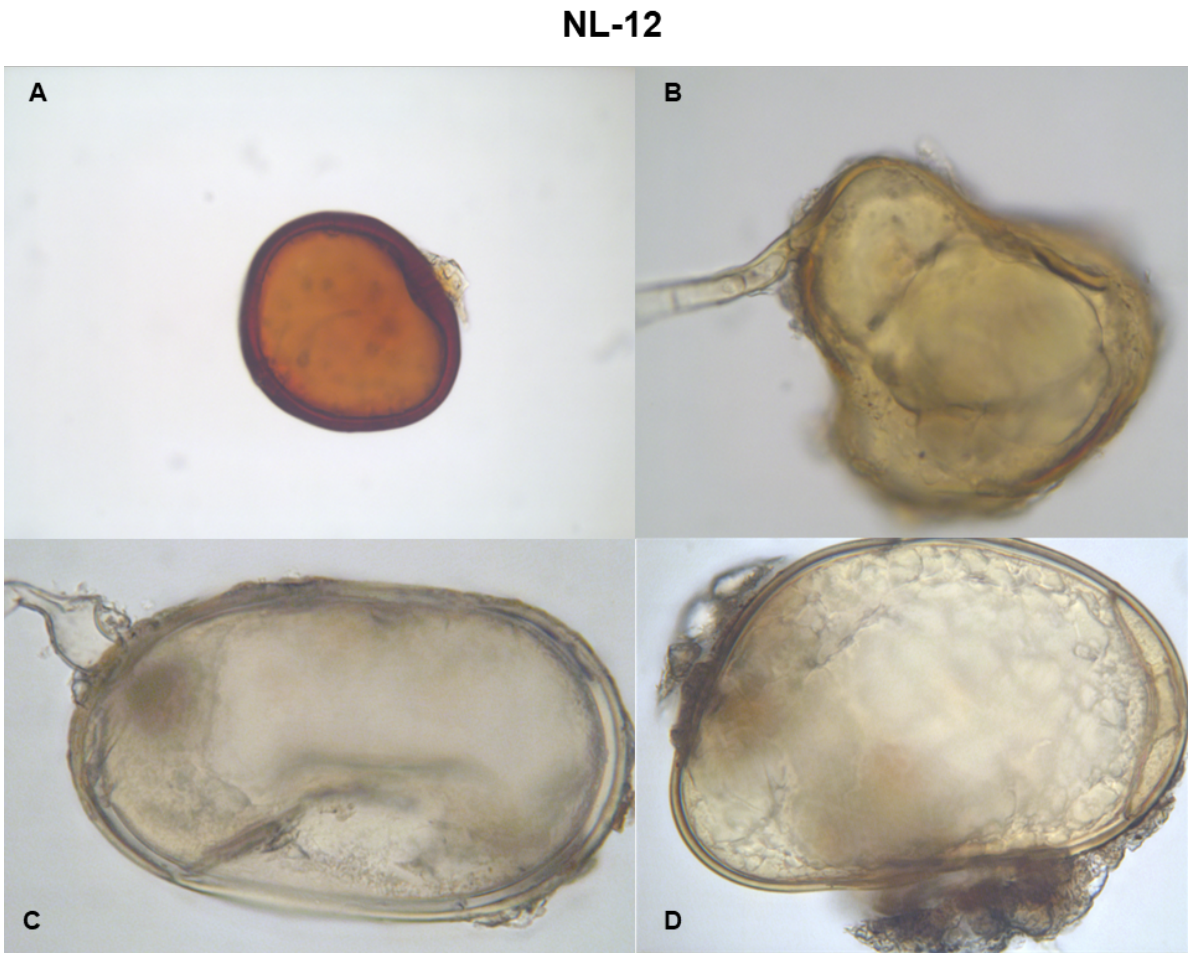

Figure 3. Representative spores of glomoid (A; B) and gigasporoid (C; D) morphotypes (NL12-n): A. NL12-2 (*Glomus badium*); B. NL12-10 (*Rhizoglomus* sp.); C-D. NL12-7 (*Scutellospora* sp.)

2) **RS_maize/13-RS_soybean/14**

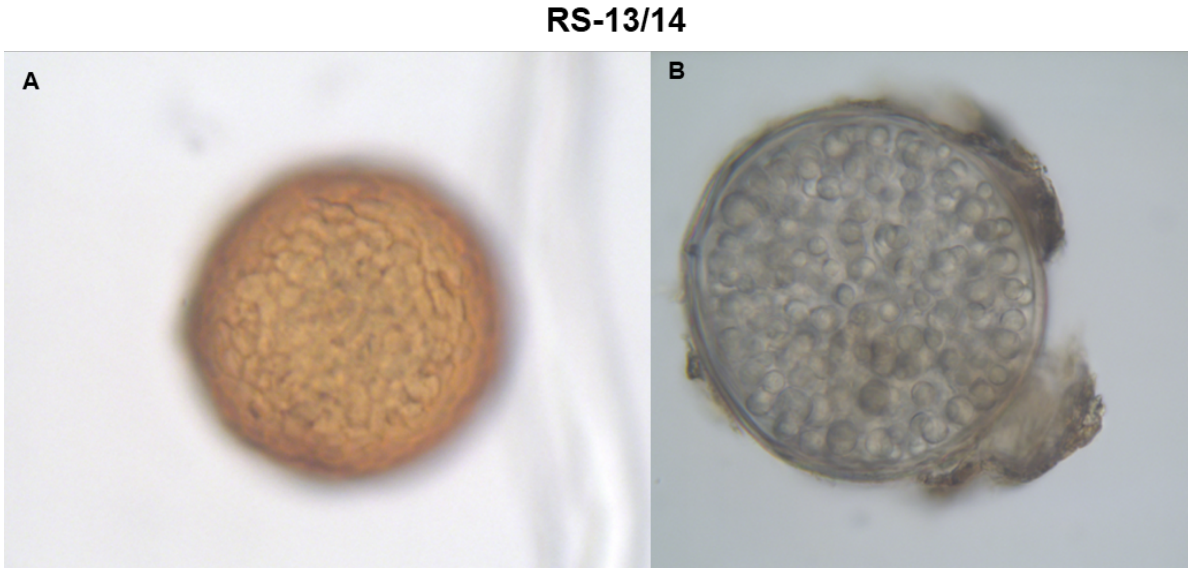

Figure 4. Representative spores of acaulosporoid (A; B) morphotypes (RS13/14-n): A. RS13/14-6 (*Acaulospora* sp.1 *rehmii*-like); B. RS13/14-8 (*Archeospora* sp.1)

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3) IT_triticale/15

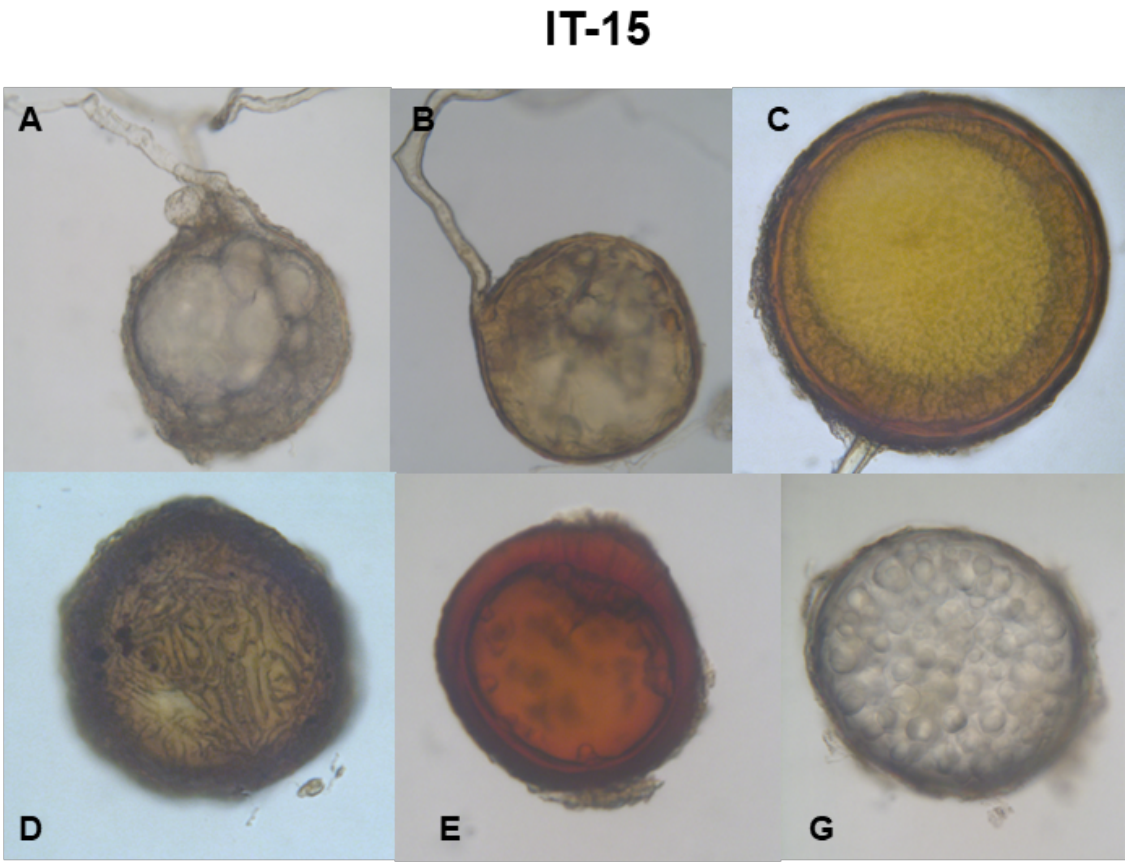

Figure 5. Representative spores of glomoid (A; B; C; D; E) and acaulosporoid (G) morphotypes (IT15-n): A. IT15-1 (*Rhizoglomus* sp.); B. IT15-2 (*Rhizoglomus* sp.2); C. IT15-3 (*Entrophospora etunicata*); D. IT15-4 (*Sieverdingia tortuosa*); E. IT15-7 (*Glomus badium*); F. IT15-10 (*Archeospora* sp1).

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4) IT_citrus/23

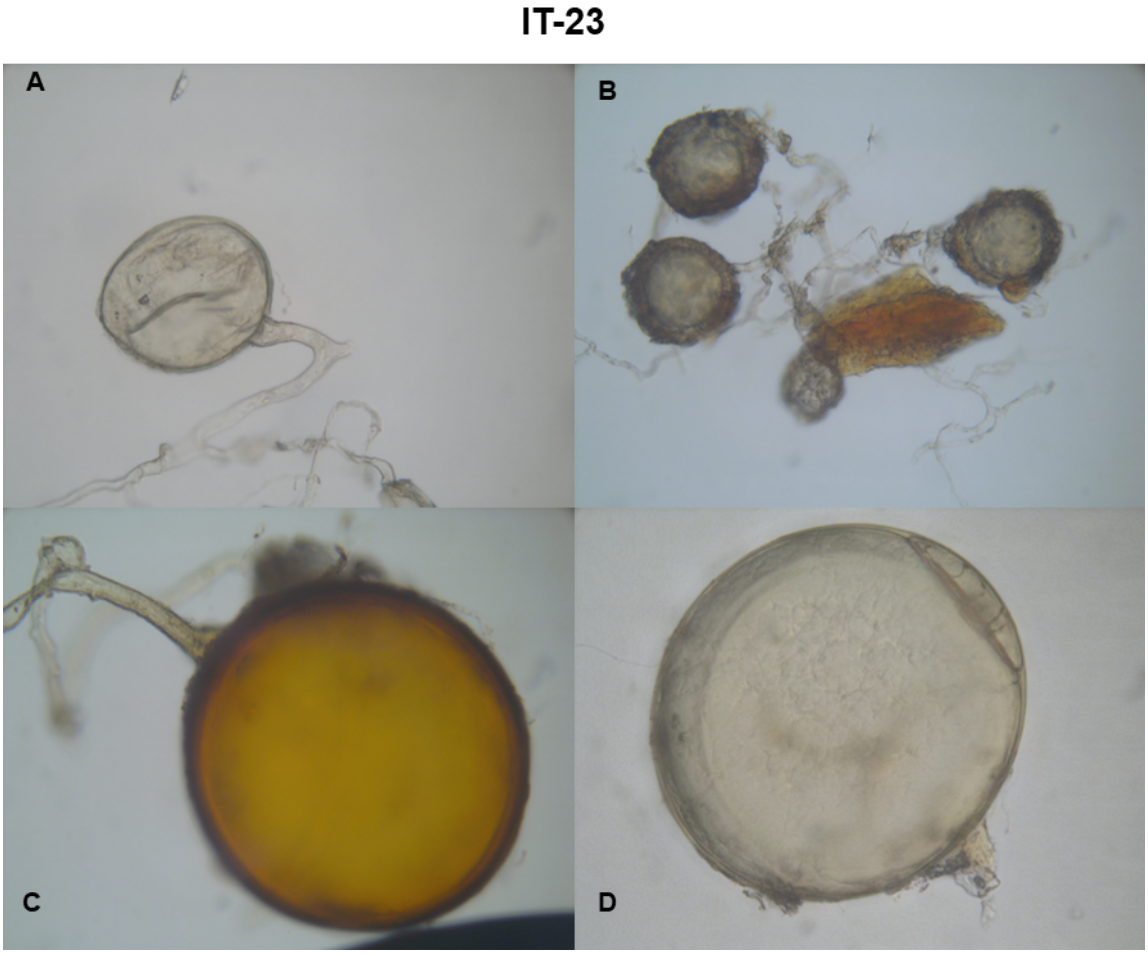

Figure 6. Representative spores of glomoid (A; B; C) and gigasporoid (D) morphotypes (IT23-n): A. IT23-2 (*Rhizoglomus* sp.); B. IT23-7 (*Septoglomus viscosum*); C IT23-10 (*Rhizoglomus* sp.); D. IT23-4 (*Scutellospora* sp.)

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5) IT_grapes/24

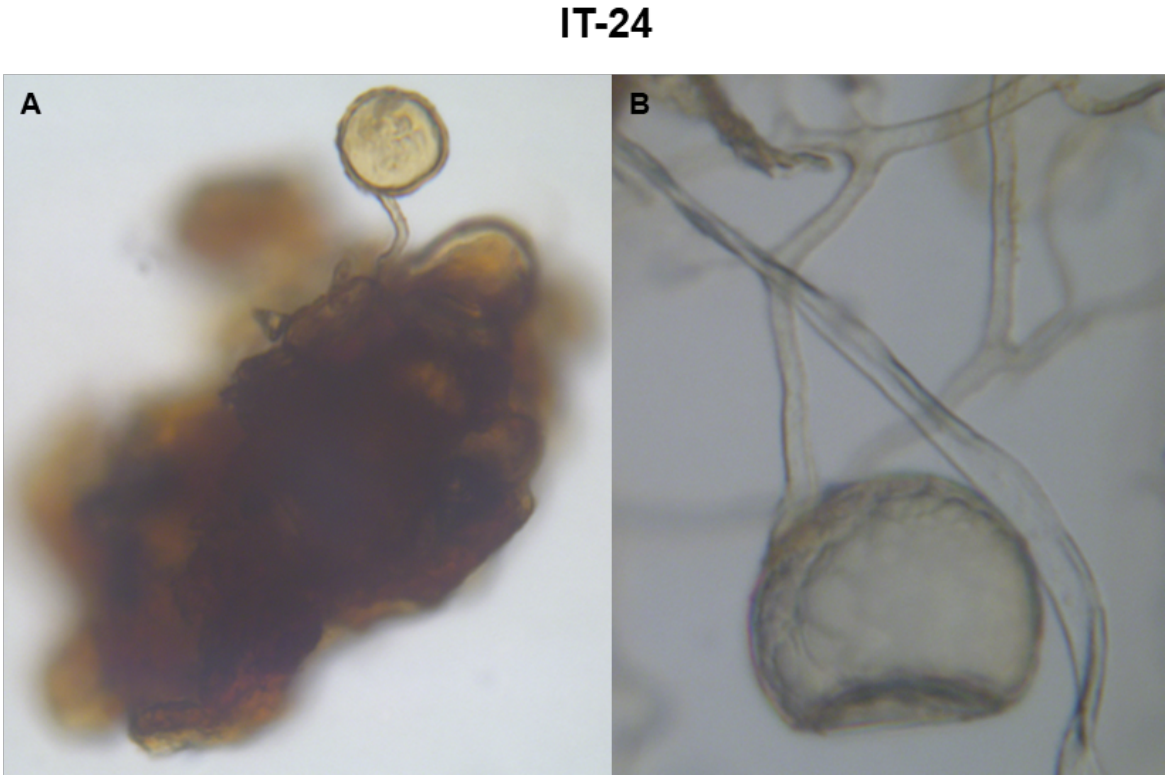

Figure 7. Representative spores of glomoid (A;B) morphotypes (IT24-n): **A**. IT24-1 (*Dominikia* sp.); **B.** IT24-5

6) GR_wheat/16

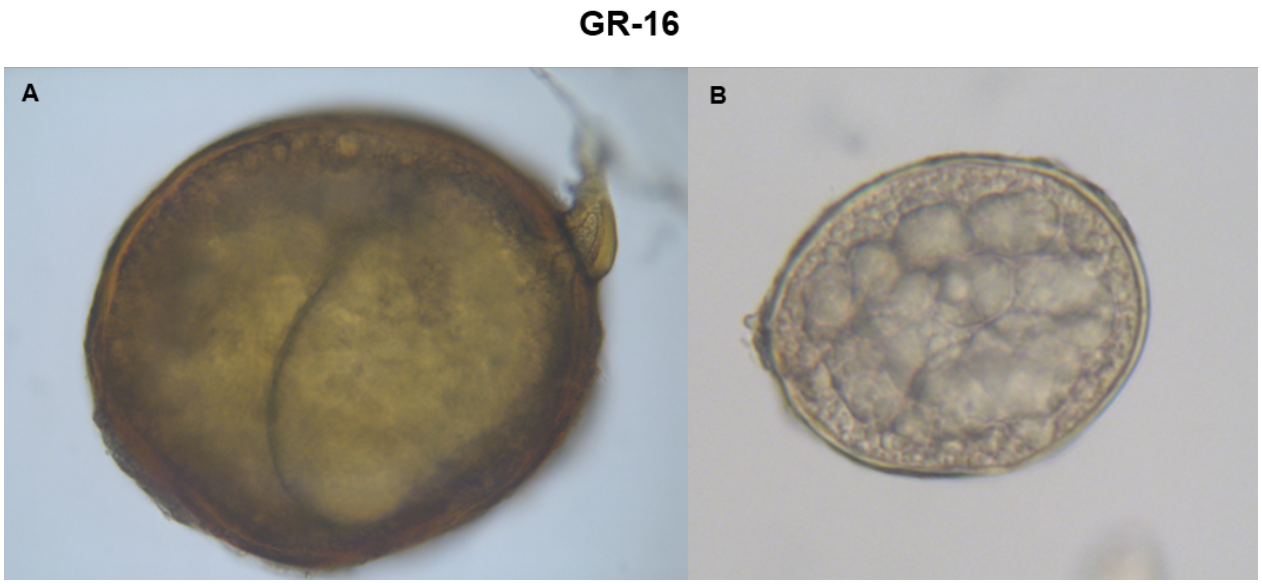

Figure 8. Representative spores of glomoid (A) and acaulosporoid (B) morphotypes (GR16-n): A. GR16-1 (*Funnelliformis* sp.); B. GR16-11 (*Archeospora* sp.2)

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7) **GR_grapes/25**

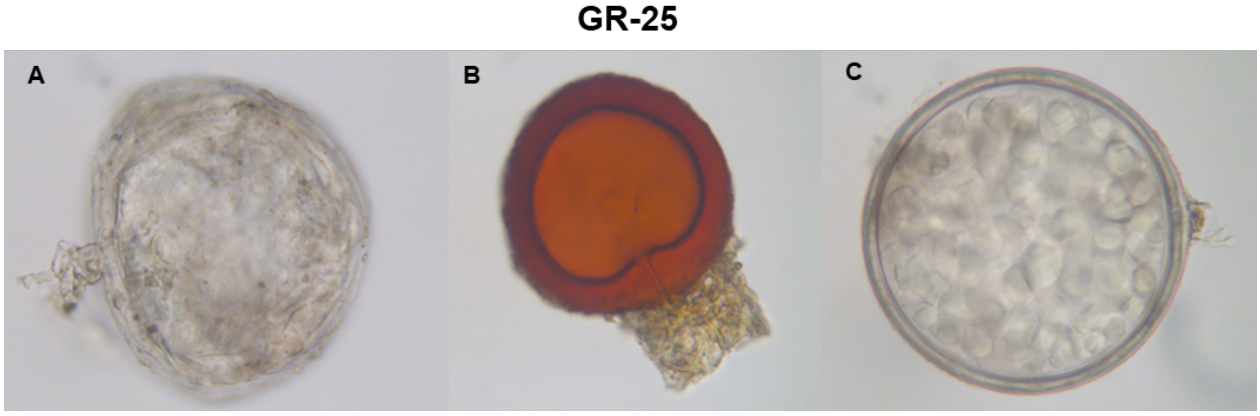

Figure 9. Representative spores of glomoid (A; B) and acaulosporoid (C) morphotypes (GR25-n): A. GR25-15 (*Paraglomus* sp.); B. GR25-1 (*Glomus badium*); C. GR25-3 (*Archeospora* sp.1)

8) **PT_cowpea/17 organic**

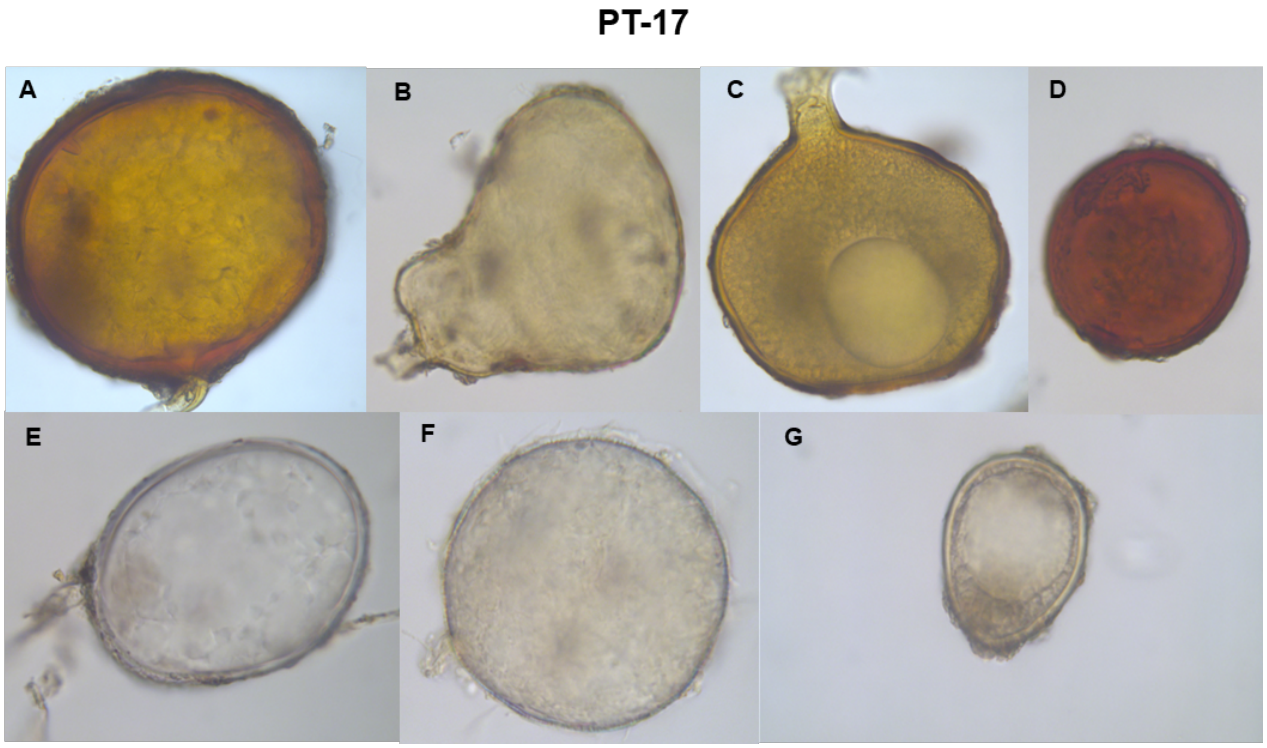

Figure 10. Representative spores of glomoid (A; B; C; D; E; F) and acaulosporoid (G) morphotypes (PT17-n): A. PT17-5 (*Entrophospora etunicata*); B. PT17-6 (*Rhizoglomus* sp.); C. PT17-12; D. PT17-14; E. PT17-16; F. PT17-19 (*Diversispora spurca*); G. PT17-7 (*Archeospora* sp2).

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9) PT_olives/22 organic

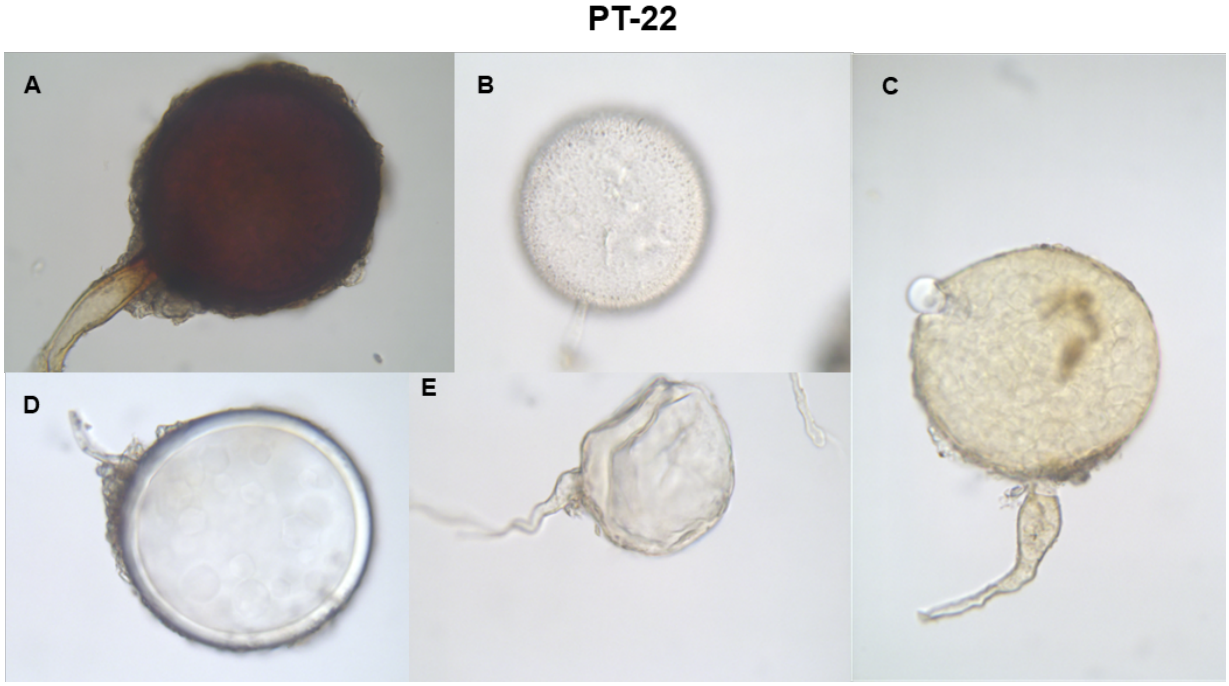

Figure 11. Representative spores of glomoid (A; B; C; D; E) morphotypes (PT22-n): A. PT22-2 (*Funnelliformis* sp.); B. PT22-5 (*Pacispora* sp.); C. PT22-15; D. PT22-8 (*Diversispora clara*); E. PT22-10 (*Rhizoglomus* sp.)

10) PT_cowpea/17 and PT_olives/22 conventional

Figure 12. Representative spore of gigasporoid (A) morphotype (PT-17/22-n): A PT17/22-5 (*Scutellospora* sp.)

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11) ES_rice/18

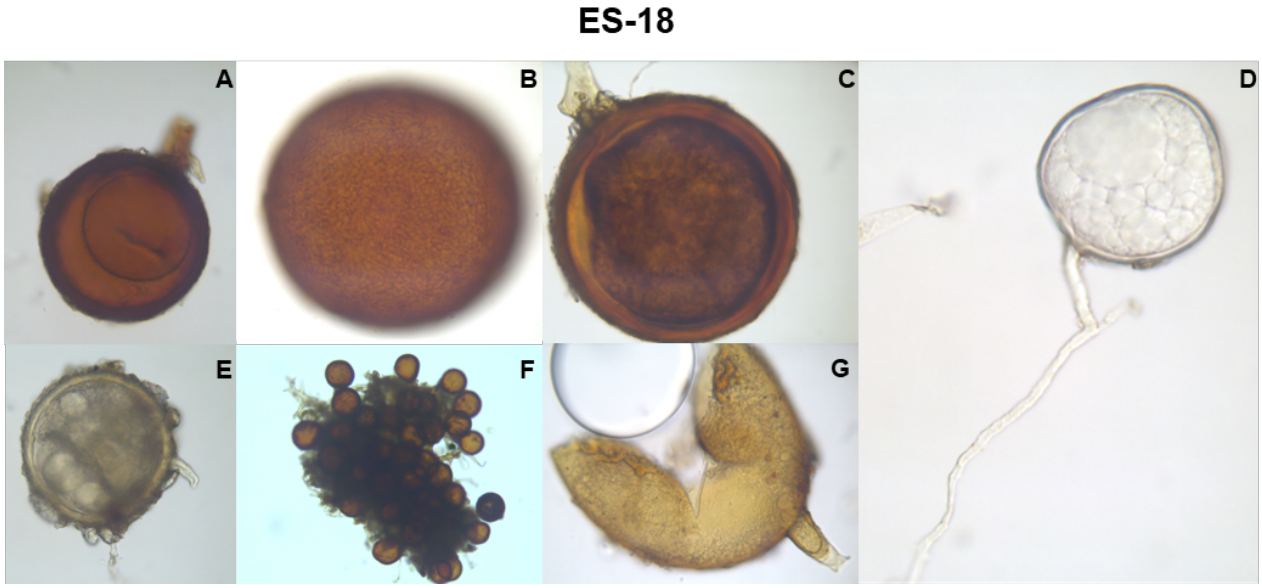

Figure 13. Representative spores of glomoid (A; B; C; D; E; F; G) morphotypes (ES18-n): A. ES18-1; B. ES18-4 (*Acaulospora* sp.); C. ES18-12 (*Entrophospora etunicata*); D. ES18-7 (*Dominikia* sp.); E. ES18-5; F. ES18-11 (*Glomus badium*); G. ESR18-2 (Glomeraceae/*Funneliformis* sp.)

12) ES_cherry/27

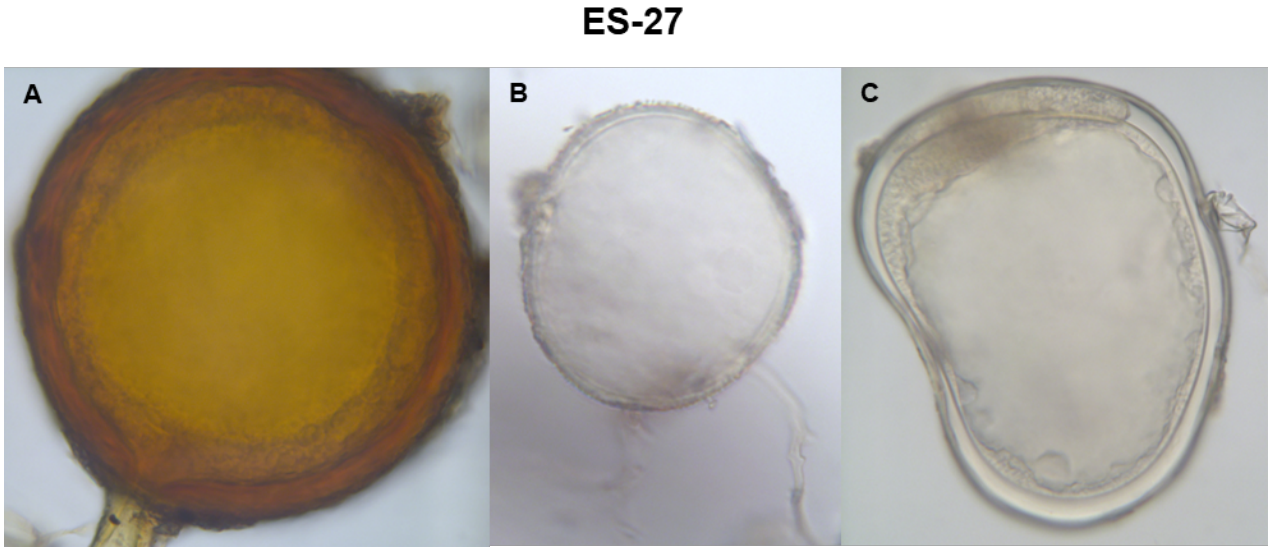

Figure 14. Representative spores of glomoid (A; B) and gigasporoid (C) morphotypes (ES27-n): A. ES27-8 (*Entrophospora etunicata*); B. ES27-15 (*Pacispora* sp.); C. ES27-1 (*Scutellospora* sp.)

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13) ES_apple-grapes/28

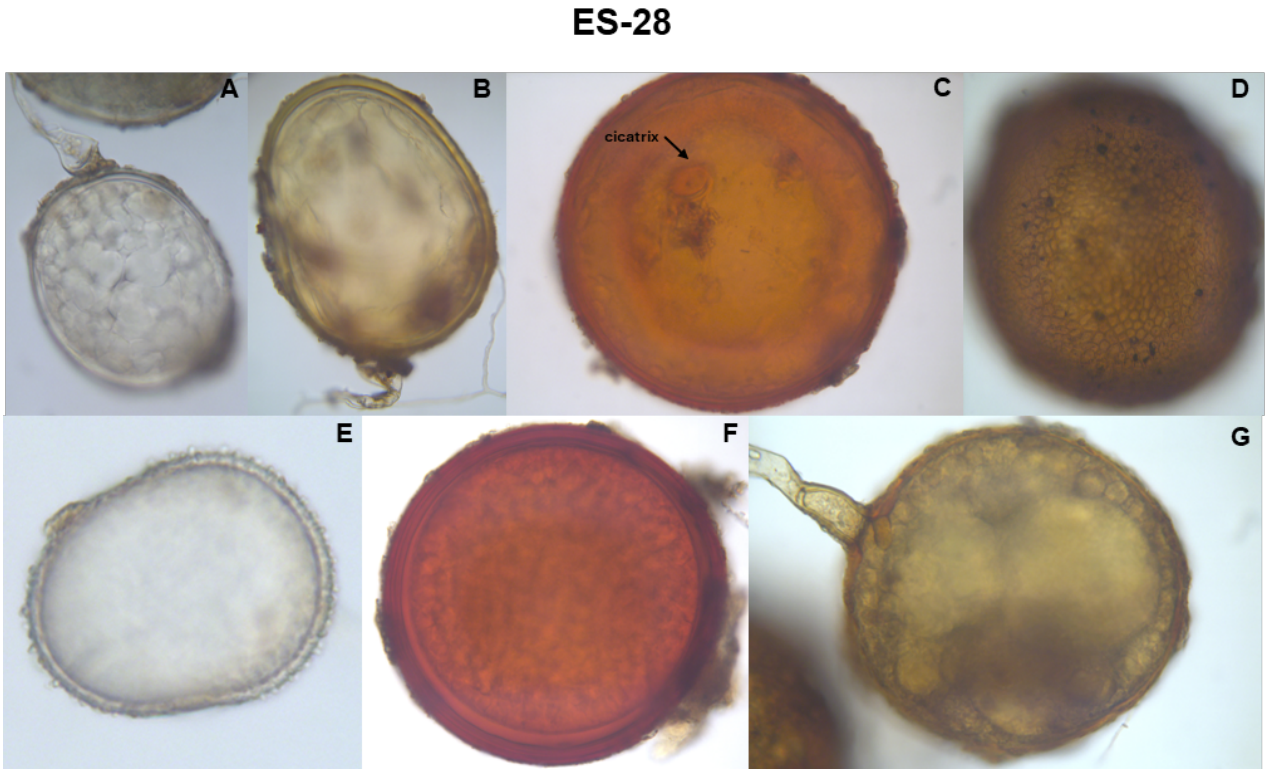

Figure 15. Representative spores of gigasporoid (A; B), acaulosporoid (C; D) and glomoid (E; F; G) morphotypes (ES28-n): A. ES28-12; B. ES28-6-bis; C. ES28-1 (*Acaulospora laevis*-like); D. ES28-7; E. ES28-15 (*Pacispora* sp.); F. ES28-2 (*Funneliformis geosporus*); G. ES28-11 (Glomeraceae/*Funneliformis* sp.)

14) CY_olives/26

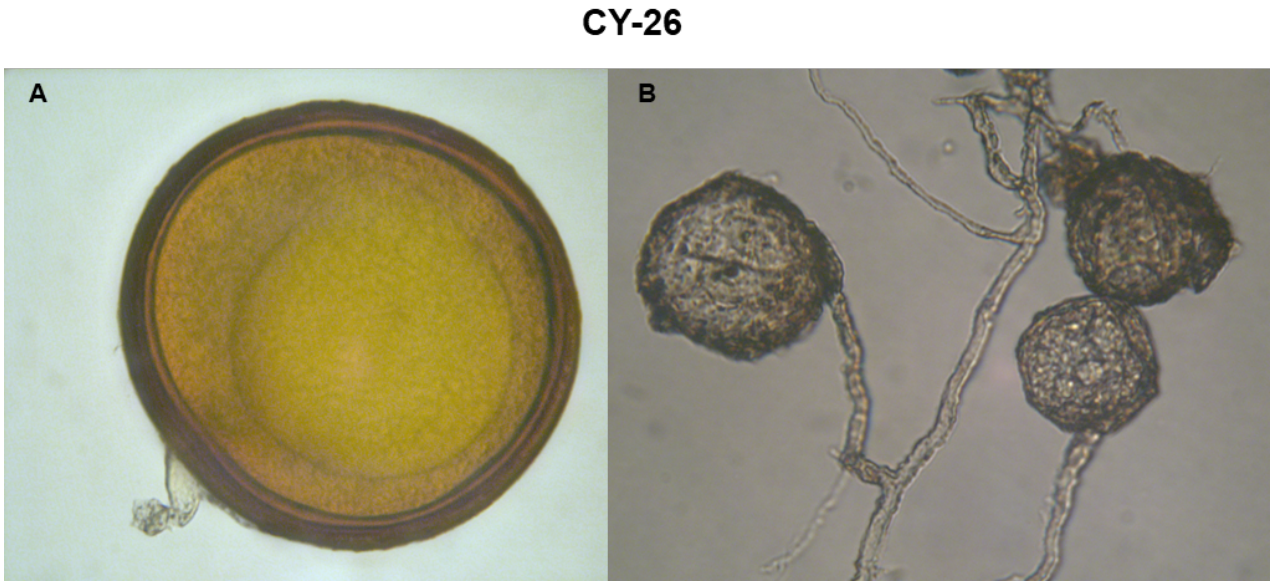

Figure 16. Representative spores of glomoid (A; B) morphotypes (CY26-n): A. CY26-5 (*Entrophospora etunicata*); B. CY26-11 (*Septoglomus viscosum*).

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3. Production of native AMF of the 14 LLs from 7 European countries, for seed inoculation

3.1 Methodology

The pooled soil samples of 14 experimental LLs from 7 European countries (the Netherlands, Serbia, Portugal, Spain, Italy, Greece and Cyprus), received in the period July-December 2023, were utilised for the reproduction of the autochthonous AMF, which was carried out in pot cultures, given the AMF status of obligate biotrophic symbionts.

Each soil type was mixed 1:1 (v:v) with sterile TerraGreen (calcinated attapulgite clay; Oil-Dri, Chicago, IL, USA) and used to fill five 2L pots. Three highly mycotrophic plant species - *Vicia villosa* (cv. Villana Rea), *Cichorium intybus* (cv. Zuccherina di Trieste) and *Medicago sativa* (cv. Messe) - were utilised as trap plants, in order to obtain a high root colonization, the production of large numbers of AMF spores and a high quantity of extraradical mycelium. The seeds of such plant species were sown in the pots and the seedlings thinned out twice, so as to obtain an even distribution of the three plant species in each pot (Fig. 17).

Figure 17. Trap pot-cultures for the production of native AMF inocula from the 14 LLs using highly mycothrophic plant species.

The 70 pots were grown in a glasshouse at the DiSAAA-a under ambient natural light and temperature conditions and supplied with tap water as needed.

Five weeks after germination, three root samples per each LLs soil were randomly selected and checked for assessing the mycorrhizal inoculum potential (MIP) of the original soils. The root systems were extracted from soil, carefully washed with tap water, cleared with 10 % KOH in water bath at 80°C for 15 min, neutralized in 2% aqueous HCl, and stained with 0.05% Trypan blue in lactic acid. Percentage of colonized root length was assessed on each root sample under a dissecting microscope (Wild, Leica, Milano, Italy) at ×25 or ×40 magnification by the gridline intersect method (Giovannetti and Mosse 1980). Colonised roots were selected, mounted on microscope slides and observed under a Reichert-Jung (Wien, Austria) Polyvar light microscope to detect intraradical fungal structures.

At the end of the production cycle (May 2024), the trap plants will be removed from the pots, root samples from each plant species will be analysed for assessing mycorrhizal colonization, following the method described above. A second MIP assay will be performed in order to assess the activity of the inocula produced from each LL.

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3.2 Results

3.2.1 MIP assay. Five weeks after germination the MIP, expressed as percentage of mycorrhizal root length, varied greatly among the different LLs soils (Table 3). The highest values were found in IT_citrus/23 and CY_olives/26 soil samples, where the autochthonous AMF successfully established mycorrhizal symbioses, showing a high root colonization, 36.7% and 33.3%, respectively. On the opposite side, the lowest values were found in NL_onion/12 (2.3%), PT_olives/22 organic (2.3%) and RS_maize/13-RS_soybean/14 (1.0%) (Fig. 18).

Table 3. Mycorrhizal Inoculum Potential (MIP), expressed as percentage of mycorrhizal root length, of the 14 LLs soils from 7 European countries, 5 weeks after seed germination.

LL code number	MIP (mean ± SE)
NL_onion/12	2.3 ± 1.3
RS_maize/13-RS_soybean/14	1.0 ± 0.0
PT_olives/22 organic	2.3 ± 1.3
PT_cowpea/17-PT_olives/22 conventional	4.3 ± 1.8
PT_cowpea/17 organic	6.0 ± 1.0
ES_apple-grape/28	16.7 ± 3.3
ES_cherry/27	23.3 ± 3.3
ES_rice/18	26.7 ± 3.3
IT_citrus/23	36.7 ± 3.3
IT_grapes/24	18.3 ± 1.7
IT_triticale/15	26.7 ± 3.3
GR_grapes/25	5.0 ± 0.0
GR_wheat/16	6.0 ± 2.1
CY_olives/26	33.3 ± 3.3

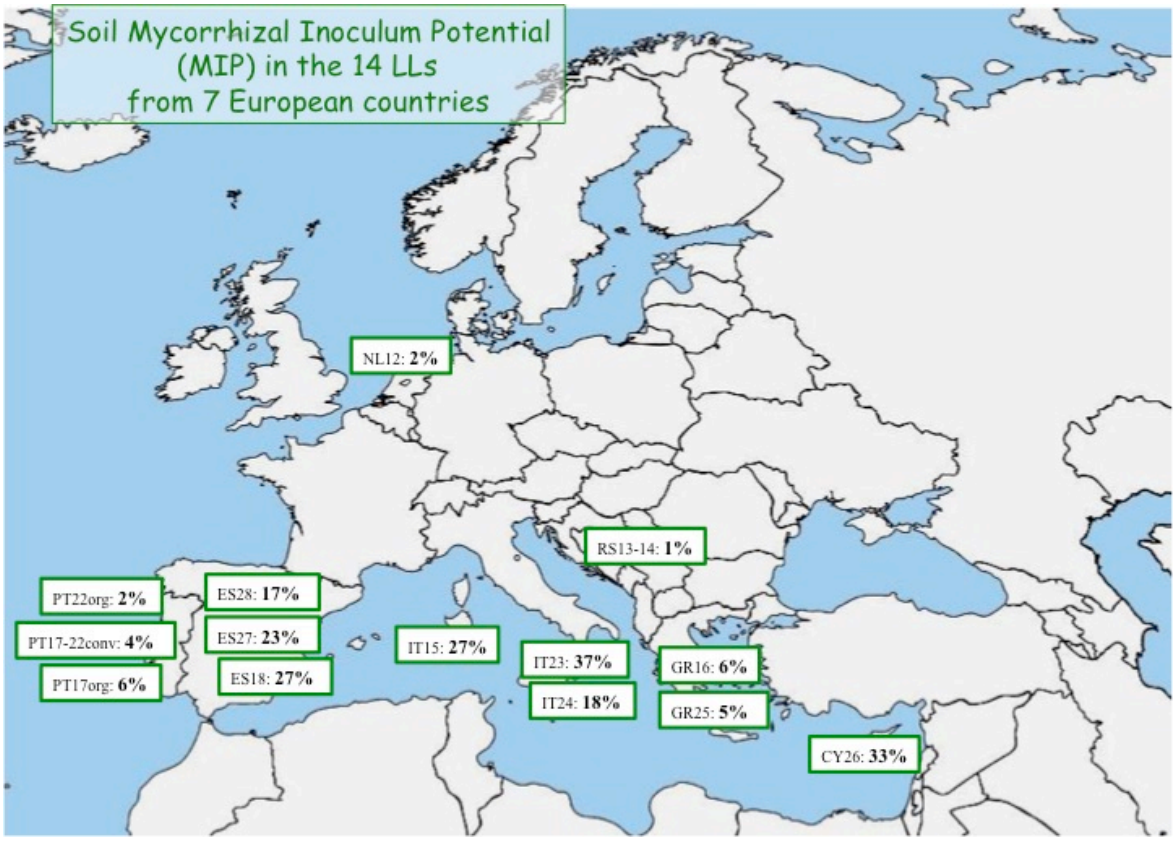

Figure 18. Mycorrhizal Inoculum Potential, expressed as percentage of mycorrhizal root length, of the 14 LLs soils from 7 European countries.

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4. Protocols and guidelines for successful seed inoculation of cover crops.

The protocol was devised in order to utilize seed inoculation as a delivery system of AMF to cover crops. The proper type and concentration of inoculum-coating material mixture were detected as crucial parameters for seed germination and viability of the AMF inoculants.
The study was focused on detecting the best and simplest protocols for on-farm application.

4.1 Methodology

4.1.1 Seeds

The protocol for AMF seed inoculation was tested on the seeds of eleven plant species sent by different LLs.
The seeds of the different plant species to be used for testing the protocol were selected on the basis of their different shape, size, weight and coat.

- Smooth small size seeds were represented by *Medicago truncatula* and *Trifolium incarnatum* from IT_triticale/15 and ES_apple-grapes/28 LLs, respectively.
- Rough small size seeds were represented by *Lolium perenne* and *Phacelia* sp. from ES_apple-grapes/28 and NL_onion/12 LLs, respectively.
- Smooth medium size seeds were represented by *Triticum durum*, *Vicia villosa* from CY_olives/26 and IT_citrus/23-IT_grapes/24 LLs, respectively.
- Rough medium size seeds were represented by *Avena sativa*, *Hordeum vulgare, Secale cereale*) from IT_citrus/23-IT_grapes/24, ES_rice/18 and PT_cowpea/17-PT_olives/22 LLs, respectively.
- Smooth large size seeds were represented by *Pisum sativum, Vicia faba minor* from CY_olives/26 and IT_citrus/23-IT_grapes/24 LLs, respectively.

4.1.2 AMF inoculum

The AMF inoculum utilized for protocol testing was represented by a consortium of 4 AMF species produced by the UNIPI partner. The four AMF species belonged to: *Funneliformis coronatus* (Giovann.) C. Walker & Schüßler (isolate IMA3), *Funneliformis mosseae* (T.H. Nicolson & Gerd.) C. Walker & A. Schüßler (isolate IMA1), *Rhizoglomus irregulare* (Błaszk., Wubet, Renker & Buscot) Sieverd., G.A. Silva & Oehl and and *Septoglomus* sp. 14W1. The crude inoculum was obtained from pot cultures maintained in the collection of the Microbiology Laboratories of the Department of Agriculture, Food and Environment, University of Pisa, Italy (International Microbial Archives, IMA), and produced in greenhouse, by growing *Medicago sativa* L. and *Cichorium intybus* in 1.0 L plastic pots containing a mixture (1:1, by volume) of soil and calcined attapulgite clay (TerraGreen Oil-Dri, Chicago, IL). The soil was a sandy loam collected at the University farm, near San Piero a Grado (Pisa). Chemical and physical characteristics of the soil used were as follows: pH (H_2O), 8.0; clay, 15.3%; silt, 30.1%; sand, 54.5%; organic matter, 2.2% (Walkley-Black); extractable P, 17.6mg kg−1 (Olsen); and extractable K, 149.6 mg kg−1. The mixture was steam sterilized (121◦C for 25 min, on two consecutive days), to kill naturally occurring AMF. Each pot was inoculated with a crude inoculum (mycorrhizal roots and soil containing spores and extraradical mycelium) of each isolate. After four months, the occurrence of AMF spores, sporocarps and mycelium was checked using the wet sieving and decanting technique, down to a mesh size of 50 µm. The mixed crude inoculum was sieved (1.25 mm sieve mesh) in order to separate roots and remove TerraGreen. The roots were chopped using a mixer and added to the sieved portion (fine inoculum). TerraGreen was wet sieved and decanted down to a mesh size of 50 µm and the material retained on the sieves was flushed into Petri dishes, then added to the fine inoculum, which was used to inoculate the seeds.

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4.1.3 Laboratory protocol development

As the shape, size, weight and coat of the seeds of the eleven plant species were very variable (Fig. 19), different quantities of inoculum and liquid adhesive material (LAM) were utilized for seed inoculation. The LAM, of green color, was obtained from Hello Nature (Italy), denominated "Coveron adesivante liquido".

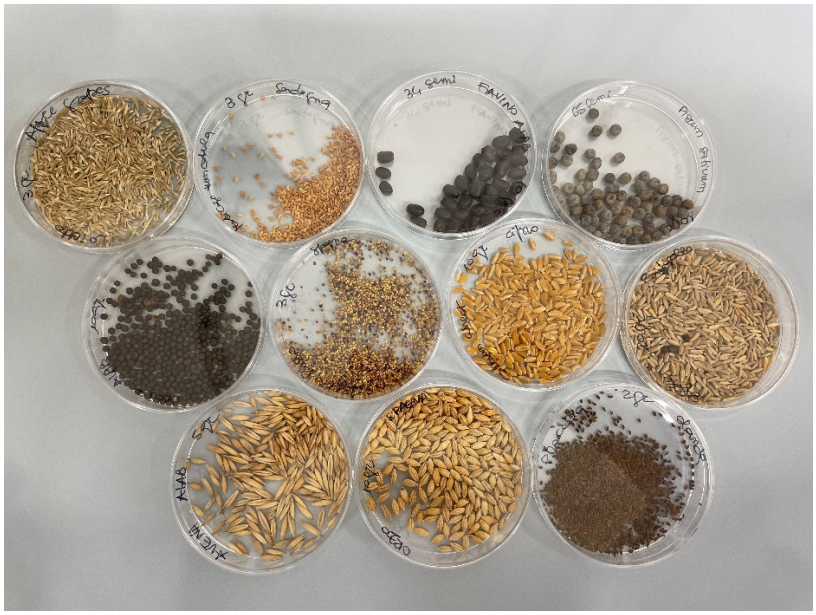

Figure 19. Seeds of eleven plant species characterized by different shape, size, weight and coat obtained from different LLs.

Seed inoculation was performed in 9 cm Petri dishes, utilizing the following inoculum and liquid adhesive material (LAM):

-smooth small size seeds (*Medicago truncatula*, *Trifolium incarnatum*)
For 3g of seeds, 20 µl LAM + 200 µl water + 600 mg AMF inoculum

-rough small size seeds (*Lolium perenne*, *Phacelia* sp.)
For 3g of *L. perenne* seeds, 20 µl LAM + 200 µl water + 900 mg AMF inoculum
For 2g of *Phacelia* sp. seeds, 20 µl LAM + 200 µl water + 600 mg AMF inoculum

-smooth medium size seeds (*Triticum durum*, *Vicia villosa*)
For 10g of *T. durum* seeds, 20 µl LAM + 200 µl water + 300 mg AMF inoculum
For 10g of *V. villosa* seeds, 10 µl LAM + 100 µl water + 300 mg AMF inoculum

-rough medium size seeds (*Avena sativa*, *Hordeum vulgare, Secale cereale*)
For 5g of *A. sativa* seeds, 20 µl LAM + 200 µl water + 300 mg AMF inoculum
For 10g of *H. vulgare, S. cereale* seeds, 20 µl LAM + 200 µl water + 300 mg AMF inoculum

-smooth large size seeds (*Pisum sativum, Vicia faba minor*)
For 10g of seeds, 10 µl LAM + 100 µl water + 300 mg AMF inoculum

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4.1.4 Methodological details

Seed inoculation was carried out in three steps:

1) Addition of LAM and water in the Petri dish;
2) Addition of seeds, covering of the Petri dish, immediately followed by vigorous shaking (few seconds);
3) Addition of AMF inoculum on the seeds, paying attention to a uniform distribution, covering of the Petri dish, immediately followed by shaking continuously until the LAM-AMF inoculum mixture is uniformly distributed and completely absorbed (visible for the uniform green color of the seeds, examples in Fig. 20).

Figure 20. Inoculated green seeds with different shape, size and coat (below), compared with non-inoculated ones (above) belonging to three different plant species.

4.2 Results

4.2.1 Seed inoculation

All the eleven species resulted uniformly covered by the AMF inoculum, as shown in Fig. 20. Pieces of roots and spores, which are privileged sources of AMF inoculum resulted attached to the seeds (see Figs 21-25).

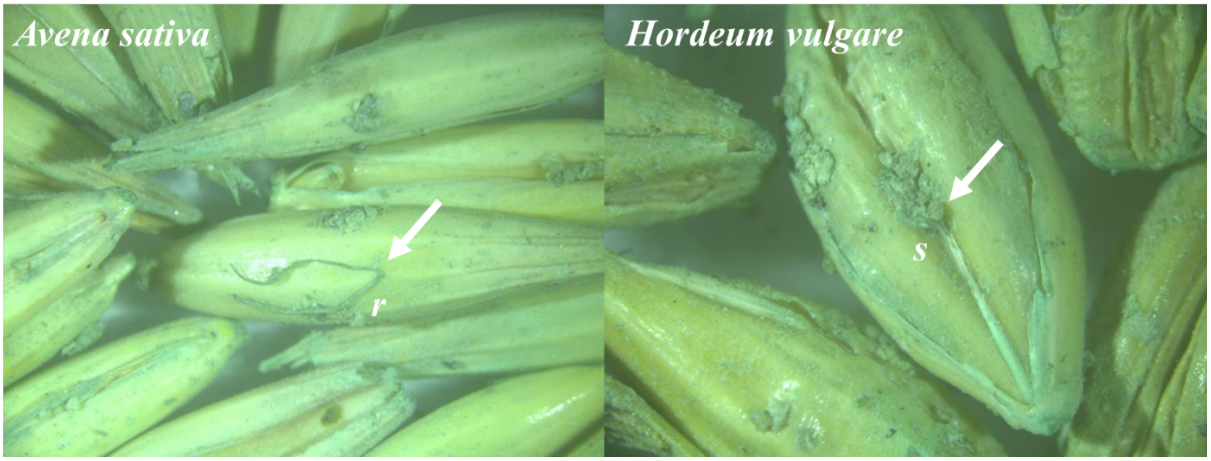

Figure 21. AMF inoculated *Avena sativa* and *Hordeum vulgare* seeds. Arrows indicate roots pieces (r) and AMF spores (s).

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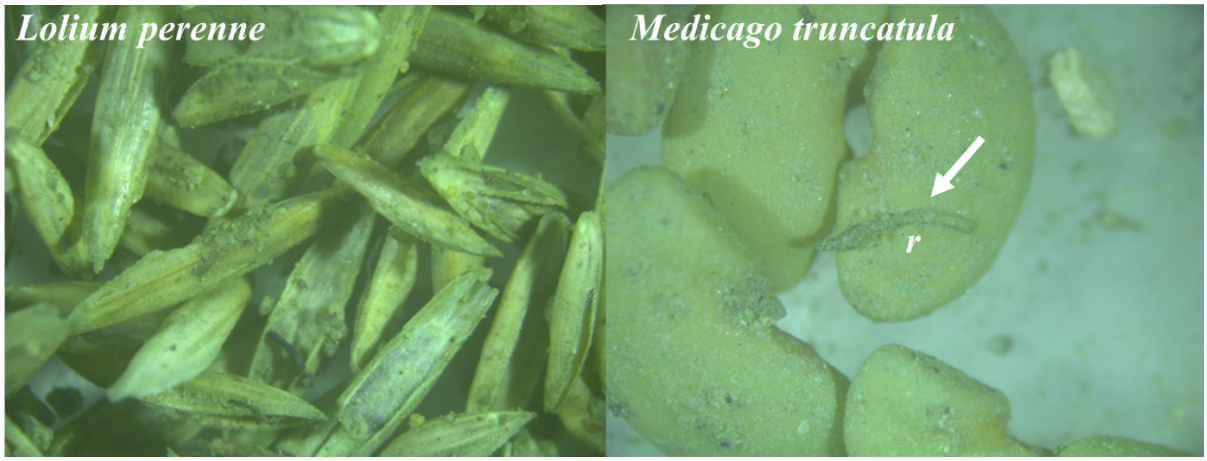

Figure 22. AMF inoculated *Lolium perenne* and *Medicago truncatula* seeds. Arrow indicates roots pieces (r).

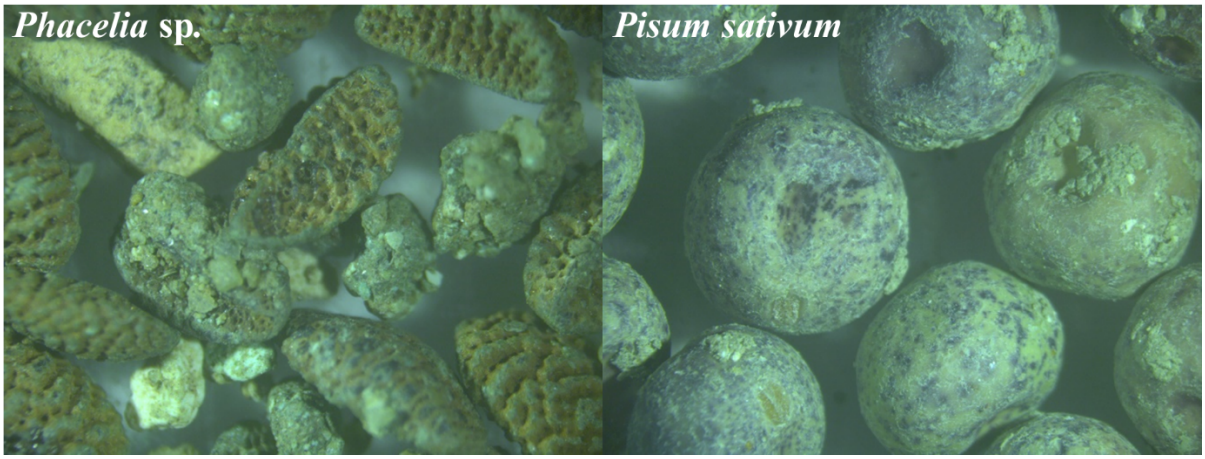

Figure 23. AMF inoculated *Phacelia sp.* and *Pisum sativum* seeds.

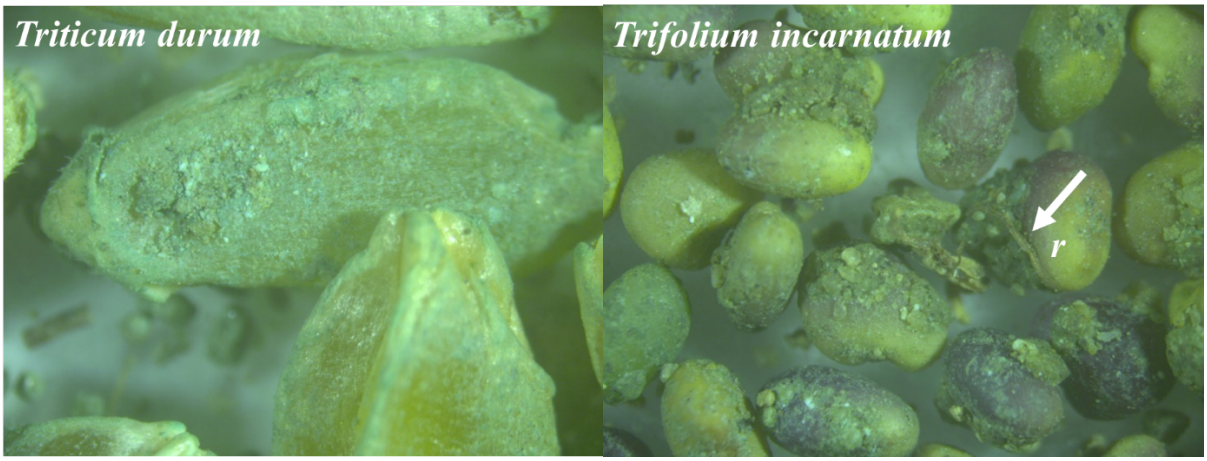

Figure 24. AMF inoculated *Triticum durum* and *Trifolium incarnatum* seeds. Arrow indicates roots pieces (r)

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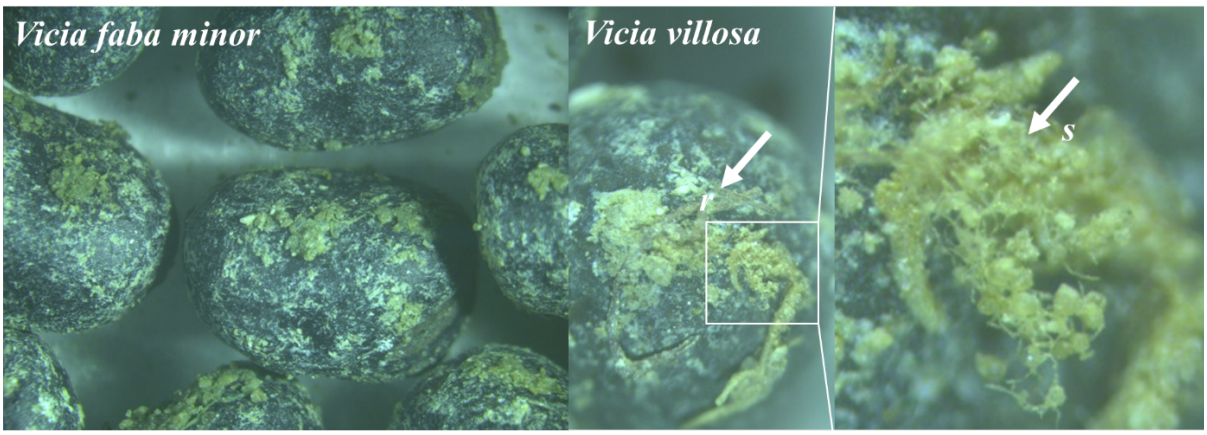

Figure 25. AMF inoculated *Vicia faba minor* and *Vicia villosa* seeds. Arrow indicates roots pieces (r) and spore clusters (s) are highlighted in the box (arrow).

4.2.2 Seed germination

The protocol utilized for seed inoculation did not affect seed germination. Five days after sowing, most seeds showed high germination percentages (80-100%), while species such as *Medicago truncatula* and *Phacelia* sp. showed low germination percentages (60%). Interestingly, two days after sowing some species, such as *Vicia villosa* and *Vicia faba minor* showed a positive effect of AMF inoculation on seed germination (Fig. 26).

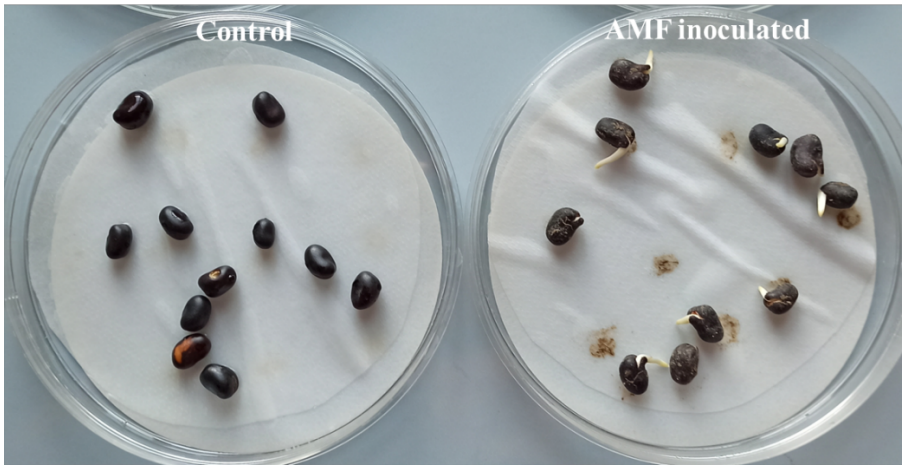

Figure 26. Non-inoculated (Control) *Vicia faba minor* seeds *vs* AMF inoculated ones, two days after sowing.

4.2.3 Rootlets elongation

The positive effect of seed inoculation was also observed on rootlets growth. Six species over eleven (*Pisum sativum, Triticum durum, Secale cereale, Vicia faba minor, Vicia villosa* and *Trifolium incarnatu*m) showed significant longer rootlets in inoculated seeds compared with control (65%, 53%, 37%, 34%, 37%, 29%, 23% longer, respectively). In Fig. 27 an example is reported.

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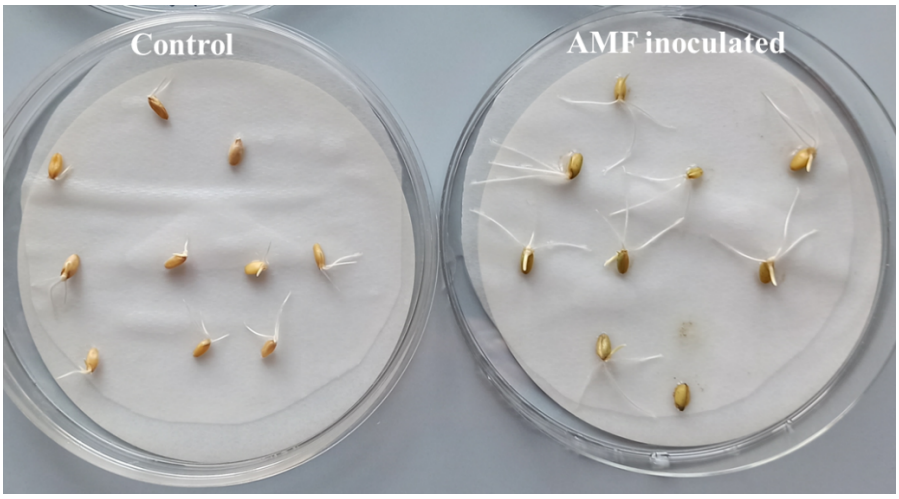

Figure 27. Non-inoculated (Control) *Triticum durum* seeds *vs* AMF inoculated ones, two days after sowing.

4.3 Equipment for seed inoculation in the LLs

The appropriate quantity of AMF inoculum and LAM to be diluted in water will be provided by UNIPI to each LL.

In due time, each LL will communicate the species of cover crop to be inoculated, in order to prepare and send the material.

Seed inoculation by LLs should be performed utilizing the appropriate container, taking into account that the containers should be filled with the seeds up to 1/3, in order to shake seeds and the inoculation material, and reach an even coating.

Examples of the type of containers with tight closure are provided in Fig. 28.

Figure 28. Examples of containers with tight closures to be utilized for seed inoculation.

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