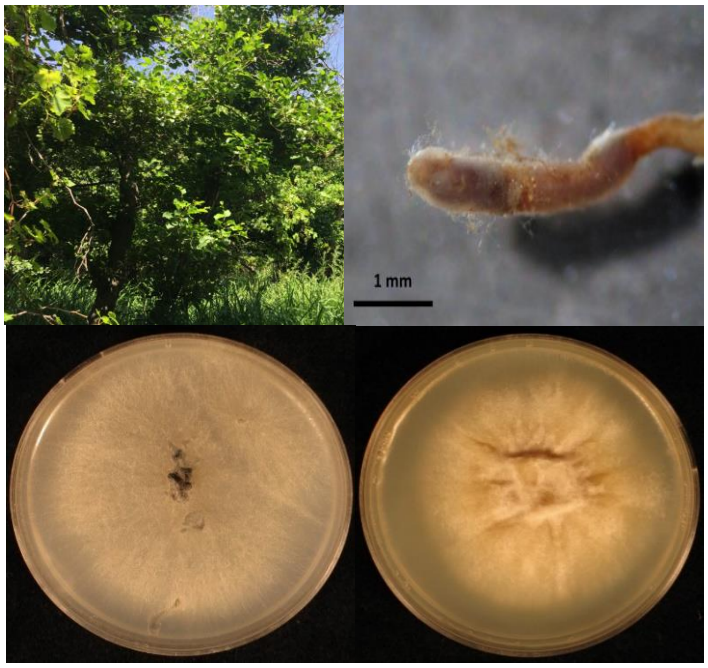
ΓΕΩΠΟΝΙΚΟ ΠΑΝΕΠΙΣΤΗΜΙΟ ΑΘΗΝΩΝ
AGRICULTURAL UNIVERSITY OF ATHENS

LIFE ANDROS PARK

LIFE ANDROS PARK

"Conservation of priority species and habitats of Andros Island protected area integrating socioeconomic considerations"



ACTION C.3

Catalogue of living pure cultures maintained for development of fungal (ECM) inocula

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AGRICULTURAL UNIVERSITY OF ATHENS

June 2021

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ΙΝΣΤΙΤΟΥΤΟ ΑΓΡΟΤΙΚΗΣ ΟΙΚΟΝΟΜΙΑΣ ΚΑΙ ΚΟΙΝΩΝΙΟΛΟΓΙΑΣ (ΙΝΑΓΡΟΚ) ΕΛΛΗΝΙΚΟΣ ΓΕΩΡΓΙΚΟΣ ΟΡΓΑΝΙΣΜΟΣ - ΔΗΜΗΤΡΑ
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Abstract

Restoration of the alluvial stands with *A. glutinosa* is one of the primary objectives of the LIFE Andros Park project. In the frame of Action A.1, collection, isolation in pure cultures and identification of alder-associated microorganisms (i.e., those growing either in plant roots or rhizosphere) was carried out in conjunction with evaluation of inoculation with ectomycorrhizal fungi (ECM) to enhance growth and/or adaptability of young alder seedlings during restoration/regeneration of deteriorated *A. glutinosa* stands. This was the outcome of field-trips performed in selected sites of Andros island during the period from 2017 to 2019. ECM mushrooms, alder roots and rhizosphere soil samples were obtained and further processed in the lab to isolate microorganisms in pure culture (e.g. ectomycorrhizas, other endophytic and soil-inhabiting fungi and actinobacteria) through the use of various techniques, and then to identify them by morphological and molecular approaches. Several hundred fungi were isolated in pure cultures, studied and grouped to provide 91 representative types of colonies which were further examined through the use of morphological criteria (colony appearance, microscopical features) and DNA sequencing.

In the frame of Action C.3 ("Ex-situ conservation actions for selected plants deriving from the priority habitat 91E0*, and of macrofungi associated with *Alnus glutinosa* trees"), 93 selected pure cultures – among those previously assessed – were further processed and maintained in the fungal Culture Collection of the Laboratory of General and Agricultural Microbiology, Agricultural University of Athens. Maintenance and storage follow the pertinent established protocols, and are implemented in the form of mycelium colonies in agar slants and as mycelium plugs submerged in distilled/sterilized water at 4 °C.

Action C.3

Deliverable titled: "Catalogue of living pure cultures maintained for development of fungal (ECM) inocula"

Introduction

Trees of the genus *Alnus* are known to form symbiotic relationships with various basidiomycetes and ascomycetes (Harley and Smith, 1983); approx. 1000 fungal species were described from ecologically diverse alder stands in Europe, and an estimated 120 species are considered to be symbiotic (Boyle 1996, Dimou et al. 2002, Polemis et al. 2012). In addition, mycocoenological studies from Europe and North America evidenced that ectomycorrhizal (ECM) fungi of *Alnus* show a remarkably high degree of host specificity compared with other tree species (Griesser, 1992; Arnolds et al., 1995, Pritsch et al., 1997).

It has been suggested that the abundant occurrence of symbiotic fungi in wet forests, such as the alder forests, is presumably an adaptation to survive adverse environmental conditions, e.g. those in water-saturated soil under oxygen poor conditions (Baar et al. 2000). Therefore, both partners (plant and fungus) depend heavily on one another for their survival and growth.

For this reason, the assessment of the diversity of *A. glutinosa* associated mycobiota was considered to be of primary importance for the successful implementation of Actions A1, C2 and C3 of the LIFE Andros Park project. The primary objectives were to collect, isolate and identify as many as possible of the alder-associated fungi (i.e. those growing either in plant roots or rhizosphere), as well as to evaluate the potential use of selected strains to serve as inocula for enhancing growth and/or adaptability of young alder seedlings in restoration/regeneration of deteriorated *A. glutinosa* stands. Moreover, a large battery of strains will be suitably prepared for long-term ex-situ conservation, which will permit the development of future pertinent applications.

In the frame of Action A.1, collection, isolation in pure cultures and identification of alder-associated microorganisms (i.e., those growing either in plant roots or rhizosphere)

was carried out in conjunction with evaluation of inoculation with ectomycorrhizal fungi (ECM) to enhance growth and/or adaptability of young alder seedlings during restoration/regeneration of deteriorated *A. glutinosa* stands.

Field-trips were performed in selected sites of Andros island during 2017-2019. ECM mushrooms, alder roots and rhizosphere soil samples were obtained and further processed in the lab to isolate microorganisms in pure culture (e.g., ectomycorrhizas, other endophytic and soil-inhabiting fungi and actinobacteria) through the use of various techniques, and then to identify them by morphological and molecular approaches. Several hundred fungi were isolated in pure cultures, studied and grouped to provide 91 representative types of colonies which were finally selected for further examination, separated according to their morphology (colony appearance, microscopical features) and subjected to molecular analysis (DNA sequencing). The majority of the materials identified were soil-borne ascomycetes which generally possess an opportunistic ecology, maneuvering between different trophic habits depending on environmental conditions.

The present deliverable is a catalogue of 93 living pure cultures which were carefully selected from the biological material presented in a previous deliverable ("Pure cultures of several ectomycorrhizal fungi growing in association with *A. glutinosa* for preparing inocula, which will be used in producing alder seedlings in the nursery"; Action A.1) to serve for the production of either single or multi-species inocula (including ECM fungi) to be used at generating alder trees suitable for large-scale restoration/reforestation projects.

Description of principles and methodologies implemented for maintaining and storage of the biological material in the fungal Culture Collection

Pure cultures of fungi were obtained from sampling performed during the period 2017 – 2019 in three mountainous (>500 m a.s.l.; Evrousies, Vourkoti, Katakaleoi), and three coastal and low-altitude (0-250 m a.s.l.; Achla, Vori and Lefka) *A. glutinosa* stands. Mushrooms as well as alder roots and rhizosphere soil samples were obtained and further processed in the lab to isolate microorganisms in pure culture (e.g. ectomycorrhizas, other endophytic and soil-inhabiting fungi and actinobacteria) through the use of various

techniques, and then to identify them by morphological and molecular approaches. Mushrooms were collected from the priority habitat, while environmental samples (alder rhizosphere soil and root fragments) were obtained from positions adjacent to *Alnus glutinosa* trees and/or from where the presence of ectomycorrhizal mushrooms was detected. Then, sampled material was placed in bags, kept in a portable fridge and transferred to AUA (Laboratory of General and Agricultural Microbiology) facilities for further processing.

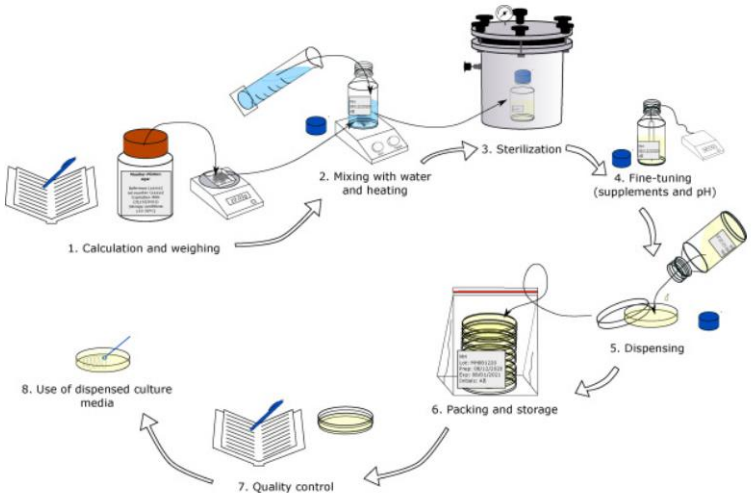


Fig. 1. Illustrated process for the preparation of culture media used for routine maintenance of fungal strains prior to storage (source: Orekan et al.; https://doi.org/10.1016/j.cmi.2021.05.016)

In the lab, different processes were implemented depending on the type of sample. Basic guidelines for preparation of nutrient media for the growth of fungi were adopted (Fig. 1) and then suitable adjustments were made as follows:

1. For obtaining pure cultures from basidiomes of ectomycorrhizal fungi, small fragments of fresh samples were transferred under aseptic conditions to petri dishes with suitable nutrient media (MMN, Hagem). In addition, spores (obtained from spore-prints) and mushroom-tissue suspensions in sterile water were used to inoculate nutrient media in petri dishes. After this process was completed, mushrooms specimens were dried at 50 °C, frozen at -80 °C for 24 hours, and further stored as dried specimens prior to their examination (for identification purposes).

2. For isolation of alder symbiotic (endophytic) microorganisms, the following procedure was adopted: Each soil sample was passed through sieves of different diameter, and then root tips and nodule-like structures were harvested and submerged into distilled water for 12 h. Each sample was then examined with a stereoscope. Different ECM morphotypes and nodule-like structures were separated, recorded and photographed. Then they were provisionally stored at 4 °C prior to further microscopic examination and isolation of microorganisms in pure culture.

2.a. Root tips from each distinct ECM morphotype was surface-sterilized (solutions of either 30% chlorine or 70% ethanol with 3-5% chlorine, v/v) fragmented into parts of 1-2 mm and placed into petri dishes with suitable nutrient media (MMN, Hagem).

2.b. Root-fragments including nodule-like structures were thoroughly washed to remove soil, transferred to flasks containing 50 ml of CTAB and agitated at 150 rpm for 30 min. After rinsing with sterilized water, they were surface sterilized with $HgCl_2$ 0.1% (w/v) or 3% H_2O_2 or with Na-hypochlorite solution, and then rinsed five times with sterilized water. The nodules were cut with a razor blade, transferred in sterile Eppendorf-tubes with 1 ml sterilized water and crushed with a sterile glass or metallic pestle. The resulting slurry was placed into petri dishes with nutrient medium (PDA).

3. For isolation of culturable fungi from the rhizosphere of *A. glutinosa*, root-fragments were gently shaken off, and the excess soil was discarded. Then the root-fragment with the closely adhering soil was placed into a flask containing sterilized tap water and stirred for 2 hours at 100 rpm. Serial soil dilutions were prepared and shaken vigorously. This method yielded dilutions of 10^{-1} to 10^{-4}, which were spread (1 ml) onto petri dishes with PDA. All plates were placed for incubation and inspected at regular intervals. The rhizosphere fungal population ranged from 1.5 X 10^5 to 3.0 x 10^6 propagules/root.

Irrespectively of whether cultures derived directly from mushrooms, ECM root tips, root nodule-like structures or rhizosphere soil, when two or more different colonies appeared, they were individually transferred to new nutrient media in petri dishes until pure cultures were established for each isolate. Then colonies and their microscopic features were studied, and photographs were obtained.

Fungal isolates were identified by molecular analysis of the internal transcribed spacer (ITS, rDNA) after performing total genomic DNA extraction from fungal mycelium. The primer set ITS1 and ITS4 targeting ITS1 and ITS2 fragments of approximately 600bp was used for polymerase chain reaction (PCR) amplification. The ITS sequence was determined by direct sequencing of the PCR product and was performed by CeMIA SA, Greece. Analysis of sequence was performed with the basic sequence alignment BLAST Program run against the database provided in the website of National Center for Biotechnology Information (web site: http://www. ncbi.nlm.nih.gov/BLAST).

Among the few hundred fungal isolates obtained in pure culture during the implementation of Action A.1, a large part was rejected from further processing due to their similar/identical morphology or because they were not related to the target fungi. For the remaining part, initial identification was based on morphological features of ECM root tips and fungal cultures, e.g., characteristics of colonies formed on nutrient media, structure of hyphae, type of sexual and asexual fructifications, microscopic characters, etc., and led to the classification of fungi up to the genus level. The use of ITS sequencing revealed the existence of several taxa (at species or genus level), while the exact identity of many isolates remains under investigation since they are associated with species complexes and/or with taxonomic groups not adequately resolved through the use of single-gene markers (in the latter case a multi-gene approach is adopted).

The evaluated biological material (representative photos of cultures and their microscopic features are presented in Fig. S1 and Fig. S2) was further processed in order to be suitable maintained and stored in the fungal Culture Collection of the Laboratory of General and Agricultural Microbiology (Agricultural University of Athens) by the methodologies described below:

In general, pure culture methodologies allow to detect, isolate, identify and maintain fungi from a wide array of environments, and to define the nutritional, chemical and other requirements for their growth and metabolism. Hence, pure cultures permit to proceed with both fundamental and applied research, as well as with the commercial exploitation of fungi. As previously stated, pure cultures were initially established in various nutrient media (e.g. MMN, Hagem and PDA), and were then transferred to PDA for long-term storage.

Although there is no universal method for storing fungal cultures, the main goal of this type of ex situ conservation is to preserve viability without contamination, genetic variation, or deterioration. In general, the technique aims either to minimize the risk of changes and eliminate frequent transfer by extending the periods between subculture or by bringing cellular activity to a halt. Selection of a method is based on its advantages or disadvantages, the amenability of the culture to preservation and its future use, and the equipment, personnel, and financial resources available. Culture collections usually employ two different types of preservation and store backup or stock off site as an added protection.

Fig. 2. Fungal cultures provisionally stored in agar slants.

In the case of our collection, serial transfers were initially performed to maintain pure cultures in the laboratory for short periods and to check their viability. Fungi were grown on agar slants that support maximum growth at intervals between transfer from old to fresh slants depending on the species, usually varying from few weeks to 3-4 months by storing them at 4 °C (Fig. 2). Transfers were performed by using either specialized propagative cells or pieces of mycelia from young, actively growing (at the margin) parts of the colony.

Long-term storage was performed by two main methods, i.e., in sterile mineral oil and submerged under water:

a. The first method is based on the extension of the storage life of an agar slant with an overlay of sterile mineral oil (Figs. 3 and 4). This technique decreases dehydration of the medium, retards metabolic activity of the culture by

reducing oxygen tension, and lessens the chance of mite infestations. Oil overlay is particularly useful for mycelial or nonsporulating forms, which cannot be freeze-dried or frozen successfully, and in small collections, where freeze-drying or cryopreservation is not economical (as it is in our case). Cultures are grown on suitable agar slants until acceptable growth or sporulation has occurred, then covered with sterile oil to 1 cm above the edge of the agar under aseptic conditions. Slants are stored in an upright position for years at refrigeration temperature. At appropriate intervals cultures must be transferred to fresh agar slants and the process repeated. More than one subculture may be necessary after retrieval from storage, because the growth rate can be reduced due to adhering oil. In more detail: High-quality mineral oil is sterilized by autoclaving at 1.1 atm pressure for 2 hours. Entrapped moisture is removed by heating the liquid in a drying oven at 170°C for 1-2 hours (optional). Fungal cultures grown on agar slants are covered with about 10 mm of oil. The entire agar surface and fungal culture are submerged completely in the oil. The tubes are kept in an upright position at 4 °C. The oil level in the tubes or vials are checked periodically, and more oil should be added, if necessary. To retrieve a culture from mineral oil, a small amount of the fungal colony is removed and placed on appropriate media after as much oil as possible has been drained. It may be necessary to subculture the colony several times to get a vigorous oil-free culture.

b. The second method is water storage in the form of submerged agar plugs placed in 2 ml Eppendorf tubes at 4 °C (Fig. 5). The method has been used successfully to preserve oomycetes (Clark and Dick 1974; Smith and Onions 1983), basidiomycetes (Ellis 1979; Richter and Bruhn 1989; Burdsall and Dorworth 1994; Croan et al. 1999), ectomycorrhizal fungi (Marx and Daniel 1976), ascomycetes (Johnson and Martin 1992), hyphomycetes (Ellis 1979), plant pathogenic fungi (Boesewinkel 1976), aerobic actinomycetes (van Gelderen de Komaid 1988), and human pathogens and yeasts (McGinnis et al. 1974). Most basidiomycetes survived for at least 2 years at 5°C (Marx and Daniel 1976; Richter and Bruhn 1989); viability decreased after 5-10 years of

storage (Burdsall and Dorworth 1994). This is considered as an inexpensive, low-maintenance method, and has numerous advantages in comparison to the previous one: culture viability or growth rate is not significantly influenced, isolates can be stored longer, genetic stability is greater, and pleomorphic changes are suppressed since the water suppresses morphological changes in most fungi. In addition, water storage is clean, quick, easy, inexpensive, and requires minimal storage space. It has been used successfully for all categories of fungi, including yeasts, ectomycorrhizal fungi, and plant and human pathogens. The most important factors influencing survival in sterile water over a long period are the selection of good sporulating cultures (although it is perfectly suitable for non-sporulating forms as well) and sufficient inoculum. Agar blocks colonized by hyphae cut from actively grown colony margins are used. The advantages of this method are that bacterial contaminants can be eliminated with an antibiotic bath, and mite infections can be eradicated or prevented from spreading during storage in the refrigerator. The procedures used for covering cultures on agar slants with oil also can be used when covering them with sterile distilled water. Alternatively, sterilized custom-made metal cork-borers (3 mm in diam.) are used to cut disks from the growing colony edge. The disks are transferred to screw-cap test tubes; to save space, small (2 ml), sterile, screw-cap cryovials are filled with several discs and topped with sterile distilled water, which are stored at 4°C. Disks are removed aseptically and transferred to fresh agar medium to retrieve cultures. An alternative method for sporulating fungi (McGinnis et al. 1974) involves inoculating agar slants of preferred media with fungal cultures and then incubating them at 25°C for several weeks to induce sporulation. Sterile distilled water (6-7 ml) is added aseptically to the culture, and the surface of the culture is scraped gently with a pipette to produce a spore and mycelial slurry. This slurry is removed with the same pipette and placed in a sterile, 2-dram glass vial (or cryovial). The cap is tightened, and the vials are stored at 25°C. To retrieve a culture, 200-300 µl of the suspension is removed from the vial and placed on fresh medium.

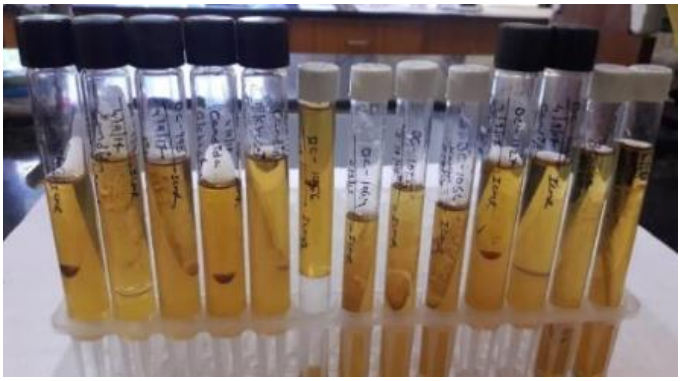


Fig. 3. Fungal cultures stored submerged in mineral oil.

Fig. 4. Fungal cultures stored in agar slants or submerged in mineral oil
(Lab. of General and Agricultural Microbiology, Agricultural University of Athens)

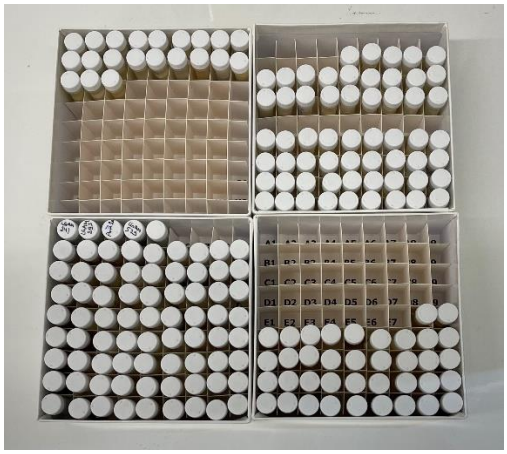


Fig. 5. Fungal cultures stored submerged in water
(Lab. of General and Agricultural Microbiology, Agricultural University of Athens)

Table 1. Catalogue of living pure cultures of fungi associated with *Alnus glutinosa*.

a/a	Species (where available) and strain code	Isolation source	Geographic origin (Andros)
1	*Fusarium solani* FAg 1	Root-fragments incl. nodule-like structures	Katakalaioi
2	*Fusarium solani* EnFAg 2	Root-fragments incl. nodule-like structures	Katakalaioi
3	*Fusarium solani* FEnPl 4	Root-fragments incl. nodule-like structures	Katakalaioi
4	*Fusarium solani* FEnPl 6	Root-fragments incl. nodule-like structures	Katakalaioi
5	*Ilyonectria radicicola* FEnPl 7	Root-fragments incl. nodule-like structures	Katakalaioi
6	*Chaetomium murorum* (syn. *Botryotrichum murorum*) FEnPl 8	Root-fragments incl. nodule-like structures	Katakalaioi
7	*Fusarium solani* FEnPl 11	Root-fragments incl. nodule-like structures	Katakalaioi
8	FEnPl 12	Root-fragments incl. nodule-like structures	Katakalaioi
9	*Fusarium solani* FEnpl 13	Root-fragments incl. nodule-like structures	Katakalaioi
10	FEnPl 14	Root-fragments incl. nodule-like structures	Achla
11	*Fusarium* sp. FEnPl 17	Root-fragments incl. nodule-like structures	Katakalaioi
12	FEnPl 19	Root-fragments incl. nodule-like structures	Achla
13	*Neurospora reticulata* FEnPl 25	Root-fragments incl. nodule-like structures	Achla
14	*Ilyonectria radicicola* FEnPl 31	Root-fragments incl. nodule-like structures	Katakalaioi
15	FEnPl 33	Root-fragments incl. nodule-like structures	Achla
16	*Fusarium solani* FEnPl 34	Root-fragments incl. nodule-like structures	Katakalaioi
17	*Fusarium* sp. [FEnPl 35	Root-fragments incl. nodule-like structures	Katakalaioi
18	*Fusarium solani* FEnPl 37	Root-fragments incl. nodule-like structures	Katakalaioi

19	*Alternaria alternata* FEnPl 38	Root-fragments incl. nodule-like structures	Katakalaioi
20	*Phomopsis* sp. (40, 92) FEnPl 39	Root-fragments incl. nodule-like structures	Katakalaioi
21	AgPF 4	Rhizosphere soil under *Paxillus olivellus* /241131.	Vori
22	AgIF 1	Rhizosphere soil under *Inocybe* sp, /251135.	Vori
23	AgIF 2	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
24	AgIF 3	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
25	*Trichoderma* sp. AgTF 1	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
26	*Phomopsis* sp. AgTF 4	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
27	AgTF 5	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
28	*Penicillium* sp. AgTF 9	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
29	AgTF 11	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
30	AgTF 12	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
31	AgN26F 3	Rhizosphere soil under *Naucoria* sp. /241126.	Vori
32	AgN26F 6	Rhizosphere soil under *Naucoria* sp. /241126.	Vori
33	AgN26F 10	Rhizosphere soil under *Naucoria* sp. /241126.	Vori
34	*Penicillium chrysogenum* AgN26F 11	Rhizosphere soil under *Naucoria* sp. /241126.	Vori
35	AgIF 10	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
36	*Talaromyces ruber* AgIF 13	Rhizosphere soil under *Inocybe*sp. /251135.	Vori
37	*Botrytis* sp. AgIF 15	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
38	AgIF 16	Rhizosphere soil under *Inocybe*s sp. /251135.	Vori

39	*Penicillium* sp. AgIF 19	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
40	AgIF 21	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
41	AgIF 23	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
42	basidiomycete AgIF 26	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
43	*Penicillium* sp. AgIF 27	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
44	AgIF 28	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
45	AgIF 30	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
46	AgIF 32	Rhizosphere soil under *Inocybe* sp. /251135.	Vori
47	AgPF 5	Rhizosphere soil under *P. olivellus* /241131.	Vori
48	AgTF 8	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
49	AgTF 10	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
50	AgTF 13	Rhizosphere soil under *Tomentella* sp. /251142.	Vori
51	AgN25F 6	Rhizosphere soil under *Naucoria* sp. /241125.	Vori
52	AgN25F 2	Rhizosphere soil under *Naucoria* sp. /241125.	Vori
53	AgN25F 1	Rhizosphere soil under *Naucoria* sp. /241125.	Vori
54	*Metacordyceps chlamydosporia* 47-1 C,D	ECM root tip	Katakalaioi.
55	*Metacordyceps chlamydosporia* 47-11 A,B	ECM root tip	Katakalaioi
56	*Phomopsis* sp. 47-31,32 (A,B)	ECM root tip	Katakalaioi
57	48-b1 A,B	ECM root tip	Katakalaioi
58	*Metacordyceps chlamydosporia* 48-b2 A,B (48-b A,B)	ECM root tip	Katakalaioi
59	48-b3 A,B	ECM root tip	Katakalaioi
60	AAL 101 A	ECM root tip	Achla
61	AAL 101 B	ECM root tip	Achla
62		ECM root tip	Achla

	AAL 101 C, 102 B		
63	*Phialocephala fortinii* AAL 101 D	ECM root tip	Achla
64	AAL 102 A	ECM root tip	Achla
65	AAL 102 C	ECM root tip	Achla
66	AAL 102 D	ECM root tip	Achla
67	AAL 103	ECM root tip	Evrousies
68	AAL 114	ECM root tip	Katakalaioi
69	*Lambertella tubulosa* AAL 142 A	ECM root tip	Vourkoti
70	*Umbelopsis* sp. AAL 142 B	ECM root tip	Vourkoti
71	AAL 142 C	ECM root tip	Vourkoti
72	*Apiognomonia lasiopetali* AAL 144	ECM root tip	Vourkoti
73	AAL 147 A	ECM root tip	Lefka
74	AAL 147 B	ECM root tip	Lefka
75	AAL 54 A	ECM root tip (under *Cortinarius* sp. /121131)	Katakalaioi
76	AAL 55	ECM root tip (under *Naucoria* sp. /121133)	Katakalaioi
77	*Cylindrocarpon* sp. AAL 56 A	ECM root tip (under *Naucoria* sp. /121133)	Katakalaioi
78	AAL 58 A, 60 B	ECM root tip (under *Naucoria* sp. /121133)	Katakalaioi
79	AAL 60 A	ECM root tip (under *Naucoria* sp. /121133)	Katakalaioi
80	AAL 75	ECM root tip (under *Inocybe* sp. /251135)	Vori
81	*Knufia* sp. AAL 83	ECM root tip (under *Naucoria* sp. /251125)	Vori
82	AAL 90 A	ECM root tip (under *Naucoria* sp. /251126)	Vori
83	AAL 91	ECM root tip (under *Naucoria* sp. /251126)	Vori
84	AAL 98	Ectomycorrhizal tip	Achla
85	Nau1	Sporocarp suspension from *Naucoria* sp. mushroom	Vori
86	Nau2	Sporocarp tissue from *Naucoria* sp. /251126.	Vori
87	Pax1	Sporocarp tissue from *P. olivellus* /11115.	Katakalaioi
88	Pax2	Sporocarp tissue from *P. olivellus* /220216.	Vori
89	Abi1	Sporocarp tissue from *Abortiporus biennis* / 021118.	Vourkoti

90	Aaj1	Sporocarp tissue from *Auricularia auricula-judae* / 220218.	Vori
91	Gad1	Sporocarp tissue from *Ganoderma adspersum* / 241117.	Vori
92	Gju1	Sporocarp tissue from *Gymnopilus junonius* / 051118.	Vori
93	Tve1	Sporocarp tissue from *Trametes versicolor* / 220218.	Vori

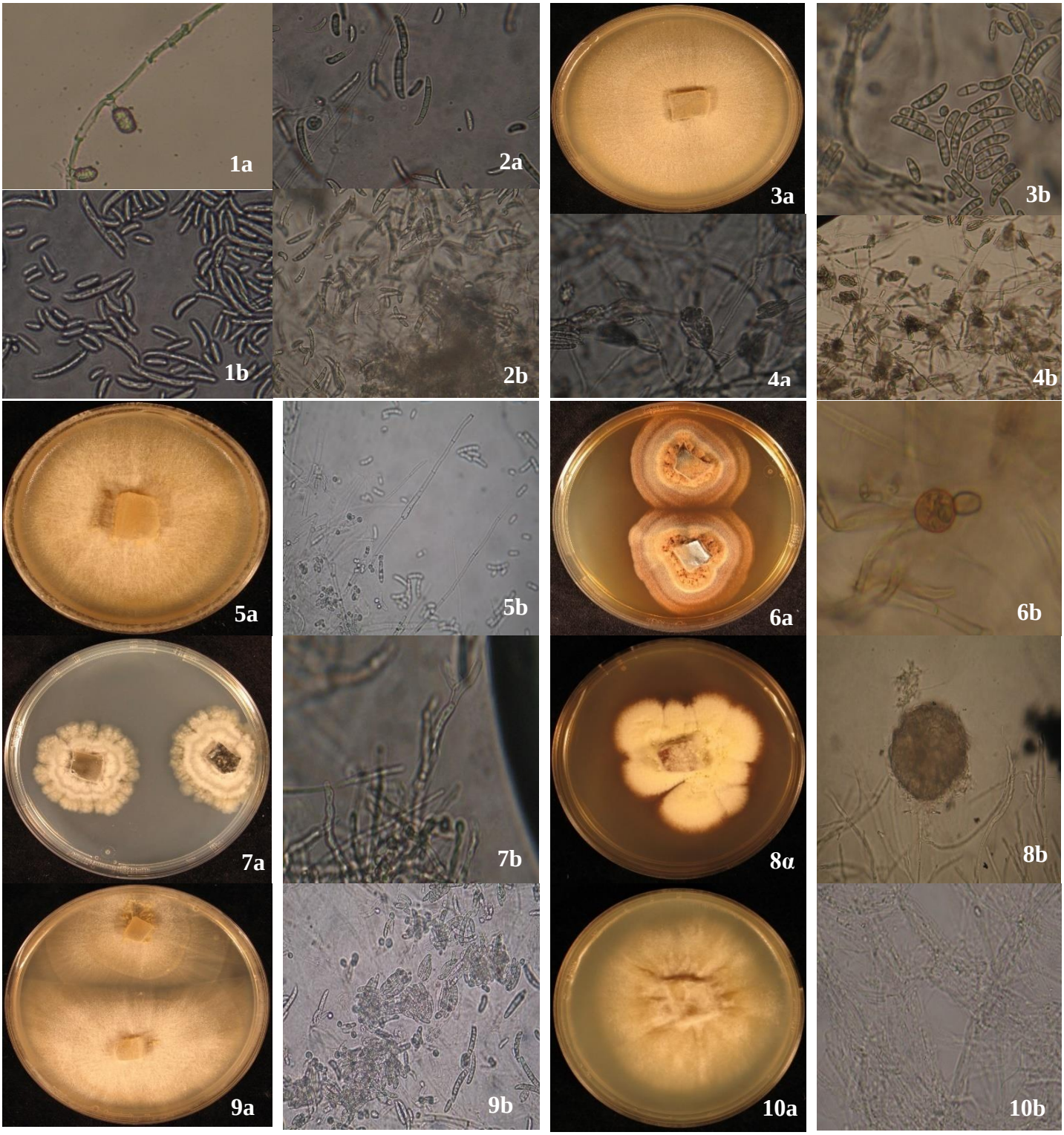


Fig. S1a: 1a) EnFAg1 chlamydospores intercalary, solitary; 1b) EnFAg1 macroconidia elongate to slightly curved; 2a) EnFAg2 macroconidia 4-celled, elongate to slightly curved; 2b) conidiophore 3a) FEnPl4 colony; 3b) FEnPl4 macroconidia 2-celled, elongate; 4a) FEnPl5bact conidiophore; 4b) FEnPl5bact conidiophore, conidia elongate; 5a) FEnPl6 colony 5b) microconidia, ellipsoidal, straight or slightly curved, hyaline, microconidiation; 6a) FEnPl7colony; 6b) FEnPl7 chlamydospore; 7a) FEnPl8 colony; 7b) FEnPl8 lateral or terminal conidiogenous cells (phialides); 8a) FEnPl12 colony; 8b) FEnPl12 pycnidia with conidiophores inside; 9a) FEnPl13 colony; 9b) FEnPl13 conidiophore with elongate conidia, 2-celled, straight or slightly curved; 10a) FEnPl14 colony; 10b) FEnPl14 hyphae septate.

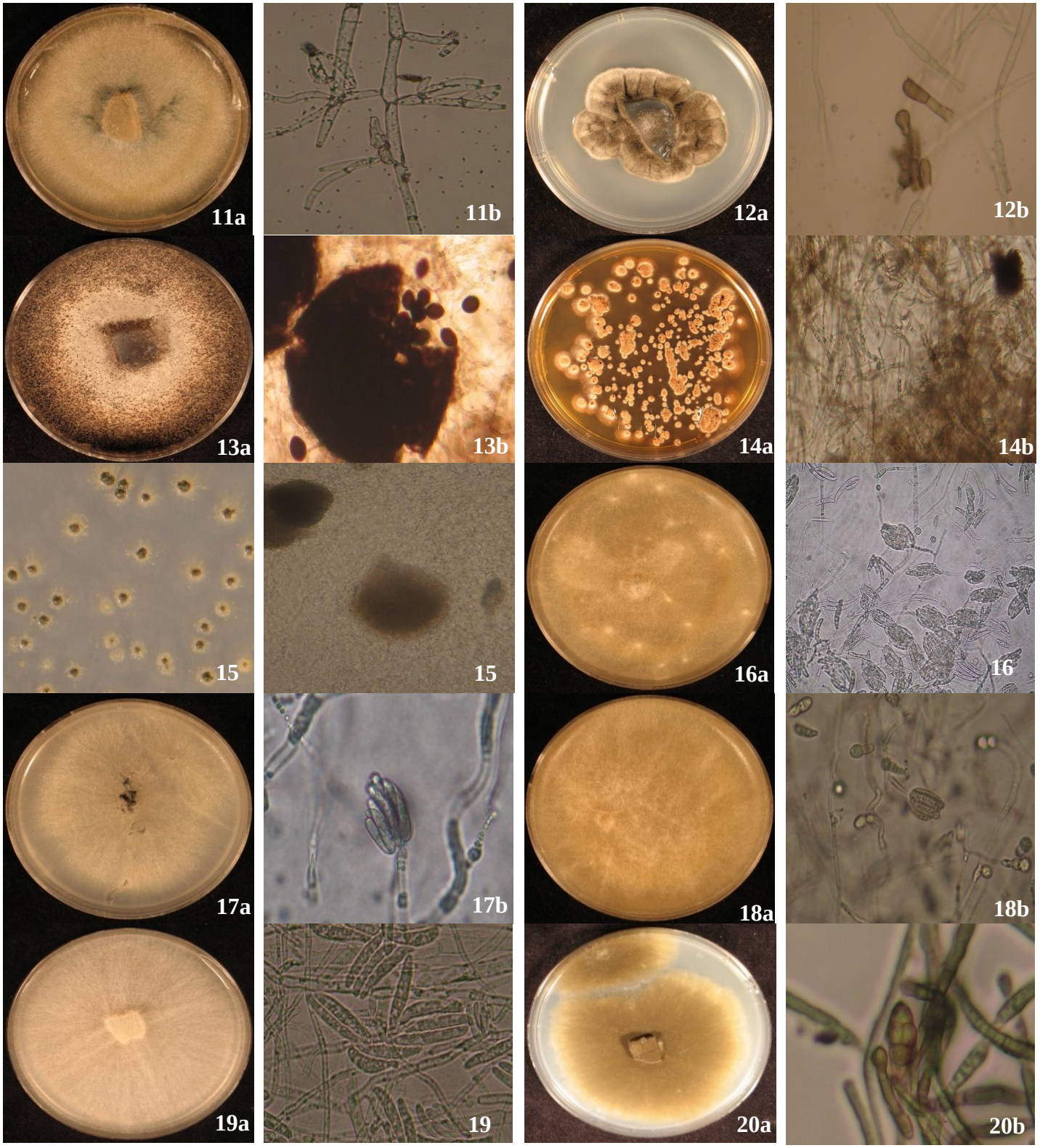


Fig. S1b: 11a) FEnPl17 colony 11b) FEnPl17 conidiophore with phialides lateral, intercalary or terminal, conidia elongate, 2-celled, straight or slightly curved; 12a) FEnPl19 colony; 12b) FEnPl19 conidia brown, hyphae septate; 13a) FEnPl25 colony; 13b) FEnPl25 perithecium asci and dark ascospores; 14a) FEnPl31 colony; 14b) FEnPl31 sporodochial conidiophore, chlamydospores; 15a) FEnPl33 colonies; 15b) FEnPl33 pycnidia, conidia hyaline (colorless); 16a) FEnPl34 colony; 16b) FEnPl34 conidiophore, macroconidia elongate, straight or slightly curved and microconidia ellipsoidal, straight or round; 17a) FEnPl35 colony 17b) FEnPl35 conidiophore, conidia elongate, straight or slightly curved; 18a) FEnPl11bact1 colony; 18b) FEnPl11bact1 conidiophore, conidia, chlamydospores; 19a) FEnPl37 colony; 19b) FEnPl37 macroconidia elongate, straight or slightly curved; 20a) FEnPl38 colony; 20b) FEnPl38 conidia cylindrical, multi-septate, brown colored;

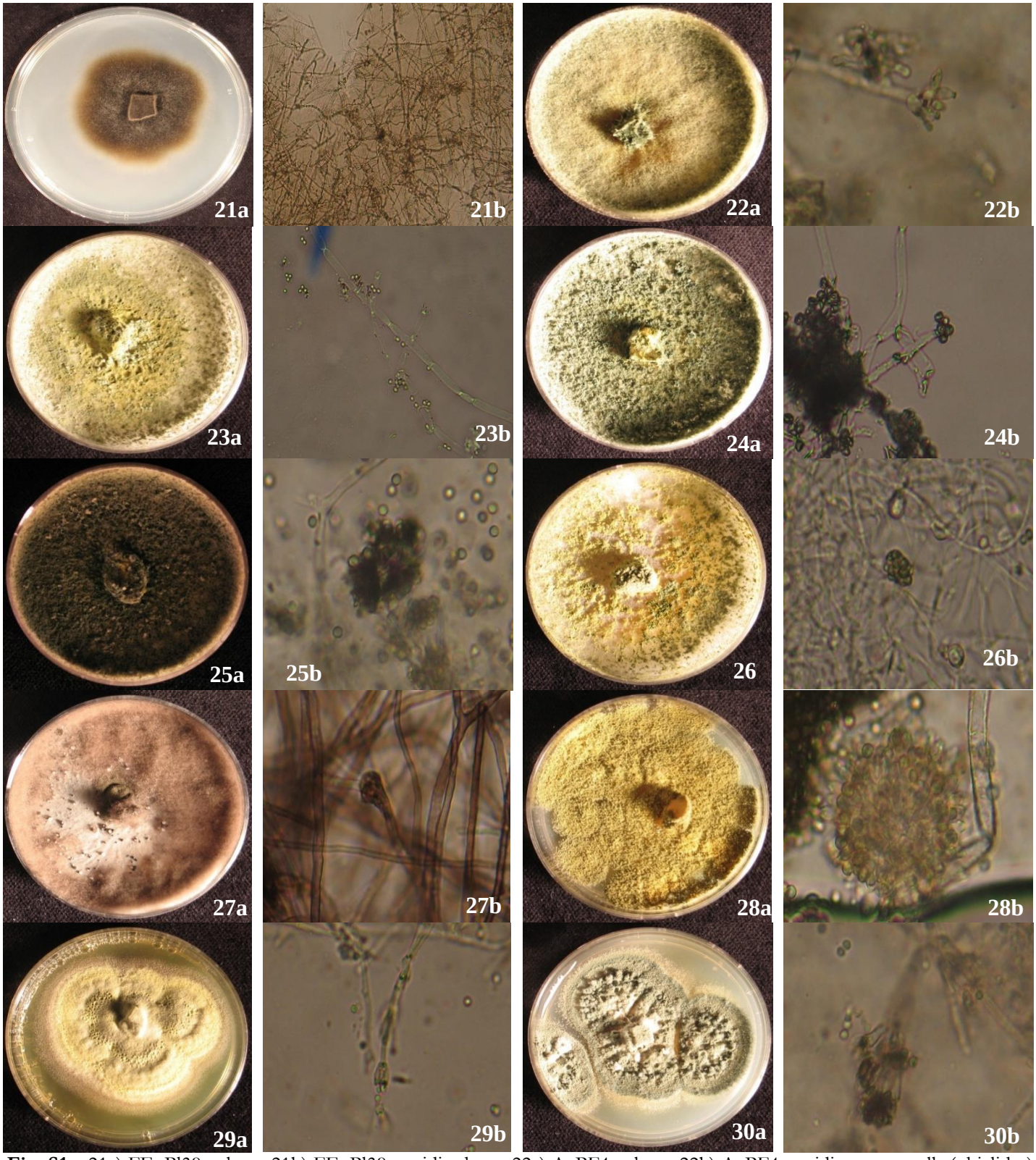


Fig. S1c: 21a) FEnPl39 colony; 21b) FEnPl39 conidiophore; 22a) AgPF4 colony; 22b) AgPF4 conidiogenous cells (phialides) with swollen bases and prominent necks, conidia spherical hyaline; 23a) AgIF1 colony; 23b) AgIF1 conidiophore, conidia round hyaline; 24a) AgIF2 colony; 24b) AgIF2 conidiophore, cluster of conidia spherical and colored; 25a) AgIF3 colony; 25b) AgIF3 conidiophore, bear cluster of conidia spherical and dark; 26a) AgTF1 colony; 26b) AgTF1 conidiophore, cluster of spherical, colored conidia, chlamydospores; 27a) AgTF4 colony; 27b) AgTF4 conidia, medium brown hyphae forming hyphae coils; 28a) AgTF5 colony; 28b) AgTF5 conidiophore, globose covered with flask shaped phialides, conidia spherical colored; 29a) AgTF9 colony; 29b) AgTF9 conidiophore with phialides and spherical, colored conidia; 30a) AgTF11 colony; 30b) AgTF11 branched conidiophores with phialides and chains of spherical, colored conidia;

Fig. S1d: 31a) AgTF12 colony; 31b) AgTF12 sporangium with sporangiospores; 32a) AgN26F3 colony; 32b) AgN26F3 intercalary phialides, 1-septate conidia; 33a) AgN26F6 colony; 33b) AgN26F6 phialides, conidia, and sporodochium; 34a) AgN26F10 colony; 34b) AgN26F10 conidiophores spherical colored conidia; 35a) AgN26F11 colony; 35b) AgN26F11 branched conidiophores with phialides and chains of spherical, colored conidia; 36a) AgIF10 colony; 36b) AgIF10 hyphae septate, clamp connections; 37a) AgIF13 colony; 37b) AgIF13 conidiophore phialidia, conidia spherical, colored; 38a) AgIF15 colony; 38b) AgIF15 conidiophores, spherical, colored conidia; 39a) AgIF16 colony; 39b) AgIF16 conidiophores, phialides, spherical, colored conidia; 40a) AgIF19 colony; 40b) AgIF19 conidiophores mostly not branched, phialides, spherical, colored conidia

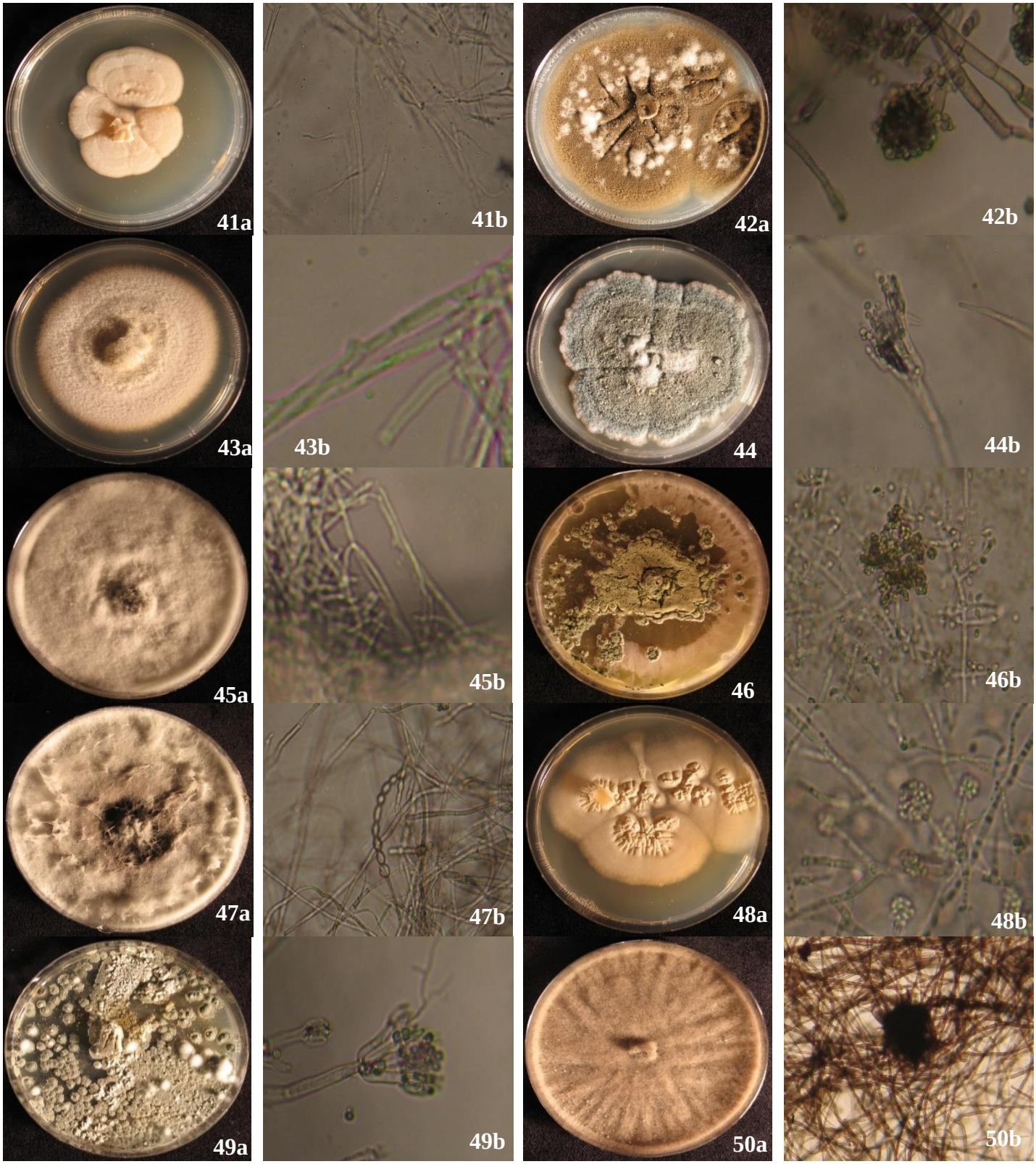


Fig. S1e: 41a) AgIF21 colony; 41b) AgIF21 hyphae with septa; 42a) AgIF23 colony; 42b) AgIF23 conidiophore, cluster of spherical, dark conidia; 43a) AgIF26 colony; 43b) AgIF26 hyphae septate, anastomized 44a) AgIF27 colony 44b) AgIF27 conidiophore, phialides, chains of spherical, dark conidia; 45a) AgIF28 colony; 45b) AgIF28 hyphae septate; 46a) AgIF30 colony; 46b) AgIF30 conidiophore with cluster of spherical, dark conidia; 47a) AgIF32 colony; 47b) AgIF32 conidiophore, conidia produced in single chain; 48a) AgPF5 colony; 48b) AgPF5 conidiophore bearing clusters of spherical conidia; 49a) AgTF8 colony; 49b) AgTF8 conidiophores branched, phialides, spherical, colored conidia; 50a) AgTF10 colony; 50b) AgTF10 chlamydospore, medium brown hyphae forming coils.

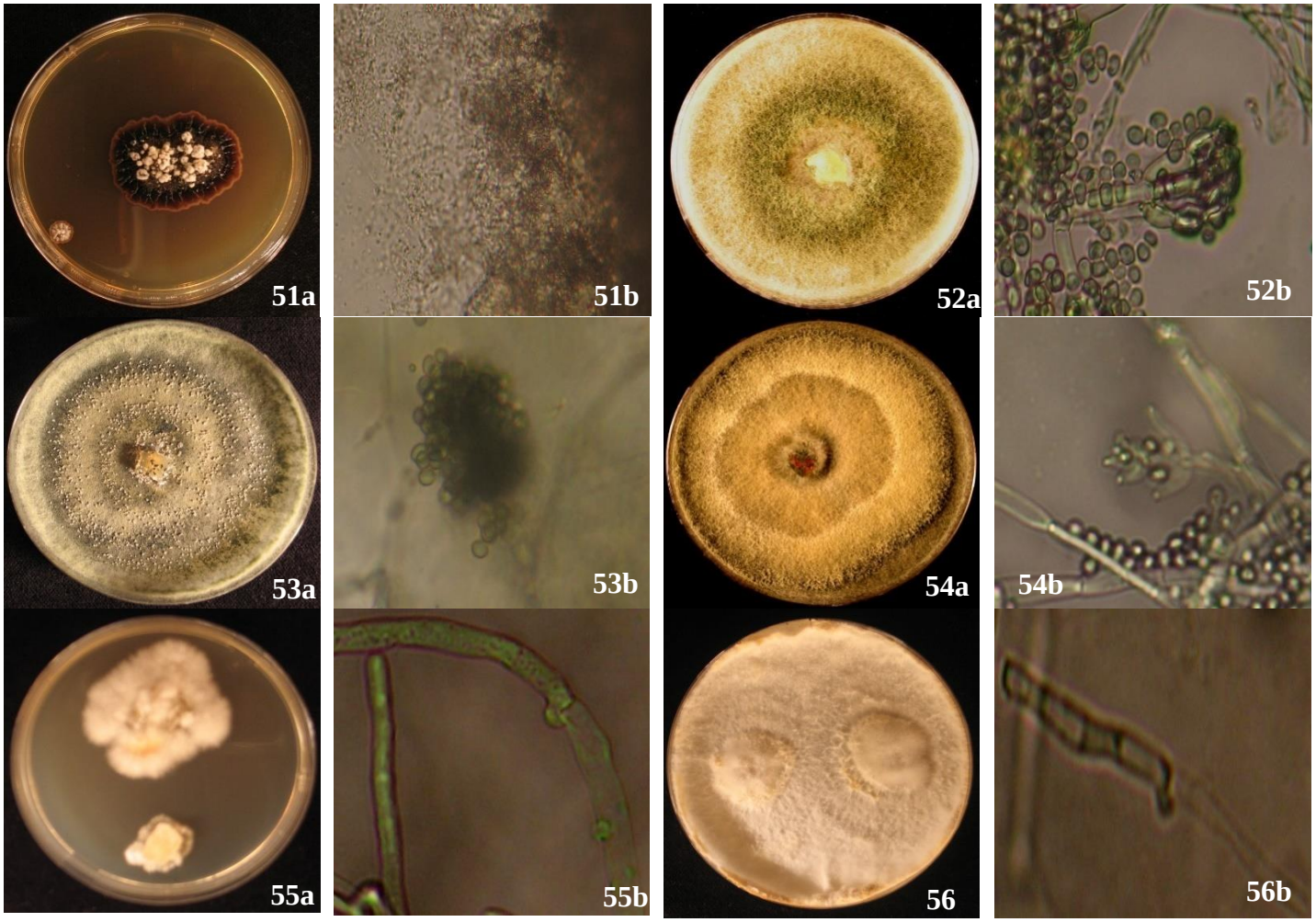
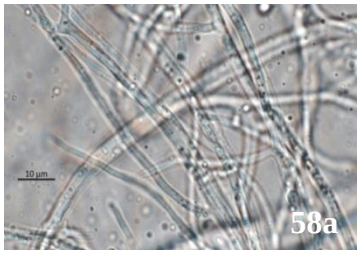
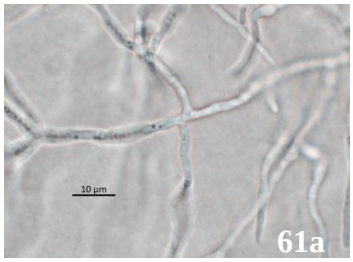


Fig. S1f: 51a) AgTF13 colony; 51b) AgTF13 fine hyphae with spores; 52a) AgN25F6 colony; 52b) AgN25F6 conidiophore, phialides with narrow neck, spherical, colored conidia; 53a) AgN25F2 colony; 53b) AgN25F2 conidiophore with spherical, dark conidia and chlamydospores; 54a) AgN25F1 colony; 54b) AgN25F1 immature conidiophore, phialides with narrow neck, spherical, colored conidia; 55a) B_2 colony; 55b) B_2 hyphae with clamp connections; 56a) B_3 colony; 56b)) B_3 hyphae with clamp connections.

Composite Fig. S1. Colonies and microscopic features of alder-associated fungi isolated from rhizosphere soil and root-fragments incl. nodule-like structures.

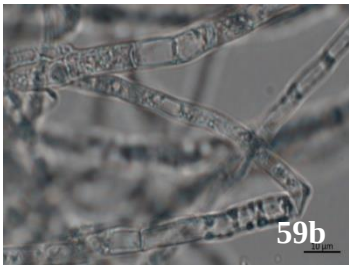


47-11 B

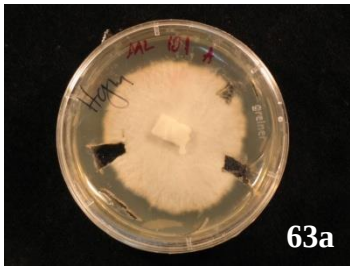


48-b2 A

47-32 B

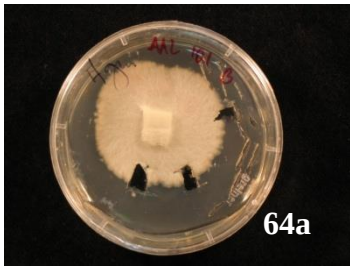


47-32 B



AAL 101 A

AAL101 A



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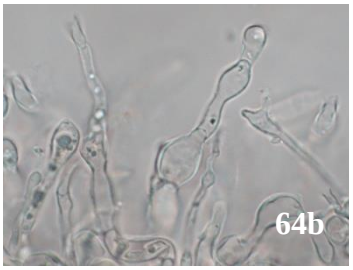

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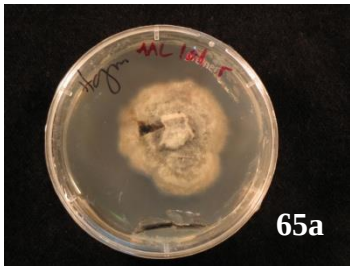

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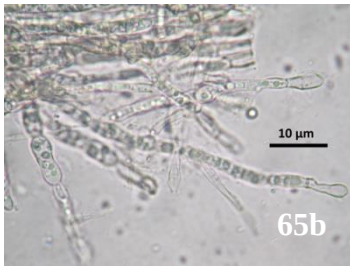

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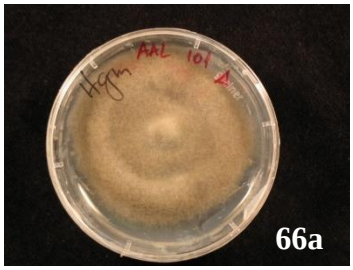
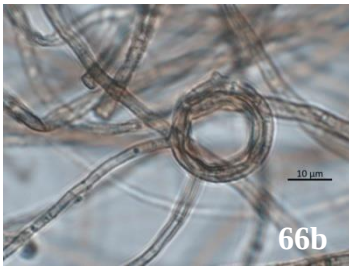


AAL 101 D



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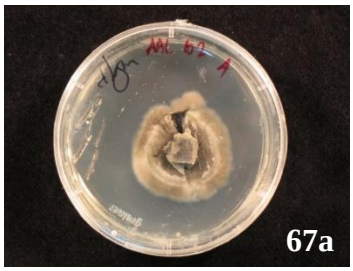

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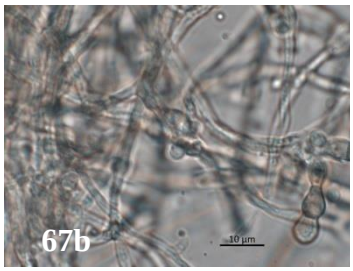
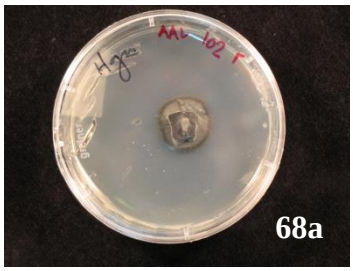


AAL 102 A



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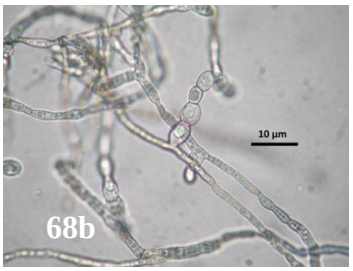

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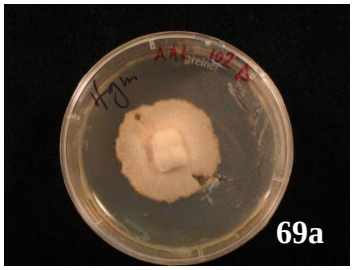
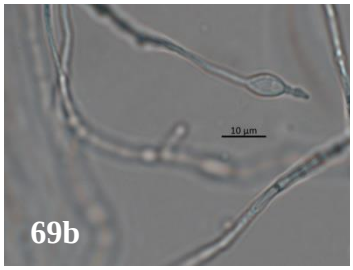
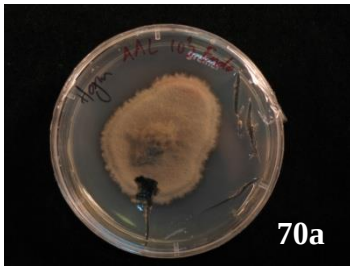


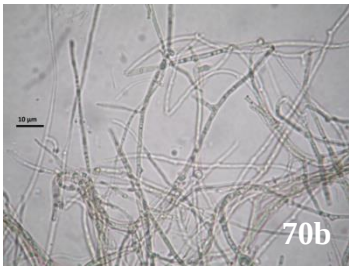
102 D



AAL 102 D

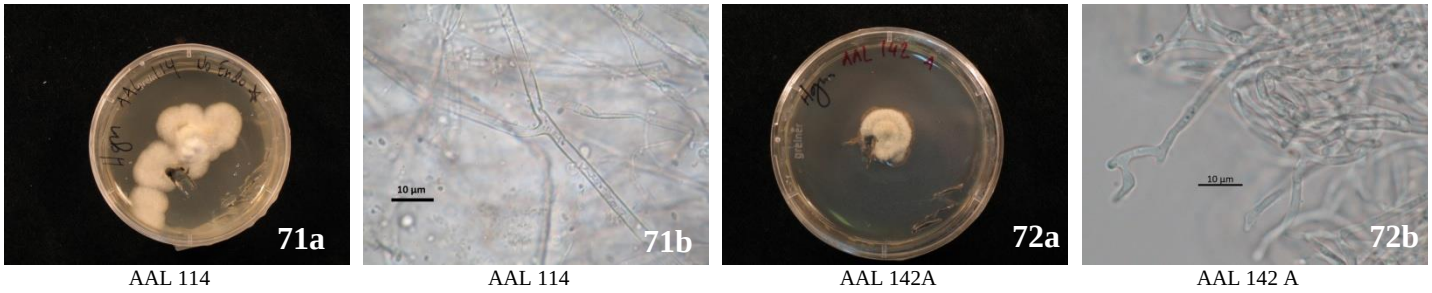


AAL 103

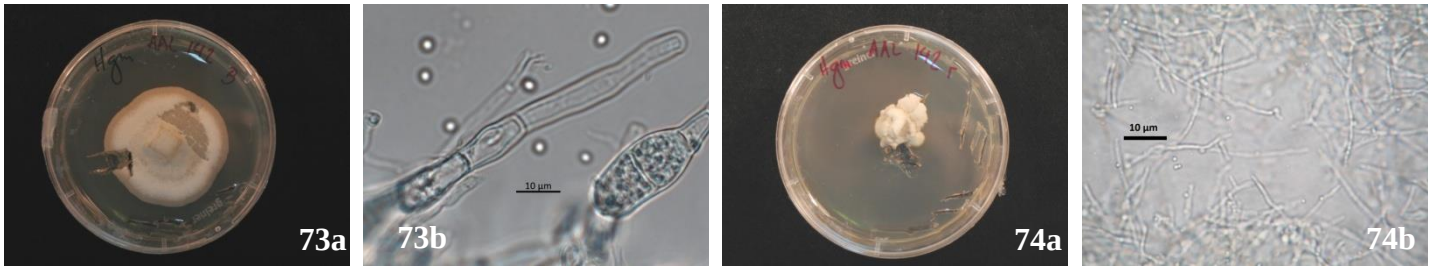


AAL 103

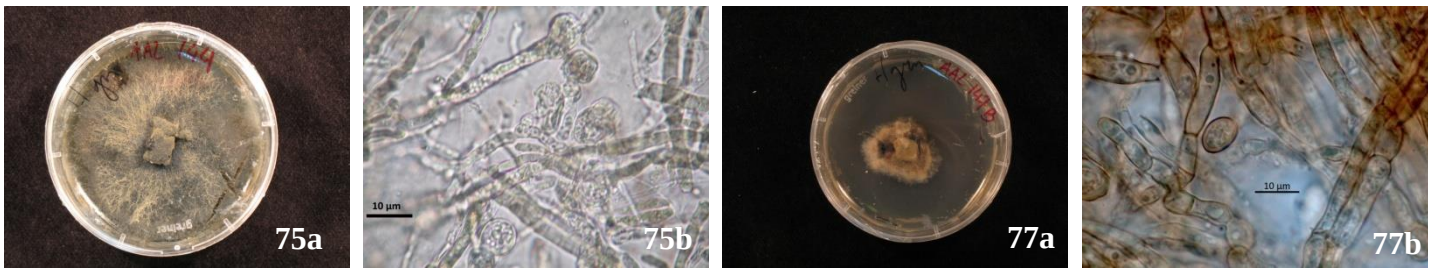
Fig. S2a: 58a) hyphae of 47 -11 B; 61a) hyphae with trifurcate ramification.; 59a,b) colony and wide hyphae of 47-32 B; 63a,b) colony and macroconidia; 64a,b) colony and inflated, phialidic hyphal cells and probably endoconidia; 65a,b) colony and endoconidia; 66a,b) dark colony and circling pigmented hyphae; 67a,b) dark colony and hyphae with lateral conidia; 68a,b) dark colony and hyphae with lateral conidia in chains; 69a,b) colony and hyphae with apical phialidic conidia; 70a,b) colony and hyphae with microconidia.



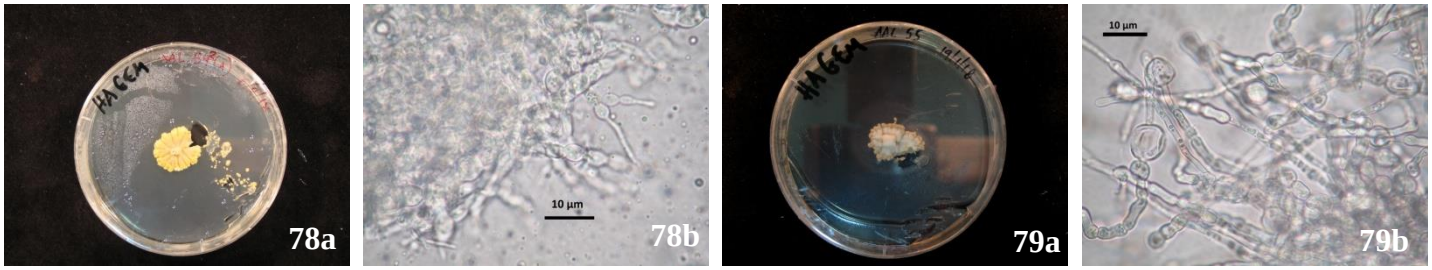
AAL 114 | AAL 114 | AAL 142A | AAL 142 A



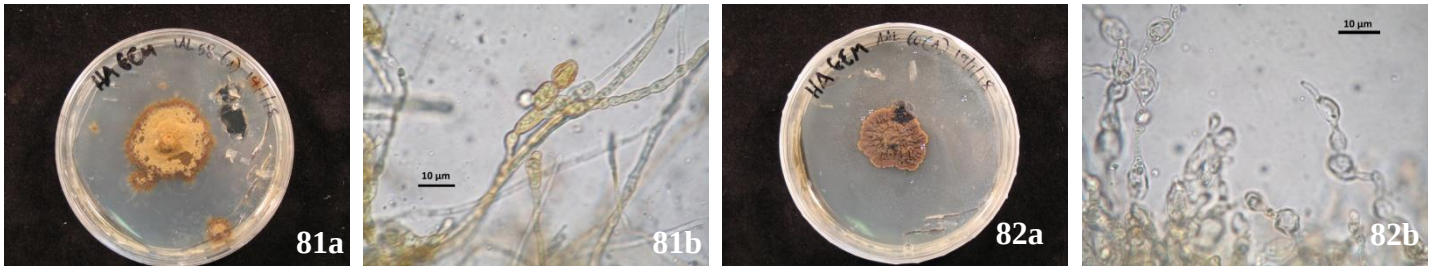
AAL 142 B | AAL 142 B | AAL 142 C | AAL 142 C



AAL 144 | AAL 144 | AAL 147 B | AAL 147 B

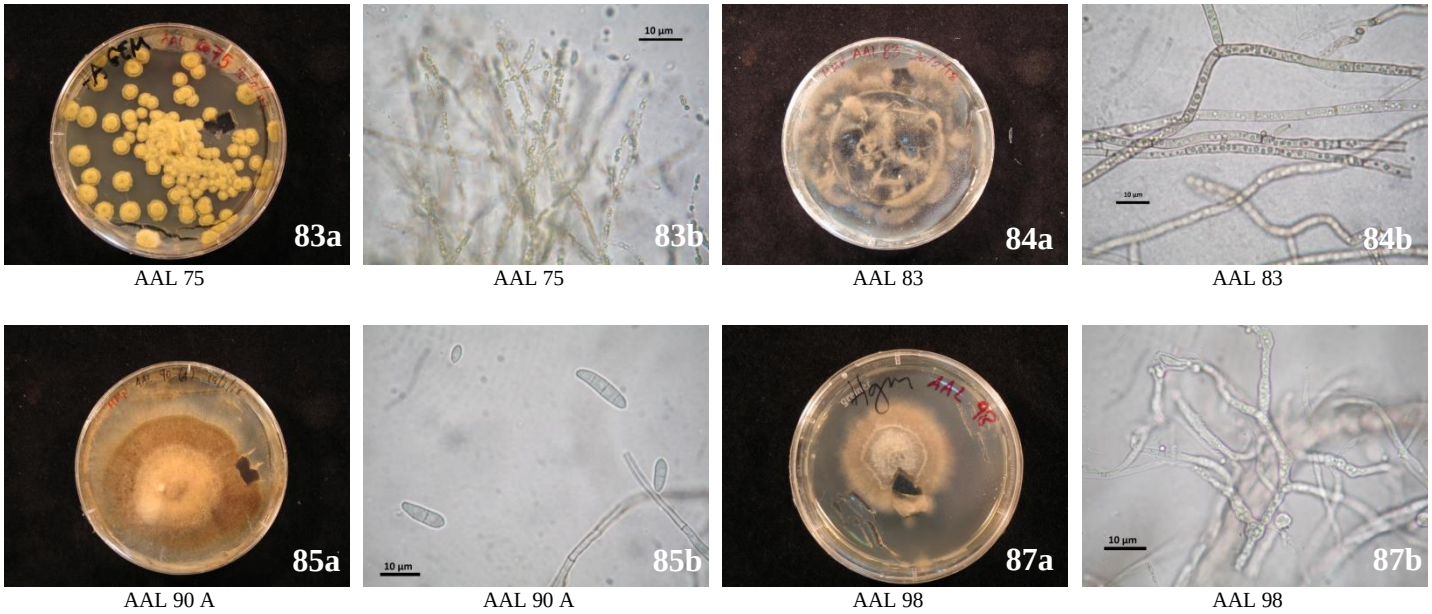


AAL 54 A | AAL 54 A | AAL 55 | AAL 55



AAL 58 A | AAL 58 A | AAL 60 A | 60 A

Fig. S2b: 71a,b) colony and hyphae; 72a,b) colony and hyphae; 73a,b) colony and wide, phialidic hyphal cells with endoconidia; 74a,b) colony and hyphae; 75a,b) dark colony and septate hyphae with conidia; 77a,b) dark colony and wide pigmented hyphae; 78a,b) colony and hyphae in yeast-like chains; 79a,b) colony and hyphae in chains with endoconidia; 81a,b) dark colony and pigmented conidiophores with conidia; 82a,b) colony and hyphae in yeast-like chains.



AAL 75 | AAL 75 | AAL 83 | AAL 83

AAL 90 A | AAL 90 A | AAL 98 | AAL 98

Fig. S2c: 83 a,b) colony and conidiophores; 84a,b) dark colony and pigmented, septate hyphae; 85a,b) dark colony and diploconidia; 87a,b) colony and hyphae.

Composite Fig. S2. Colonies and microscopic features of fungal isolates deriving from ECM root tips and mushrooms.