Optimizing conditions of mycelial inoculum immobilized in Ca-alginate beads: a case study in ectomycorrhizal fungus Astraeus odoratus

Yanisa Punsung
Chulalongkorn University
Pawara Pachit
Chulalongkorn University
Teeratas Kijpornyongpan
Purdue University
Chanita Paliyavuth
Chulalongkorn University
Karn Imwattana
Chulalongkorn University
Jittra Piapukiew
jittra.k@chula.ac.th
Chulalongkorn University

Research Article

Keywords: Ectomycorrhiza, Forest restoration, Mycelial entrapment, Vegetative inoculum

Posted Date: February 15th, 2024

DOI: https://doi.org/10.21203/rs.3.rs-3953078/v1

Additional Declarations: No competing interests reported.

Version of Record: A version of this preprint was published at World Journal of Microbiology and Biotechnology on June 11th, 2024. See the published version at https://doi.org/10.1007/s11274-024-03962-8.

Abstract

Ectomycorrhizal inoculum has emerged as a critical tool for forest restoration, especially under challenging climate change conditions. The inoculation of selective ectomycorrhizal fungi can enhance seedling survival and subsequent growth in the field. Entrapment of vegetative inocula within alginate beads has proven to be the most suitable method for seedling application in nurseries and plantations. This study optimized the liquid media for mycelial growth of *Astraeus odoratus* strain K1 and the sodium alginate solution composition for enhanced mycelial viability after entrapment. Using Modified Melin-Norkrans as the optimal media for mycelial cultivation and 2% sodium alginate supplemented with Czapek medium, 0.25% activated charcoal, 5% sucrose, and 5% sorbitol in the alginate solution yielded the highest viability of *A. odoratus* mycelia. Preservation in distilled water and 10% glycerol at 25°C for 60 days proved to be the most effective storage condition for the alginate beads. Both fresh and preserved alginate beads were tested for colonizing on *Hopea odorata* Roxb. seedlings, showing successful colonization and ectomycorrhizal root formation, with over 49% colonization. This study fills a crucial gap in biotechnology and ectomycorrhizal inoculum, paving the way for more effective and sustainable forest restoration practices.

Introduction

Astraeus is a member of the Diplocystidiaceae family (Boletales, Agaricomycetes, Basidiomycota) and mainly occurs in sandy soils in forests across Asia, Africa, North and South America and Europe (Cunningham 1944; Nouhra and Toledo 1998; Phosri et al. 2004; Fangfuk et al. 2010; Pavithra et al. 2015; Ahmadzai et al. 2023). The *Astraeus* species are ectomycorrhizal (ECM) fungi and can establish symbiotic relationships with a broad range of forest tree species, including the trees in dipterocarp forests (Wilson et al. 2012; Phosri et al. 2013; Karun and Sridhar 2014; Pavithra et al. 2015). Tender basidiomata of *Astraeus* spp. are widely recognized as a highly prized edible mushroom in several parts of Asia (Mortimer et al. 2012), and are harvested in the wild and marketed in many countries such as India, Japan, Laos, and Thailand (Sanmee et al. 2003; Phosri et al. 2004, 2007; Dell et al. 2005; Butkrachang et al. 2007; Karun and Sridhar 2014; Ahmadzai et al. 2023). In Thailand, *A. odoratus* is found in dry dipterocarp forests, particularly in the northern and northeastern regions (Phosri et al. 2004, 2007).

Climate change is a well-established reality that results in extreme weather events impacting our daily lives (IPCC 2023; Nunes et al. 2020). Forests play a critical role in mitigating climate change by absorbing CO_2 and storing carbon in their biomass and soils (Waring et al. 2020; Sterck et al. 2021). While forest trees absorb CO_2 molecules, they do not do this solely by themselves. Ectomycorrhizal (ECM) fungi can help the trees absorb CO_2 more rapidly, and it is estimated that sixty percent of trees on earth have symbiotic relationships with ECM fungi. Furthermore, ECM fungi possess the capacity to obstruct the organic decomposition process, which is responsible for the release of carbon from forest soils into the atmosphere (Averill et al. 2018; Rudawska and Leski 2019). Increased CO_2 concentrations, rising temperatures, reduced rainfall and other climate change-related conditions are exogenous key factors in the association between ECM fungi and their plant companions. Previous researches have

indicated that when water is limited and photosynthesis is consequently reduced, the production of ectomycorrhizal biomass, specifically external mycelium also declines (Hagenbo et al. 2021; Sapes et al. 2021), resulting in reducing the ability of mycelium to form connections between trees (Rudawska and Leski 2019; Fernandez et al. 2023). In addition, soil moisture decrease caused by climate change affects the growth and survival of ectomycorrhizal fungi (Coleman et al. 1989; Widden and Parkinson 2011). Ectomycorrhiza refers to a mutualistic relationship between ECM fungi and the roots of higher plants. The fungi support the host plants by providing water and nutrients, while the host plants reciprocate by supplying carbon to the fungi through their root system (Smith and Read 2008). ECM fungi are crucial for forest ecosystems as they contribute to the nutrient cycle and enhance the growth of host plants by increasing water and mineral uptake through an expanded root surface area (Futai et al. 2008; Itoo and Reshi 2013). In the soil food webs, ECM fungi also play a vital role as entry points for carbon. Carbon from their host plants allocates to the persistence of ECM fungi throughout forests, which are characterized by their extensive mycelial networks, often referred to as the “wood wide web”. As a result, these fungi contribute to the modulation of the global climate through their influence on terrestrial soils (Castro-Delgado et al. 2020; Hawkins et al. 2023). Additionally, ECM fungi protect plant roots from soil pathogens and improve their growth, especially in stressed soil conditions (Lehto and Zwiazek 2011; Hachani et al. 2020; Yu et al. 2020).

Dry deciduous dipterocarp forests (DDF) represents an important natural resource of Asia, covering tropical to subtropical regions in Southeast Asia (SE Asia). These areas are characterized by extreme temperatures as well as unique seasonal precipitation patterns. DDF is a distinct form of forest ecosystem that supports a wide range of rare and endangered species. Several tree species in DDF require ECM association for a successful establishment (Lee et al. 2008; Brearley 2011; Helbert et al. 2019; Suwannasai et al. 2020). Climate change and human activities have caused DDF to undergo degradation and transformation for centuries (Sodhi et al. 2010; Koh et al. 2013). Ironically, *A. odoratus*, an ECM fungus themselves, has become a significant contributor to anthropogenic fires in Thailand’s DDF due to the popular belief that fire enhances mushroom production. The fact that *Astraeus* fruit bodies were found in both burned and unburned areas indicates that fire was not the factor that stimulated the formation of sporocarps (Kennedy et al. 2012). Excessive burning is harmful to the environment because it destroys primary forests, increases grassy ground plants, changes the ground flora species and decreases soil nutrients, all of these effects contributed to the loss of biodiversity within the area (Kafle 2006). To restore the disturbed DDF forest ecosystems, ECM inocula are needed to promote host plants and reestablish soil fertility. ECM inoculation has become a routine practice in nurseries; thus, the establishment of methods for inoculum production at an industrial scale is necessary (Brundrett et al. 2005; Rossi et al. 2007). Various inoculum types and formulations have been developed for the application (Repáč 2011). The ideal ECM inoculum must contain sufficient numbers of active propagules, retain viability throughout storage and transportation, and preserve their infectivity for several months after production. Additionally, the inoculum should be user-friendly, free of contamination, and the production process should be economically efficient (Rossi et al. 2007; Charya and Garg 2019).

Due to their numerous benefits, ECM fungal inoculation has been widely used in forest restoration, including reforestation and afforestation, particularly in soils lacking native ECM fungi or having low species diversity (Marx et al. 2002; Bois et al. 2005; Pineiro et al. 2013; Onwuchekwa et al., 2014; Karlsen-Ayala et al., 2022). Seedlings inoculated with specific ECM fungi not only improve their survival upon transplantation but also enhance their subsequent growth in the field, even under harsh environmental conditions (Pineiro et al. 2013; Sebastiana et al. 2013). Furthermore, ECM inoculation in both seedlings and the field contributes to the regeneration of healthy soil structure by increasing soil aggregates and microbial diversity (Miller and Jastrow 1992; Sousa et al. 2014).

Mycelium inoculum has been considered the most suitable inoculation method due to the availability of previously selected isolates with high efficiency in promoting plant growth (Rossi et al. 2007). Pure cultures of ECM fungi are grown in suitable solid substrates, such as a mixture of peat and vermiculite, supplemented with a nutritive solution. Another way to prepare the inocula is through submerged cultivation, followed by mycelia entrapped within calcium alginate beads or other types of polymeric gels (Repáč 2011; Charya and Garg 2019). Among a variety of biopolymers, alginate is the most widely used due to its biodegradability and suitability for all types of microorganisms. Moreover, several additives are incorporated into the polymer to impart appropriate properties, such as improving the structure of the beads and enhancing encapsulation efficiency. The most used fillers include minerals, organic materials, and osmoprotectants (Szopa et al. 2022). This method provides a microenvironment that supports mycelial viability and offers more advantages than other types of inocula due to the high biomass loading capacity of the beads, protection of mycelia from adverse environmental conditions, high efficiency in storage and transportation, and a high survival rate of fungi (Kuek et al. 1992; Friel and McLoughlin 1999; Szopa et al. 2022). According to nursery studies, ECM inoculum immobilized within alginate beads was proved to be more efficient than solid-state fermentation in the formation of ECM roots and the growth of seedlings (Tacon et al. 1985; Mortier et al. 1990). However, the low growth rates of ECM fungi in culture and the lack of information on physiology and kinetics of growth remain limitations for their application and mass production on an industrial scale (Oliveira et al. 2006; Rossi et al. 2007). In addition, the conditions to maintain the viability of mycelia entrapped within a calcium alginate bead need to be investigated for each ECM fungal species (Rodrigues et al. 1999).

Numerous experiments demonstrated that the presence of ECM fungi on the roots of dipterocarp seedlings enhances seedling growth, though primarily in nursery conditions. In reforestation programs, it has been suggested that seedlings ought to be inoculated before being released into the natural environment (Walker 1999; Menkis et al. 2007; Martínez et al. 2012; Bauman et al. 2013). Recent and continuous ECM inoculation strategies focus mainly on the *Scleroderma*, *Pisolithus*, and *Tomentella* genera (Turjaman et al. 2005, 2006; Ogawa 2006; Lee et al. 2008). *A. odoratus* has been used for producing ECM inoculum; spore or hyphal suspension, due to its abundance of spores and availability for vegetative cultivation, and not only for enhancing the establishment of dipterocarp seedlings in reforestation programs but also for establishing dipterocarp plantations for mushroom production (Kaewgrajang et al. 2013; Kaewgrajang et al. 2019). Despite being a popular delicacy in northern Thailand, attempts at the artificial cultivation of *A. odoratus* have proven unsuccessful due to the

limitations of the seasonal blooming of sporocarp and the limited functions of some types of culture media, such as the low shelf life and high sensitivity of contamination in the storage of mycelium on solid culture media (Brundrett et al. 2005; Repác 2011). Moreover, the application of *A. odoratus* inoculum with calcium alginate bead has never been examined so given the potential of *A. odoratus* as an inoculum, this study aimed to optimize the conditions for producing mycelial inoculum from *A. odoratus* entrapped with calcium alginate and determine its storage conditions. Additionally, the study also investigated the effect of al ginate-entrapped mycelial inoculum on the root colonization of dipterocarp seedlings. Acknowledging the importance of ECM fungi inoculum such as optimizing production methods, and comprehension of ecological factors influencing inoculum success needs to be the focus for fostering sustainability.

Materials and methods

Fungal strain

The ECM fungus *A. odoratus* Phosri, Watling, Martín and Whalley, strain K1, was used in this study. This fungus was isolated from a basidiocarp collected from a dipterocarp forest in Srisawat District, Kanchanaburi Province. The culture of this fungal strain was kept in a fungal culture collection at the Mycology Laboratory, Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand. The strain was maintained on Modified Melin-Norkrans (MMN) agar medium at room temperature (30 ± 2°C) and subcultured onto fresh medium every month.

Effect of media on mycelial growth of A. odoratus strain K1

Four different kinds of culture broth media were tested: Pridham-Gottlieb medium (PG) modified by Kuek (Kuek, 1996), MMN (Marx 1969), Biotin-Aneurin-Folic Acid medium (BAF) as described by Águeda et al. (2008), and Potato Dextrose Broth (PDB) for mycelial growth of the fungus strain K1. All culture broth media were supplemented with 0.25% activated charcoal and adjusted to pH 5.5. A piece of 7 mm diameter mycelial disk from 20-day old culture on MMN agar was transferred aseptically to a 250 mL Erlenmeyer flask containing 100 mL of liquid medium. The flasks were incubated statically for 30 days at room temperature in the dark with three replicates per treatment. The dry weight of the mycelia was measured every five days. The mycelial growth was determined in term of the dry weight. The best growth medium was selected for further study.

Optimization of conditions for preparing A. odoratus alginate bead inoculum

Alginate solution

The fungal mycelia were encapsulated in calcium alginate beads in a manner similar to that described by Mauperin et al. (1987). MMN medium, Czapek medium (Visagie et al. 2014), and sterile distilled water, were used for preparing sodium alginate solution. Mycelium (approximately 10 g fresh weight) was mixed in 100 mL of sodium alginate solution containing 2% sodium alginate (Glentham Life Sciences

Ltd., United Kingdom) and 0.25% activated charcoal and then fragmented by hand blender for 10 seconds. The mixture was dropped into a 0.1 M $CaCl_2$ solution to entrap the mycelium particles into polymerized alginate beads. After 30 minutes of curing in the $CaCl_2$ solution, the beads were washed with sterile distilled water three times. The viability of mycelia entrapped in calcium alginate beads was assessed directly after the encapsulation process by placing 100 beads onto PDA in Petri dishes in the dark at room temperature, and mycelial development was checked every 5 days for a period of 20 days. Each treatment had ten replications. The beads mycelial growth corresponded to germination and, therefore, viability. The percentage of viable beads was recorded. The treatment showing the highest percentage of viability was selected and used for subsequent experiments.

Sodium alginate concentration

The mycelium of *A. odoratus* strain K1 was entrapped in calcium alginate as described above with different sodium alginate concentrations: 1.5%, 2%, 2.5%, and 3%. The concentration yielding the highest percentage of viable beads was selected for next experiments.

Protectant additives

The alginate solution was supplemented with different sugars glucose (G), sucrose (S), and trehalose (T) at the concentrations of 1% and 5% and different protectant additives: 5% sorbitol (s) and 5% polyethylene glycol 8000 (p). The alginate solution without supplemented sugar and protectant was used a control. The alginate beads were preserved in sterile distilled water at 25°C for 15 days. Fresh and preserved beads were evaluated for their efficacy by percentage of viable beads as described above. The treatment with the highest percentage of viability was used in further studies.

Preservation solution and temperature

The alginate beads were prepared in the optimal conditions according to previous experiments. They were preserved in various sterile solutions: distilled water, 5% and 10% glycerol, 5% and 10% dimethyl sulfoxide (DMSO) and 0.07 M $CaCl_2$. All treatments were kept at 4°C and 25°C for up to 2 months. Every 15 days, the alginate beads were placed on a PDA plate and incubated at room temperature to check their viability. The survival rate was measured as following the formula below modified from Oberoi et al. (2021):

$$\text{Survival rate } (\%) = \frac{\text{Number of germinated alginate beads after preservation}}{\text{Number of germinated alginate beads before preservation}} \times 100$$

Effectiveness of alginate beads on A. odoratus colonization

The viability of mycelia inoculum and its infectivity on the roots of seedlings are important properties for application. Thus, alginate beads with different storage time under selected preservation condition were applied to *H. odorata* seedlings, in order to determine the efficacy of *A. odoratus* colonization in terms of root formation and colonization rate.

Effectiveness of alginate beads on the formation of ECM roots

Seed wings of *H. odorata* were removed, and then the seeds were surface-sterilized with 5% sodium hypochlorite for 10 minutes before being thoroughly rinsed with sterile water three times consecutively. The sterile seeds were incubated in a zip-lock bag at room temperature for 5 days. Each individual seedling with a 3–4 cm root length was transplanted into a rhizobox, which was filled with sterile perlite and then wrapped with aluminum foil. A rhizobox was constructed with a plastic box (16 cm × 32 cm × 2 cm), and one of the short edges was removed to facilitate plant growth. After that, the rhizoboxes with seedlings were inclined by 60 degrees to encourage the roots to grow along the lid of the box and were daily watered with distilled water.

Six-month-old, uniformly healthy, non-mycorrhizal seedlings were selected for fungal inoculation under the following regimes: In treatments 1–3, seedlings were inoculated with 50 fresh alginate beads of *A. odoratus* strain K1, equivalent to 5 grams, alginate beads preserved for 1 and 2 months, respectively. Additionally, seedlings were inoculated with 5 grams of K1 mycelial disc cut from the actively growing margin of *A. odoratus* colonies on the PDA for a positive control, and non-inoculated seedlings were used for a negative control. Three replications were prepared for each fungal inoculation treatment. ECM colonization in each treatment was observed every 10 days for 2 months. The development of ECM roots was examined based on morphology and recorded as stages 1–4 (Table 1), which is modified from Péret et al. (2009). Then, few representative ECM root tips were collected and identified using molecular techniques, as shown in the next experiment, to prove that *A. odoratus* was the cause of the infection.

Table 1
Developmental stages of the ECM root of *A. odoratus*

(adapted from Péret et al. (2009))

Stage	Description
1	Mycelia of *A. odoratus* germinated from alginate beads
2	Attachment of mycelia to the root surface and presence of swelling roots
3	Presence of a dense mantle and extraradical mycelia
4	Presence of sclerotia

Effectiveness of calcium alginate beads on colonization rate

Seeds of *H. odorata* were surface sterilized and incubated using the same methods as in the previous experiment. Each germinating seedling was individually transplanted into a polyethylene bag (5 cm × 15 cm) filled with autoclaved commercial potting soil. After 3 months, each non-mycorrhizal seedling was transplanted into a new polyethylene bag (10 cm × 20 cm) filled with autoclaved commercial potting soil

and inoculated with mycelia of *A. odoratus*, following the same treatments as in the previous experiment. Each fungal inoculation treatment had ten replications, resulting in 50 seedlings in total. All seedlings were watered daily and maintained in the nursery for 45 days.

After the inoculation period, seedlings were removed, and their root systems were gently washed over a 0.85 mm mesh sieve with tap water. Then, 200 root tips of each seedling were randomly selected and investigated under a stereomicroscope (SZ2-ST Olympus, Tokyo, Japan). ECM roots of *A. odoratus* were sorted based on their surface color, texture, and branching pattern, as described by Kaewgrajang et al. (2019). The colonization rate was measured as the percentage of the number of root tips colonized by *A. odoratus* divided by the total number of root tips (Brundett et al. 1996).

Moreover, to confirm the infection of *A. odoratus*, few representative tips of ECM roots and mycelia of *A. odoratus* were performed DNA extraction using cetyltrimethylammonium bromide (CTAB) with a protocol from Zhou et al. (1999). Molecular identification was operated by PCR-sequencing of the Internal Transcribed Spacer (ITS) region using the fungus-specific primer pairs ITS1F (Gardes and Bruns 1996) and ITS4 (White et al. 1990). The PCR reactions were performed as described in Pachit et al. (2020). The PCR products were purified and sequenced at Celemics, Inc., Korea, using the Barcode-Tagged Sequencing method, Platform illumina pair-end read. The acquired sequences were manually edited in MEGA version 11 (Tamura et al. 2021) and were subsequently compared with reference ITS sequences using the BLASTn algorithm against the GenBank and the UNITE databases. DNA sequences that were at least 97% similar to reference sequences in the database (Kaewgrajang et al. 2019; Nilsson et al. 2019) were confirmed to belong to the *A. odoratus* species. The nucleotide sequences of ECM root tips and mycelia were submitted under NCBI accession numbers OQ916937-OQ916939.

Statistical analysis

The experiment was conducted using the completely randomized design (CRD). All data obtained from the experiments were subjected to one-way ANOVA, means ± SE showing statistical significance followed by Duncan's New Multiple Range Test (DMRT) at $P < 0.05$ using IBM SPSS Statistics 16 program.

Results

Effect of media on mycelial growth of A. odoratus strain K1

The result of growing *A. odoratus* strain K1 mycelium for 30 days in four liquid media (PG, MMN, BAF and PDB) showed the highest dry weight of mycelium growth in MMN medium with 0.345 g/100 ml (Fig. 1), which was significantly higher ($P < 0.05$) than the mycelial dry weight in other liquid cultural media. The mycelial growth of *A. odoratus* in all tested media increased continuously until the end of the experiment with a significant mycelium dry weight difference in MMN cultural medium starting at Day 20. There was no significant difference among the four kinds of cultural media at the beginning (Day 5).

MMN medium supplemented with activated charcoal was the most suitable for mycelial growth of strain K1.

Optimization of mycelial entrapment with calcium alginate

Effect of different alginate solutions on the viability of mycelia entrapped in alginate beads was evaluated. The viability of mycelia entrapped in alginate beads composed of Czapek and MMN media, and distilled water was 100%, 93% and 67%, respectively (Fig. 2a). The viability was not significant difference between Czapek and MMN medium. Mycelia entrapped in beads composed of Czapek and MMN alginate solutions germinated within 5 days of placing on the PDA plate, while mycelium entrapped in distilled water alginate beads required 10 days. The mycelia that grew from the beads made of Czapek medium produced dense aerial mycelia growing on both the surface of the beads and the PDA, whereas the aerial mycelia that germinated from the beads made of MMN medium were compact on the beads but loose and fluffy on the surface of the PDA. The mycelia slightly germinated from the beads made of distilled water alginate solution and formed appressed mycelia on the PDA. Mycelia also produced a yellowish to dark brown pigment that diffused through the PDA, especially from beads made of MMN and distilled water alginate solution (Fig. 2b). According to this result, the most optimal alginate solution for mycelial entrapment was Czapek medium.

The viability of strain K1 mycelia was tested with four different concentrations of sodium alginate: 1.5%, 2%, 2.5%, and 3%. The viability was highest at 1.5% concentration (96%), then at 2% sodium alginate (94%). There was no significant difference between the two concentrations. The lowest viability was found in 3% sodium alginate with only 49% (Fig. 3a).

The shape and texture of the alginate beads at each sodium alginate concentration varied. The alginate beads of 1.5% concentration were soft and not spherical. After placing on the PDA for several days, the beads shrank due to water loss. However, they exhibited the fastest germination only on Day 3 after placing on PDA medium, and the germinated aerial mycelia grew well. For the 2% concentration, the alginate beads were spherical and germinated on Day 5 after placing on the PDA with well-developed aerial mycelia. For the 3% sodium alginate beads, the morphology was round and quite rigid; the beads were too solid for mycelia to germinate, and mycelia germinated after 12 days on PDA medium (Fig. 3b). Therefore, considering the aesthetics and productivity of the alginate beads, a concentration of 2% was selected for the following experiment.

The viability of *A. odoratus* K1 mycelia entrapped in fresh alginate beads ranged from 40 to 97.5%, with the highest percentage from the fresh alginate beads adding 1% sucrose and 5% sorbitol (1Ss). However, the highest viability was not significantly different from the control condition. The viability of mycelia entrapped in fresh beads adding 5% sucrose and 5% sorbitol (5Ss) was 96.25% (Fig. 4), growing very fine on the PDA. The lowest germination rate of 40% was the testing result of 5% trehalose and polyethylene glycol (5Tp) alginate bead formula, and the mycelia cultured on solid medium were thin and not aerial

(Online Resource 1). Moreover, *A. odoratus* mycelia that germinated from alginate beads composed of different additives produced different pigments, such as reddish, yellowish, or dark brown.

After storing the fresh alginate beads in distilled water at 25°C for 15 days, the viability of mycelia in most treatments significantly decreased range from 27.5 to 88.75%, except for the mycelia entrapped in the bead containing 1% sucrose and 5% polyethylene glycol (1Sp) (Fig. 4). However, the mycelia entrapped in alginate beads containing 1% sucrose and 5% sorbitol (1Ss) still had the highest viability (88.75%), with no significant difference from the mycelia entrapped in alginate beads containing 5% sucrose and 5% sorbitol (5Ss) (86.25%). Both treatments showed significantly higher viability than the control condition. The mycelial cultures on PDA medium in both treatments formed dense aerial mycelia and grew well. The lowest viability (27.5%) was also from the alginate beads containing trehalose and polyethylene glycol. Both 1Ss and 5Ss alginate beads were selected for the next step of the experiment.

Different regular letters showed significant differences ($P < 0.05$) in the viability of mycelia entrapped in fresh alginate beads, and different italic letters showed significant differences ($P < 0.05$) in the viability after preservation for 15 days (Error bar: SE)

The survival rate of *A. odoratus* K1 mycelia entrapped in 1Ss and 5Ss alginate beads was evaluated under various preservation conditions by periodically examining the beads on the PDA medium every 15 days. After 15 days of preservation at 4°C, the viability of all treatments decreased significantly (Fig. 5a, b). The 5Ss treatment preserved in 0.7 M $CaCl_2$ had the maximum survival rate (13%) after 60 days of storage. It was significantly higher than the other treatments. The second-best treatment was the 5Ss alginate beads stored in distilled water with 10% survival rate (Fig. 5a, b, Online

Resource 2). There was no germination in the remaining treatments. At 25°C in preservation conditions, the maximum survival rate was found in 5Ss alginate beads stored in distilled water and 5% glycerol, with 86% and 85% survival rates, respectively. The treatment of 1Ss alginate beads preserved in distilled water and 5% glycerol was the highest, with survival rates of 70% and 64%, respectively (Fig. 5c, d). In addition, the 60-day survival rate decreased significantly in all treatments.

Comparing the survival rate of the optimal solution for preservation, which was distilled water and 5% glycerol. The 5Ss were able to maintain a survival rate of 86% and 85% in distilled water and 5% glycerol, respectively, which was significantly higher than the survival of the 1Ss when stored at 25°C for 60 days, as shown in Fig. 5c, d, and Online Resource 2.

The optimal conditions for mycelia entrapment in calcium alginate beads of *A. odoratus* were 2% sodium alginate supplemented with amended Czapek medium, 5% sucrose, and 5% sorbitol.

Effectiveness of alginate beads on A. odoratus colonization

The *A. odoratus* K1 mycelia germinated from mycelial discs and alginate beads to contact the root surface of *H. odorata* seedlings within 20 and 30 days after inoculation, respectively (Table 2). The mycelia completely covered the roots and formed ECM roots with extraradical mycelia within Day 40 in

both treatments. The observed ECM roots had a brown to dark brown color, a smooth surface, and monopodial-pinnate branching. (Fig. 6a, b). Young to mature dark brown sclerotia (Fig. 6c, d) were also found in the root system of *H. odorata* within 40 and 50 days after inoculation with mycelial discs and fresh alginate beads, respectively. Additionally, delayed processes in root colonization of *A. odoratus* mycelia were demonstrated in *H. odorata* seedlings inoculated with 1- and 2-month-old alginate beads preserved in distilled water at 25°C. The treatment with 1-month-old preserved beads showed mycelial germination and subsequent attachment to the root surface at Day 50 and 60 after inoculation, respectively. However, only mycelial germination was observed in the treatment with 2-month-old preserved alginate beads within the 60-day follow-up period. No ECM colonization was observed in the negative control treatment (non-inoculation).

Table 2
The effectiveness of alginate beads preserved for different durations on *A. odoratus* colonization in *H. odorata* seedlings in terms of ECM root development (Experiment I) and colonization rate (Experiment II)

Treatment	Experiment I: ECM root development						Experiment II: colonization rate (%)
	Day 10	Day20	Day 30	Day 40	Day 50	Day 60	at Day 45
non-inoculation	nd	nd	nd	nd	nd	nd	0 ± 0 [d]
mycelial disc	nd	stage I	stage II	stage III, IV	stage III, IV	stage III, IV	70.20 ± 2.19 [a]
fresh alginate bead	nd	nd	stage I	stage II, III	stage III, IV	stage III, IV	59.35 ± 1.11 [b]
1-month-preserved alginate bead	nd	nd	nd	nd	stage I	stage II	56.66 ± 1.89 [b]
2-month-preserved alginate bead	nd	nd	nd	nd	nd	stage I	49.37 ± 2.15 [c]

Note Abbreviations: "nd" indicates no differentiation, and different letters indicate significant differences ($P < 0.05$) in the percentage of colonization

Based on molecular identification, the ECM root sequence alignment (accession number OQ916937) showed a 100% match with the ITS sequence of the strain K1 submitted in the GenBank databases (accession number OQ916939).

The colonization rate of *A. odoratus* entrapped in alginate beads under different durations of preservation on the roots of *H. odorata* seedlings is presented in Table 2. The mycelial disc inoculum as the positive control exhibited the significantly highest average colonization rate, at 70.20%. Subsequently, the treatment with fresh alginate beads and 1-month-old preserved beads demonstrated average colonization rates of 59.35% and 56.66%, respectively, with no significant difference between them. In

contrast, the seedlings inoculated with 2-month-old preserved alginate beads displayed the lowest colonization rate at 49.37%. Notably, the non-inoculation treatment showed no ECM root colonization.

Furthermore, the sequence of the ECM root (accession number OQ916938) exhibited a 100% identity match with the K1 mycelial ITS sequences (accession number OQ916939).

Discussion

The production of ECM fungal inoculants necessitates the undertaking of studies to investigate the optimal culture conditions of these ECM fungi for the mass production of mycelia. Culture medium is an important factor that needs to be studied in order to determine the optimal growth required for achieving such production. Regarding the kind of liquid media tested for the mycelial growth of *A. odoratus* strain K1 in this study, we found that the mycelial growth of *A. odoratus* strain K1 was varied in different culture media (Fig. 1). This result was corroborated by the findings of several previous studies indicating that changes in the culture medium significantly affect the mycelial growth of ECM fungi in pure cultures (Brundrett et al. 1996; Xu et al. 2008; Kumla et al. 2011; Rossi and Oliveira 2011; Suwannasai et al. 2020). The MMN medium significantly yielded the highest mycelial dry weight, indicating a suitable growth medium for this fungal strain similar to those reported by Coleman et al. (1989), Torres and Honrubia (1991), and Curguz et al. (2010), who observed the highest growth for various species of *Suillus* in MMN medium. Vuorinen et al. (2015) also found that the majority (65%) of the twenty tested ECM fungal strains that typically colonize Norway spruce seedlings grew best on modified MMN medium with reduced sugar content (½ MMN). Furthermore, Rossi et al. (2017) studied the growth of ECM fungi including *Scleroderma, Rhizopogon, Pisolithus, Chondrogaster*, and *Scleroderma* spp., in MMN liquid medium to acquire a large quantity of mycelia for use in large scale inoculant production. MMN medium is probably recognized as one of the most commonly employed media broadly for experimental procedures (e.g. Kibar and Peksen 2011; Murata et al. 2012; Ramos and Tad-awan 2018; Wang et al. 2019; Kumar and Satyanarayana 2020). Suwannasai et al. (2020) found that the pure culture of *Astraeus sirindhorniae* grew best on MNC medium; similarly, *Astraeus hygrometricus* was cultivated in MNC medium prior to testing for ECM synthesis in *Pinus densiflora* seedlings (Fangfuk et al. 2010). According to the previously mentioned, the most suitable culture medium for the mycelial growth of ectomycorrhizal fungi was therefore dependent not only on the fungal species but also on the strain of that species.

Another crucial consideration is the fact that many fungi have the capacity to create secondary metabolites. We noticed significant color changes in the growing media during cultivation of *A. odoratus* strain K1. These changes are evidence of variations in metabolite production. However, pigmentation could be a sign of the limitations of growing conditions (Rossi and Oliveira 2011). Therefore, methods for mitigating the effects of these compounds are essential. Activated charcoal has proven very helpful for removal of several toxic compounds produced by fungus itself (Mussatto and Roberto 2004; Chandel et al. 2007). The fragmentation of several fungi before immobilization with sodium alginate resulted in a loss of viability, possibly due to the release of residues that created a toxic environment in the mycelial

suspension (Rossi et al. 2017). Rossi and colleagues (2017) demonstrated that mycelial suspensions of various fungal isolates without activated charcoal lost their viability within 24 hours. Activated charcoal should also provide suitable conditions in culture media for the preservation and evaluation of several characteristics of the microorganism in culture (Oliveira et al. 2006; Rossi et al. 2017).

Based on the results, the most effective formulation of alginate solution for the entrapment of *A. odoratus* strain K1 mycelia was 2% sodium alginate supplemented with Czapek medium, 0.25% activated charcoal, 5% sucrose, and 5% sorbitol. The addition of various components to the alginate solution aimed to improve mycelial viability, affecting aspects such as nutrition and protection (Szopa et al. 2022). This advantage of the alginate bead procedure provides tremendous application flexibility (Lalaymia et al. 2014).

In this study, the addition of MMN and Czapek media filled with alginate solution significantly improved the viability of *A. odoratus* mycelia compared to the control treatment without additives. These results suggest that the additional cultural media as a nutritional source enhanced the viability of mycelia after alginate entrapment. Despite the lack of reports about using media as additives for alginate beads in ECM fungal inocula. Culture media were applied for alginate entrapment as artificial spawn in mushroom production (Friel et al. 1999). The mycelial growth of *Pleurotus ostreatus* and *Agaricus bisporus* from alginate beads with culture media was significantly greater than on beads without media after incubation on the PDA medium. Moreover, the suitable nutrient formulation in beads might vary with different fungal species and required evaluation for each species (Ortiz et al. 2017).

Czapek medium is the optimal solution for alginate encapsulation of *A. odoratus* strain K1 mycelia because it promotes more vigorous mycelial germination and results in less production of dark brown secondary metabolites than MMN medium-formed alginate beads. Czapek is a synthetic medium that consists of low nutrient content with sole carbon and nitrogen sources (Basu et al. 2015; Jian et al. 2019). Some endophytic fungi also exhibited a slow growth rate and low production of secondary metabolites when cultured in Czapek (Vandermolen et al. 2013). Furthermore, half-strength MMN, which is widely used to culture several ectomycorrhizal fungi (Erland et al. 1990; Plett et al. 2020; Stuart et al. 2023), was also of interest to test its efficacy in preserving the viability of mycelia entrapped in calcium-alginate beads.

The concentration of the main component, sodium alginate, is crucial to the immobilization method. According to a systematic review by Szopa and colleagues (2022), concentrations below 1% of sodium alginate inhibited crosslinking and necessitated the presence of an additional component, such as bentonite. On the other hand, concentrations above 3% resulted in significant viscosity, preventing the solution from forming beads properly. The most commonly used concentration is 2%, as observed in other studies involving the entrapment of ECM mycelia (Rodrigues et al. 1999; Oliveira et al. 2006; Repáč 2007; Rossi et al. 2017; Costa et al. 2019). In this study, 2% sodium alginate was the optimal concentration, providing the highest viability of *A. odoratus* mycelia and proper bead formation.

The optimal types and concentrations of sugar and protectants depended on fungal species and the water potential (Magan and Lynch 1986). The addition of sugar and protectants to the sodium alginate solution created a hypertonic environment where the external solution had a higher concentration than the cell solution. As a result, water moved out of the cell through osmosis until equilibrium was reached (Lefa 2015). While sucrose served as an osmoprotectant, it also sustained the viability of the mycelia entrapped in the alginate beads by providing a carbon source. Moreover, sucrose can inhibit pigment production (Tseng et al. 2000). This pigment is a secondary metabolite produced by *A. odoratus* mycelium and has negative effect on mycelium cell growth. According to Lin and Demain (1991), the effect of carbon type on growth was found to differ depending on the fungal species; for example, sucrose gave maximum mycelial growth of golden chanterelle (*Cantharellus cibarius*) (Deshaware et al. 2021). This study demonstrated that 5% sucrose and 5% sorbitol were the most suitable additives for *A. odoratus*. Furthermore, the sodium alginate solution supplemented with 1% sucrose and 5% polyethylene glycol effectively maintained the viability of mycelia after storage for 15 days, despite slightly lower viability in fresh beads.

Additionally, a salt solution with a concentration of 0.85–0.9% was found to have the appropriate osmotic pressure to protect fungi and bacteria (Rossi et al. 2017; Cesari et al. 2020) and according to Deshaware (2021), increasing sucrose concentration up to 5% leads to a decrease in mycelial growth. Similarly, Yuan et al. (2012) and Itoo and Reshi (2013) documented a mycelial growth decrease as the concentration of the carbon source increases. So, the combination of these two protectants and their suitable concentrations was intriguing and should be examined in further studies.

After the mycelia of *A. odoratus* strain K1 were entrapped in alginate beads, the storage condition was considered important. Preserving the original properties of the fungi, including their colonization capability, required storing the fungal strains under conditions that slow down their metabolism (Lalaymia et al. 2014). Adequate consideration should be given to temperature and humidity during the storage of alginate beads (Rodrigues et al. 1999) as the suitable preservation conditions may vary among ECM fungal species. The viability of *Paxillus involutus* mycelia was nearly 100% when the alginate beads were kept in sterile water, 0.7 M CaCl2, and on filter paper at 25°C for 60 days. The suitable condition for the preservation of *Pisolithus tinctorius* immobilized in alginate beads was CaCl2 solution at 25°C as well (Rodrigues et al. 1999). Alginate beads of *Rhizopogon nigrescens*, preserved in 0.85% saline solution at 8°C, exhibited 100% viability even after 12 months (Oliveira et al. 2006), similar to beads of *Rhizopogon vulgaris* and *Pisolithus microcarpus* stored in distilled water at the same temperature (Rossi et al. 2017). Mycobeads of *Laccaria laccata* and *Hebeloma westralinese*, stored in deionized water at 4°C, still maintained over 90% viability (Kuek 1992). According to our results, the optimal condition for preservation of *A. odoratus* strain K1 mycelia entrapped in alginate beads was storage in distilled water and glycerol at 25°C, which presented an over 80% survival rate after 60 days. The proper temperature for preservation may correspond to the habitat of this tropical ECM fungus, such as the deciduous dipterocarp forest, where the underground temperature ranges from 19°C to 32°C (Intanil et al. 2018). The cultivation of *A. sirindhorniae* mycelia at different temperatures revealed that

30°C and room temperature were the optimal conditions, providing the highest mycelial biomass (Suwannasai et al. 2020).

Verifying the effectiveness of ECM mycelial immobilization requires investigating ECM colonization on plants. In this study, we examined the efficacy of *A. odoratus* colonization in terms of ECM root development and colonization rate. The experiment, conducted in the rhizobox of *H. odorata* seedlings, showed that mycelia germinated from fresh alginate beads within 30 days. A similar result was also demonstrated in *Eucalyptus dunnii* inoculated with *Pisolithus microcarpus* entrapped in alginate beads supplemented with activated charcoal (Rossi et al. 2017). However, the colonization and ECM root formation of mycelia germinated from fresh alginate beads were slightly delayed when compared with the colonization of mycelia germinated from discs. The initial step of ECM root colonization involves the recognition of signal molecules released by both host plants and ECM fungi. Various root exudates induced spore germination and enhance the mycelial growth of ECM fungi (Garcia et al. 2015). Nevertheless, the hydrogel structure of alginate beads forms a temporary barrier between the inside organism and the external environment (Szopa et al. 2022), which may require time for degradation and the reach of root exudate to the ECM mycelia entrapped in the beads. The slow development of *A. odoratus* mycelia was also distinctly observed in preserved alginate beads, emphasizing the effect of preservation conditions on the efficiency of ECM root development.

In our study, the colonization rate of *A. odoratus* in *H. odorata* seedlings showed high efficacy compared to other studies using alginate bead inocula of ECM fungi. The ECM colonization rates in *Picea abies* (Repáč 2007) and *Pinus taeda* (Oliveira et al. 2006) seedlings inoculated with alginate beads were 36% and 39%, respectively. Additionally, Kaewgrajang (2019) reported colonization rates of *A. odoratus* in the form of spores and mycelial suspension in two other dipterocarp species, *Dipterocarpus tuberculatus* and *Shorea roxburghii*, within the range of 32–60%. However, within 45 days, *A. odoratus* colonized the roots by more than 49%, even though the percentage of colonization decreased when the mycelia were entrapped in alginate beads and preserved for 2 months. This result indicates the efficiency and applicability of *A. odoratus* alginate beads as a high-performance inoculum with the potential for large-scale production.

Conclusion

Ectomycorrhizae are a crucial factor in forest productivity. The establishment and growth of the most important plant species utilized in reforestation programs and forest plantation are dependent on ECM fungi. The development of production technology for ECM inoculants is an important achievement in advancing the widespread utilization of ECM fungi in forest nurseries. In this study, the mycelia of the ECM fungus *A. odoratus* strain K1 were successfully entrapped in calcium alginate beads with a high viability. This study clearly highlights the potential of alginate gel as an efficient formulation. These alginate beads used as inoculum presented a high survival rate and a significant infectivity in relation to *H. odorata* seedlings after 2 months of storage. This indicates a high potential for commercial application of the inoculum in large scale dipterocarp seedling production. The findings of this study

open new perspectives for enriching plantation forest research where ectomycorrhizal associations can flourish to protect the decline of ECM mushroom and dipterocarp forests in Thailand due to severe climate change effects. However, a future study should also focus on optimizing alginate matrix composition in terms of the highest survival rate of this fungus, and additional research involving the scale-up of the encapsulation process is also being developed.

Declarations

Acknowledgments

The authors are highly grateful to Thailand Science Research and Innovation Fund Chulalongkorn University (CU_FRB65_dis (12) 100_23_30) for providing financial grant for the conduct of research. The authors are also thankful to Mr. Nopporn Nontapa for providing seeds and seedlings for conducting the experiment. Additionally, many thanks to Dr. Kansinee Hungsaprug for linguistic support and proof-reading the earlier versions of the manuscript, and Mr. Phobthum Kosolwattana for his photos provision.

Funding

This work was supported by Thailand Science Research and Innovation Fund Chulalongkorn University (CU_FRB65_dis (12) 100_23_30).

Competing Interests

All authors have no relevant financial or non-financial interests to disclose.

Author Contributions

Yanisa Punsung and Jittra Piapukiew contributed to the research conceptualization and design of the experiment. The funding acquisition was performed by Jittra Piapukiew. Material preparation, experimentation, data collection and analysis were performed by Yanisa Punsung. Pawara Pachit provided a guidance on molecular identification for the experiment and analysis. The first draft of the manuscript was written by Yanisa Punsung, and all authors commented and edited on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

References

1. Águeda B, Parladé J, Fernández-Toirán LM, Cisneros Ó, de Miguel AM, Modrego, MP, Martínez-Peña F, Pera J (2008). Mycorrhizal synthesis between Boletus edulis species complex and rockroses

(*Cistus* sp.). Mycorrhiza18:443-449. https://doi.org/10.1007/s00572-008-0192-3

2. Ahmadzai AS, Ejtehadi H, Farzam M, Bashirzadeh M (2023). A new record of *Astraeus hygrometricus* (Pers.) Morgan (Boletales, Basidiomycota) from Afghanistan. MycoAsia. https://doi.org/10.59265/%20mycoasia.2023-02
3. Averill C, Dietze MC, Bhatnagar JM (2018). Continental-scale nitrogen pollution is shifting forest mycorrhizal associations and soil carbon stocks. Global change biology 24:4544-4553. https://doi.org/10.1111/gcb.14368
4. Basu S, Bose C, Ojha N, Das N, Das J, Pal M, Khurana S (2015). Evolution of bacterial and fungal growth media. Bioinformation 11:182-184. https://doi.org/10.6026/97320630011182
5. Bauman JM., Keiffer CH, Hiremath S, Mccarthy BC (2013). Soil preparation methods promoting ectomycorrhizal colonization and American chestnut Castanea dentata establishment in coal mine restoration. J. Appl. Ecol 50:721–729. https://doi.org/10.1111/1365-2664.12070
6. Bois G, Piché Y, Fung YP, Khasa DP (2005). Mycorrhizal inoculum potentials of pure reclamation materials and revegetated tailing sands from the Canadian oil sand industry. Mycorrhiza 15:149-158. https://doi.org/10.1007/s00572-004-0315-4
7. Brearley FQ (2011). The Importance of Ectomycorrhizas for the Growth of Dipterocarps and the Efficacy of Ectomycorrhizal Inoculation Schemes. In: Rai M and Varma A (eds) Diversity and Biotechnology of Ectomycorrhizae, Springer Berlin Heidelberg, pp 3-17 https://doi.org/10.1007/978-3-642-15196-5_1
8. Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996). Working with Mycorrhizas in Forestry and Agriculture. Australian Centre for International Agricultural Research. https://doi.org/10.13140/2.1.4880.5444
9. Brundrett M, Malajczuk, N, Mingqin G, Daping X, Snelling S, Dell B (2005). Nursery inoculation of Eucalyptus seedlings in Western Australia and Southern China using spores and mycelial inoculum of diverse ectomycorrhizal fungi from different climatic regions. Forest Ecology and Management 209:193-205. https://doi.org/10.1016 /j.foreco.2005.01.031
10. Butkrachang S, Boonchieng E, Sardsud U, Sukchotiratana M., Plikomol A, Chairote G, Narongchai P (2007). Wild mushroom database of Chiang Mai community forest. The Asian Journal of Biology Education 3:65-70. https//doi.org/10.57443/ajbe.3.0_65
11. Castro AL, Elizondo S, Valladares Y, Rivera W (2020). Wood Wide Web: communication through the mycorrhizal network. Tecnología en Marcha 33:114-125. https://doi.org/10.18845/tm.v33i4.4601
12. Cesari AB, Paulucci NS, Yslas EI, Dardanelli MS (2020). Immobilization of *Bradyrhizobium* and *Azospirillum* in alginate matrix for long time of storage maintains cell viability and interaction with peanut. Appl Microbiol Biotechnol104:10145-10164. https://doi.org/10.1007/s00253-020-10910-7
13. Chandel AK, Kapoor RK, Singh A, Kuhad RC (2007). Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. Bioresource Technology 98:1947-1950. https://doi.org/10.1016/j.biortech.2006.07.047

14. Charya LS, Garg S (2019). Chapter 19 - Advances in methods and practices of ectomycorrhizal research. In: Meena SN and Naik MM (eds) Advances in Biological Science Research, Academic Press, pp 303-325. https://doi.org/10.1016/B978-0-12-817497-5.00019-7
15. Coleman MD, Bledsoe CS, Lopushinsky W (1989). Pure culture response of ectomycorrhizal fungi to imposed water stress. Canadian Journal of Botany 67:29-39. https://doi.org/10.1139/b89-005
16. Costa LS, Grazziotti PH, Silva AC, Fonseca AJ, Gomes ÂLF, Grazziotti DCFS, Rossi MJ (2019). Alginate gel entrapped ectomycorrhizal inoculum promoted growth of cuttings of Eucalyptus clones under nursery conditions. Canadian Journal of Forest Research 49: 978-985. https://doi.org/10.1139/cjfr-2018-0129
17. Cunningham GH (1944) The Gasteromycetes of Australia and New Zealand. Dunedin, New Zealand
18. Curguz V, TabakovicM, Veselinovic M, Raicevic V, Drazic D, Jovanovic L, Kikovic D (2010). The influence of heavy metals on the growth of ectomycorrhizal fungi. Minerva Biotecnologica 22:17-22.
19. Dell B, Sanmee R, Lumyong P, Lumyong S (2005) Ectomycorrhizal fungi in dry and wet dipterocarp forests in northern Thailand - Diversity and use as food. Proceedings of the 8th Round Table Conference on Dipterocarps, Ho Chi Minh, Vietnam.
20. Deshaware S, Marathe SJ, Bedade D, Deska J, Shamekh S (2021). Investigation on mycelial growth requirements of *Cantharellus cibarius* under laboratory conditions. Archives of Microbiology 203:1539-1545. https://doi.org/10.1007/s00203-020-02142-0
21. Erland s, soderstrom B, Andersson S (1990). Effects of liming on ectomycorrhizal fungi infecting *Pinus sylvestris* L. New Phytologist 115:683-688. https://doi.org/10.1111/j.1469-8137.1990.tb00500.x
22. Fangfuk W, Okada K, Petchang R, To-anun C, Fukuda M, Yamada A (2010). In vitro mycorrhization of edible *Astraeus* mushrooms and their morphological characterization. Mycoscience 51:234-241. https://doi.org/10.1007/S10267-009-0031-1
23. Fernandez CW, Mielke L, Stefanski A, Bermudez R, Hobbie SE, Montgomery RA, Kennedy PG (2023). Climate change–induced stress disrupts ectomycorrhizal interaction networks at the boreal–temperate ecotone. Proceedings of the National Academy of Sciences 120: e2221619120. https://doi.org/10.1073/pnas.2221619120
24. Friel MT, McLoughlin AJ (1999). Immobilisation as a strategy to increase the ecological competence of liquid cultures of *Agaricus bisporus* in pasteurised compost. FEMS Microbiology Ecology 30:39-46. https://doi.org/10.1016/S0168-6496(99)00037-9
25. Futai K, Taniguchi T, Kataoka R (2008). Ectomycorrhizae and Their Importance in Forest Ecosystems. In: Siddiqui ZA, Akhtar MS, Futai K (eds) Mycorrhizae: Sustainable Agriculture and Forestry. Springer, Netherlands, pp. 241-285. https://doi.org/10.1007/978-1-4020-8770-7_11
26. Garcia K, Delaux PM, Cope KR, Ané JM (2015). Molecular signals required for the establishment and maintenance of ectomycorrhizal symbioses. New Phytologist 208:79-87. https://doi.org/10.1111/nph.13423

27. Gardes M, Bruns TD (1996). Community structure of ectomycorrhizal fungi in a *Pinus muricata* forest: above- and below-ground views. Canadian Journal of Botany 74:1572-1583. https://doi.org/10.1139/b96-190
28. Hachani C, Lamhamedi M, Cameselle C, Gouveia S, Abidine A, Khasa D, Bejaoui Z (2020). Effects of Ectomycorrhizal Fungi and Heavy Metals (Pb, Zn, and Cd) on Growth and Mineral Nutrition of *Pinus halepensis* Seedlings in North Africa. Microorganisms 8:1-16. https://doi.org/10.3390/microorganisms8122033
29. Hagenbo A, Piñuela Y, Castaño C, Martínez J, Miguel S, Alday JG, Bonet JA (2021) Production and turnover of mycorrhizal soil mycelium relate to variation in drought conditions in mediterranean *Pinus pinaster*, *Pinus sylvestris* and *Quercus ilex* forests. New Phytol 230:1609–1622. https://doi.org/10.1111/nph.17012
30. Hawkins HJ, Cargill RIM, Nuland ME, Hagen SC, Field KJ, Sheldrake M, Soudzilovskaia NA, Kiers ET (2023). Mycorrhizal mycelium as a global carbon pool. Current Biology 33:560-573. https://doi.org/10.1016/j.cub.2023.02.027
31. Helbert M, Nara K (2019). Ectomycorrhizal fungal communities of secondary tropical forests dominated by Tristaniopsis in Bangka Island, Indonesia. PLoS One 14:e0221998. https://doi.org/10.1371/journal.pone.0221998
32. Itoo ZA, Reshi ZA (2013). The Multifunctional Role of Ectomycorrhizal Associations in Forest Ecosystem Processes. The Botanical Review *79*:371-400. https://doi.org/10.1007/s12229-013-9126-7
33. Jian Q, Li T, Wang Y, Zhang Y, Zhao Z, Zhang X, Gong L, Jiang Y (2019). New insights into fumonisin production and virulence of *Fusarium proliferatum* underlying different carbon sources. Food Research International 116:397-407. https://doi.org/10.1016/j.foodres.2018.08.053
34. Kaewgrajang T, Sangwanit U, Iwase K, Kodama M, Yamato M (2013). Effects of ectomycorrhizal fungus *astraeus odoratus* on dipterocarpus alatus seedlings. Journal of Tropical Forest Science 25:200-205. http://www.jstor.org/stable/23617034
35. Kaewgrajang T, Sakolrak B, Sangwanit U (2019). Growth Response of *Dipterocarpus tuberculatus* and *Shorea roxburghii* Seedlings to *Astraeus odoratus*. Environment and Natural Resources Journal 17:80-88. https://doi.org/10.32526/ennrj.17.3.2019.25
36. Kafle SK (2006). Effects of forest fire protection on plant diversity in a tropical deciduous dipterocarp-oak forest, Thailand. Int Forest Fire News 34:64-71.
37. Karlsen E, Smith ME, Askey BC, Gazis R (2022). Native ectomycorrhizal fungi from the endangered pine rocklands are superior symbionts to commercial inoculum for slash pine seedlings. Mycorrhiza 32:465-480. https://doi.org/10.1007/s00572-022-01092-3
38. Karun NC, Sridhar KR (2013) Occurrence and distribution of Termitomyces (Basidiomycota, Agaricales) in the Western Ghats and on the west coast of India. Czech Mycology 65:233– 254. https://doi.org/10.33585/cmy.65207

39. Kennedy K, Maxwell J, Lumyong S (2012). Fire and the production of *Astraeus odoratus* (Basidiomycetes) sporocarps in deciduous dipterocarp-oak forests of northern Thailand. Maejo International Journal of Science and Technology 6:483-504. https://doi.org/10.14456/mijst.2012.35
40. Kibar B, Peksen A (2011). Nutritional and environmental requirements for vegetative growth of edible ectomycorrhizal mushroom *Tricholoma terreum*. Zemdirbyste 98:409-414.
41. Koh LP, Kettle CJ, Sheil D, Lee TM, Giam X, Gibson LG, Clement GR (2013). Biodiversity state and trends in Southeast Asia. Encyclopedia of biodiversity. In: Levin S (ed) Encyclopedia of biodiversity, 2nd edn. Academic Press, Amsterdam, pp 509-527. http://dx.doi.org/10.1016/B978-0-12-384719-5.00357-9
42. Kuek C, Tommerup IC, Malajczuk N (1992). Hydrogel bead inocula for the production of ectomycorrhizal eucalypts for plantations. Mycological Research 96:273-277. https://doi.org/10.1016/S0953-7562(09)80937-4
43. Kuek C (1996). Shake-flask culture of *Laccaria laccata*, an ectomycorrhizal basidiomycete. Applied Microbiology and Biotechnology45:319-326. https://doi.org/10.1007/s002530050690
44. Kumar S, Satyanarayana T (2002). Production of Inoculum of Ectomycorrhizal Fungi. In: Mukerji KG, Manoharachary C, Chamola BP (eds) Techniques in Mycorrhizal Studies, Springer, Netherlands, pp 143-166. https://doi.org/10.1007/978-94-017-3209-3_8
45. Kumla J, Danell E, Bussaban B, Lumyong S (2011). Suitable growth conditions and nutrition factors on in vitro culture of Phlebopus portentosus (Boletales). Chiang Mai Journal of Science 38:156-159.
46. Lalaymia I, Cranenbrouck S, Declerck S (2014). Maintenance and preservation of ectomycorrhizal and arbuscular mycorrhizal fungi. Mycorrhiza 24:323-337. https://doi.org/10.1007/s00572-013-0541-8
47. Lefa B (2015). The movement of water molecules in response to solute concentration. Journal of Science Education 1: 14.
48. Lehto T, Zwiazek, JJ (2011). Ectomycorrhizas and water relations of trees: a review. Mycorrhiza 21:71-90. https://doi.org/10.1007/s00572-010-0348-9
49. Lee SS, Patahayah M, Chong WS, Lapeyrie FF (2008) Successful ectomycorrhizal inoculation of two dipterocarp species with a locally isolated fungus in Peninsular Malaysia. J Trop for Sci 20:237–247
50. Lin TF, Demain AL (1991). Effect of nutrition of *Monascus* sp. on formation of red pigments. Applied Microbiology and Biotechnology 36:70-75. https://doi.org/10.1007/BF00164701
51. Magan N, Lynch J (1986). Water Potential, Growth and Cellulolysis of Fungi Involved in Decomposition of Cereal Residues. Microbiology 132:1181-1187. https://doi.org/10.1099/00221287-132-5-1181
52. Martínez J, Fischer C, Bonet JA, Olivera A, Oliach D, Colinas C (2012). Economically profitable post fire restoration with black truffle (*Tuber melanosporum*) producing plantations. New Forests 43:615-630. https://doi.org/10.1007/s11056-012-9316-x
53. Marx DH (1969). The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. II. Production, identification, and biological activity of antibiotics produced by

Leucopaxillus cerealis var. piceina. Phytopathology 59:411-417.

54. Marx DH, Marrs LF, Cordell CE (2002). Practical use of the mycorrhizal fungal technology in forestry, reclamation, arboriculture, agriculture, and horticulture. Dendrobiology 47:27-40
55. Mauperin C, Mortier F, Garbaye J, Tacon FL, Carr G (1987). Viability of an ectomycorrhizal inoculum produced in a liquid medium and entrapped in a calcium alginate gel. Canadian Journal of Botany, 65:2326-2329. https://doi.org/10.1139/b87-316
56. Menkis A, Vasiliauskas R, Taylor AFS, Stenlid J., Finlay R (2007). Afforestation of abandoned farmland with conifer seedlings inoculated with three ectomycorrhizal fungi - Impact on plant performance and ectomycorrhizal community. Mycorrhiza 17:337–348. https://doi.org/10.1007/s00572-007-0110-0
57. Miller RM, Jastrow JD (1992). The Role of Mycorrhizal Fungi in Soil Conservation. In: Bethlenfalvay GJ, Linderman RG (eds) Mycorrhizae in Sustainable Agriculture, American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, pp. 29-44. https://doi.org/10.2134/asaspecpub54.c2
58. Mortier F, Tacon F, Garbaye J (1990). Effect of dose and formulation of *Laccaria laccata* inoculum on mycorrhizal infection and growth of Douglas fir in a nursery. Agriculture, Ecosystems and Environment 28:351-354. https://doi.org/10.1016/0167-8809(90)90062-I
59. Mortimer PE, Karunarathna SC, Li Q *et al* (2012) Prized edible Asian mushrooms: ecology, conservation and sustainability. Fungal Diversity 56:31–47. https://doi.org/10.1007/s13225-012-0196-3
60. Murata H, Yamada A, Maruyama T, Endo N, Yamamoto K, Ohira T, Shimokawa T (2013). Root endophyte interaction between ectomycorrhizal basidiomycete *Tricholoma matsutake* and arbuscular mycorrhizal tree Cedrela odorata, allowing in vitro synthesis of rhizospheric “shiro”. Mycorrhiza 23:235-242. https://doi.org/10.1007/s00572-012-0466-7
61. Mussatto SI, Roberto IC (2004). Optimal Experimental Condition for Hemicellulosic Hydrolyzate Treatment with Activated Charcoal for Xylitol Production. Biotechnology Progress 20:134-139. https://doi.org/10.1021/bp034207i
62. Nguyen TT, Baker PJ (2016). Structure and composition of deciduous dipterocarp forest in Central Vietnam: patterns of species dominance and regeneration failure. *Plant Ecology and Diversity*, *9*(5-6), 589-601. https://doi.org/10.1080/17550874.2016.1210261
63. Nilsson RH, Larsson KH, Taylor AFS, Bengtsson J, Jeppesen TS, Schigel D, Kennedy P, Picard K, Glöckner FO, Tedersoo L, Saar I, Kõljalg U, Abarenkov K (2018). The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. Nucleic Acids Research, 47:259-264. https://doi.org/10.1093/nar/gky1022
64. Nouhra ER, Toledo DL (1998) The first record of *Astraeus hygrometricus* from Argentina. Mycologist 12:112–113.
65. Nunes LJR, Meireles CIR, Pinto CJ, Almeida NMC (2020). Forest Contribution to Climate Change Mitigation: Management Oriented to Carbon Capture and Storage. Climate 8:21.

https://doi.org/10.3390/cli8020021

66. Oliveira LP, Rossi MJ, Furigo A, Silva GN, Oliveira VL (2006). Viability and infectivity of an ectomycorrhizal inoculum produced in an airlift bioreactor and immobilized in calcium alginate. Brazilian Journal of Microbiology 37:251-255. https://doi.org/10.1590/S1517-83822006000300011
67. Ogawa M (2006) Inoculation methods of Scleroderma column are onto dipterocarps. In: Suzuki K, Ishii K, Sakurai S, Sasaki S (eds) Plantation technology in tropical forest science. Springer-Verlag, Tokyo, Japan, pp 185–197
68. Onwuchekwa NE, Zwiazek J J, Quoreshi A, Khasa DP (2014). Growth of mycorrhizal jack pine (*Pinus banksiana*) and white spruce (*Picea glauca*) seedlings planted in oil sands reclaimed areas. Mycorrhiza 24:431-441. https://doi.org/10.1007/s00572-014-0555-x
69. Ortiz G, Colavolpe MB, Alberto E (2017). Artificial spawn generation based on alginate encapsulated mycelium as inoculum for mushroom cultivation. African Journal of Biotechnology 16:1776-1783. https://doi.org/10.5897/AJB2017.16065
70. Pachit P, Disyatat NR, Piapukiew J (2020). Temporal changes in enzyme activities associated with ectomycorrhizas and soil from secondary deciduous dipterocarp forest fragments. Pedobiologia 81-82:150661. https://doi.org/10.1016/j.pedobi.2020.150661
71. Pavithra M, Greeshma AA, Karun NC, Sridhar KR (2015). Observations on the *Astraeus* spp. of Southwestern India. Mycosphere 6:421-432. https://doi.org/10.5943/mycosphere%2F6%2F4%2F4
72. Péret B, Svistoonoff S, Laplaze L (2009). When Plants Socialize: Symbioses and Root Development. In: Beeckman T (ed) Annual Plant Reviews Volume 37: Root Development, pp 209-238. https://doi.org/10.1002/9781444310023.ch9
73. Pérez J, Guerin A, Rinaldi AC, Yu F, Verbeken A, Hernández F, Martínez M (2021). Edible mycorrhizal fungi of the world: What is their role in forest sustainability, food security, biocultural conservation and climate change? Plants, people, planet 3:471-490. https://doi.org/10.1002/ppp3.10199
74. Phosri C, Watling R, Martín MP, Whalley AJS (2004). The genus *Astraeus* in Thailand. Mycotaxon 89:453-463.
75. Phosri C, Martín MP, Sihanonth P, Whalley AJS, Watling R (2007). Molecular study of the genus *Astraeus*. Mycological Research 111:275-286. https://doi.org/10.1016/j.mycres.2007.01.004
76. Phosri C, Martín MP, Watling R (2013). *Astraeus*: hidden dimensions. IMA fungus 4:347-356.
77. Piñeiro J, Maestre FT, Bartolomé L, Valdecantos A (2013). Ecotechnology as a tool for restoring degraded drylands: A meta-analysis of field experiments. Ecological Engineering 61:133-144. https://doi.org/10.1016/j.ecoleng.2013.09.066
78. Plett KL, Singan VR, Wang M, Ng V, Grigoriev IV, Martin F, Plett JM, Anderson IC (2020). Inorganic nitrogen availability alters Eucalyptus grandis receptivity to the ectomycorrhizal fungus *Pisolithus albus* but not symbiotic nitrogen transfer. New Phytologist 226:221-231. https://doi.org/10.1111/nph.16322
79. Ramos WJ, Tad-awan BS (2018). Mycelial growth of *Scleroderma* sp. as Affected by Culture Media. Mountain Journal of Science and Interdisciplinary Research, 78:49-60.

80. Repáč I (2007). Ectomycorrhiza formation and growth of *Picea abies* seedlings inoculated with alginate-bead fungal inoculum in peat and bark compost substrates. Forestry: An International Journal of Forest Research 80:517-530. https://doi.org/10.1093/forestry/cpm036
81. Repáč I (2011). Ectomycorrhizal Inoculum and Inoculation Techniques. In: Rai M, Varma A (eds) Diversity and Biotechnology of Ectomycorrhizae, Springer Berlin, Heidelberg, pp 43-63. https://doi.org/10.1007/978-3-642-15196-5_3
82. Rodrigues L, Megumi MC, Borges AC (1999). Viability of ectomycorrhizal fungus mycelium entrapped in calcium alginate gel. Mycorrhiza 8:263-266. https://doi.org/10.1007/s005720050243
83. Rossi MJ, Furigo A, Oliveira VL (2007). Inoculant Production of Ectomycorrhizal Fungi by Solid and Submerged Fermentations. Food Technology and Biotechnology 45:277-286.
84. Rossi MJ, Oliveira VL (2011). Growth of the Ectomycorrhizal Fungus *Pisolithus Microcarpus* in different nutritional conditions. Brazilian Journal of Microbiology, 42:624-632. https://doi.org/10.1590/s1517-838220110002000027
85. Rossi MJ, Nascimento FX, Brandão PMFDR, Camelini CM, Giachini AJ (2017). Activated Charcoal Increases the Viability of Ectomycorrhizal fungi During the Stages of Cultivation Aiming the Production of Large-Scale Inocula via Submerged Cultivation. Advances in Biotechnology and Microbiology 6:1-9. https://doi.org/10.19080/AIBM.2017.06.555692
86. Rudawska M, Leski T (2019). The Impact of Climate change on ectomycorrhizal fungi. ACADEMIA-The magazine of the Polish Academy of Sciences 44-47.
87. Sanmee R, Dell B, Lumyong P, Izumorid K, Lumyong S (2003) Nutritive value of popular wild edible mushrooms from Northern Thailand. Food Chemistry 82:527–532. https://doi.org/10.1016/S0308-8146(02)00595-2
88. Sapes GP, Demaree Y, Lekberg A (2021) Plant carbohydrate depletion impairs water relations and spreads via ectomycorrhizal networks. New Phytol 229:3172–3183. https://doi.org/10.1111/nph.17134
89. Sebastiana M, Pereira VT, Alcântara A, Pais MS, Silva AB (2013). Ectomycorrhizal inoculation with *Pisolithus tinctorius* increases the performance of *Quercus suber* L. (cork oak) nursery and field seedlings. New Forests 44:937-949. https://doi.org/10.1007/s11056-013-9386-4
90. Smith SE, Read D (2008). Mycorrhizal Symbiosis, 3rd edn. Academic Press. https://doi.org/10.1016/B978-0-12-370526-6.X5001-6
91. Sodhi NS, Posa MRC, Lee TM, Bickford D, Koh LP, Brook BW (2010). The state and conservation of Southeast Asian biodiversity. Biodiversity and Conservation, 19:317-328.
92. Sousa NR, Ramos MA, Marques APGC, Castro PML (2014). A genotype dependent-response to cadmium contamination in soil is displayed by *Pinus pinaster* in symbiosis with different mycorrhizal fungi. Applied Soil Ecology 76:7-13. https://doi.org/10.1016/j.apsoil.2013.12.005
93. Sterck F, Vos M, Hannula SE, Goede S, Vries W, Ouden J, Nabuurs GJ, Putten W, Veen C (2021). Optimizing stand density for climate-smart forestry: A way forward towards resilient forests with enhanced

carbon storage under extreme climate events. Soil Biology and Biochemistry, 162:108396. https://doi.org/10.1016/j.soilbio.2021.108396

94. Stuart EK, Singan V, Amirebrahimi M, Na H, Ng V, Grigoriev IV, Martin F, Anderson IC, Plett JM, Plett KL (2023). Acquisition of host-derived carbon in biomass of the ectomycorrhizal fungus *Pisolithus microcarpus*
is correlated to fungal carbon demand and plant defences. FEMS Microbiology Ecology 99. https://doi.org/10.1093/femsec/fiad037
95. Suwannasai N, Dokmai P, Yamada A, Watling R, Phosri C. (2020). First ectomycorrhizal syntheses between *Astraeus sirindhorniae* and *Dipterocarpus alatus* (Dipterocarpaceae), pure culture characteristics, and molecular detection. Biodiversitas Journal of Biological Diversity 21. https://doi.org/10.13057/biodiv/d210130
96. Szopa D, Mielczarek M, Skrzypczak D, Izydorczyk G, Mikula K, Chojnacka K, Witek A. (2022). Encapsulation efficiency and survival of plant growth-promoting microorganisms in an alginate-based matrix – A systematic review and protocol for a practical approach. Industrial Crops and Products 181:114846. https://doi.org/10.1016/j.indcrop.2022.114846
97. Tacon FL, Jung G, Mugnier J, Michelot P, Mauperin C (1985). Efficiency in a forest nursery of an ectomycorrhizal fungus inoculum produced in a fermentor and entrapped in polymeric gels. Canadian Journal of Botany 63:1664-1668. https://doi.org/10.1139/b85-229
98. Tamura K, Stecher G, Kumar S (2021). MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Molecular Biology and Evolution 38:3022-3027. https://doi.org/10.1093/molbev/msab120
99. Torres P, Honrubia M (1991). Growth dynamics and characterization of some ectomycorrhizal fungi in culture. Cryptogamie, Mycologie 12:183-192.
100. Tseng YY, Chen MT, Lin CF (2000). Growth, pigment production and protease activity of *Monascus purpureus* as affected by salt, sodium nitrite, polyphosphate and various sugars. Journal of Applied Microbiology 88:31-37. https://doi.org/10.1046/j.1365-2672.2000.00821.x
101. Turjaman M, Tamai Y, Segah H, Limin SH, Cha JY, Osaki M, Tawaraya K (2005) Inoculation with the ectomycorrhizal fungi Pisolithus arhizus and Scleroderma sp. improves early growth of Shorea pinanga nursery seedlings. New For 30:67–73
102. Turjaman M, Tamai Y, Segah H, Limin SH, Osaki M, Tawaraya K (2006) Increase in early growth and nutrient uptake of *Shorea seminis* inoculated with two ectomycorrhizal fungi. J Trop For Sci 18:243–249
103. Vandermolen KM, Raja HA, El-Elimat T, Oberlies NH (2013). Evaluation of culture media for the production of secondary metabolites in a natural products screening program. AMB Express 3:71. https://doi.org/10.1186/2191-0855-3-71
104. Visagie CM, Houbraken J, Frisvad JC, Hong SB, Klaassen CHW, Perrone G, Seifert KA, Varga J, Yaguchi T, Samson R A (2014). Identification and nomenclature of the genus Penicillium. Studies in Mycology 78:343-371. https://doi.org/10.1016/j.simyco.2014.09.001

105. Vuorinen I, Hamberg L, Müller M, Seiskari P, Pennanen T (2015). Development of growth media for solid substrate propagation of ectomycorrhizal fungi for inoculation of Norway spruce (*Picea abies*) seedlings. Mycorrhiza 25:311-324. https://doi.org/10.1007/s00572-014-0611-6
106. Walker RF (1999). Reforestation of an eastern sierra nevada surface mine with containerized jeffrey pine: seedling growth and nutritional responses to controlled release fertilization and ectomycorrhizal inoculation. J. Sustain. Fo 9:127–147. https://doi.org/10.1300/J091v09n03_06
107. Wang Z, Jiang Y, Deane DC, He F, Shu W, Liu Y (2019). Effects of host phylogeny, habitat and spatial proximity on host specificity and diversity of pathogenic and mycorrhizal fungi in a subtropical forest. New Phytologist 223:462-474. https://doi.org/10.1111/nph.15786
108. Waring B, Neumann M, Prentice IC, Adams M, Smith P, Siegert M (2020). Forests and Decarbonization – Roles of Natural and Planted Forests [Perspective]. Frontiers in Forests and Global Change 3. https://doi.org/10.3389/ffgc.2020.00058
109. White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ, White TJ (eds) PCR Protocols: A Guide to Methods and Applications, Academic Press, pp 315-322. https://doi.org/10.1016/B978-0-12-372180-8.50042-1
110. Widden P, Parkinson D (2011), The effects of a forest fire on soil microfungi. Soil Bio and Biochem 78:125-138. https://doi.org/10.1016/0038-0717(75)90010-3
111. Wilson AW, Binder M, Hibbett DS (2012). Diversity and evolution of ectomycorrhizal host associations in the Sclerodermatineae (Boletales, Basidiomycota). New Phytologist 194:1079-1095. https://doi.org/10.1111/j.1469-8137.2012.04109.x
112. Xu Ml, Zhu J, Kang H, Xu A, Zhang J, Li F. (2008). Optimum conditions for pure culture of major ectomycorrhizal fungi obtained from *Pinus sylvestris* var. mongolica plantations in southeastern Keerqin sandy lands, China. Journal of Forestry Research 19:113-118. https://doi.org/10.1007/s11676-008-0019-2
113. Yuan B, Chi X, Zhang R (2012). Optimization of exopolysaccharides production from a novel strain of Ganoderma lucidum CAU5501 in submerged culture. Brazilian journal of microbiology 43:490-497. https://doi.org/10.1590/S1517-83822012000200009
114. Zhou Z, Miwa M, Hogetsu T (1999). Analysis of genetic structure of a *Suillus grevillei* population in a *Larix kaempferi* stand by polymorphism of inter-simple sequence repeat (ISSR). New Phytologist 144:55-63. https://doi.org/10.1046/j.1469-8137.1999.00504.x

Figures

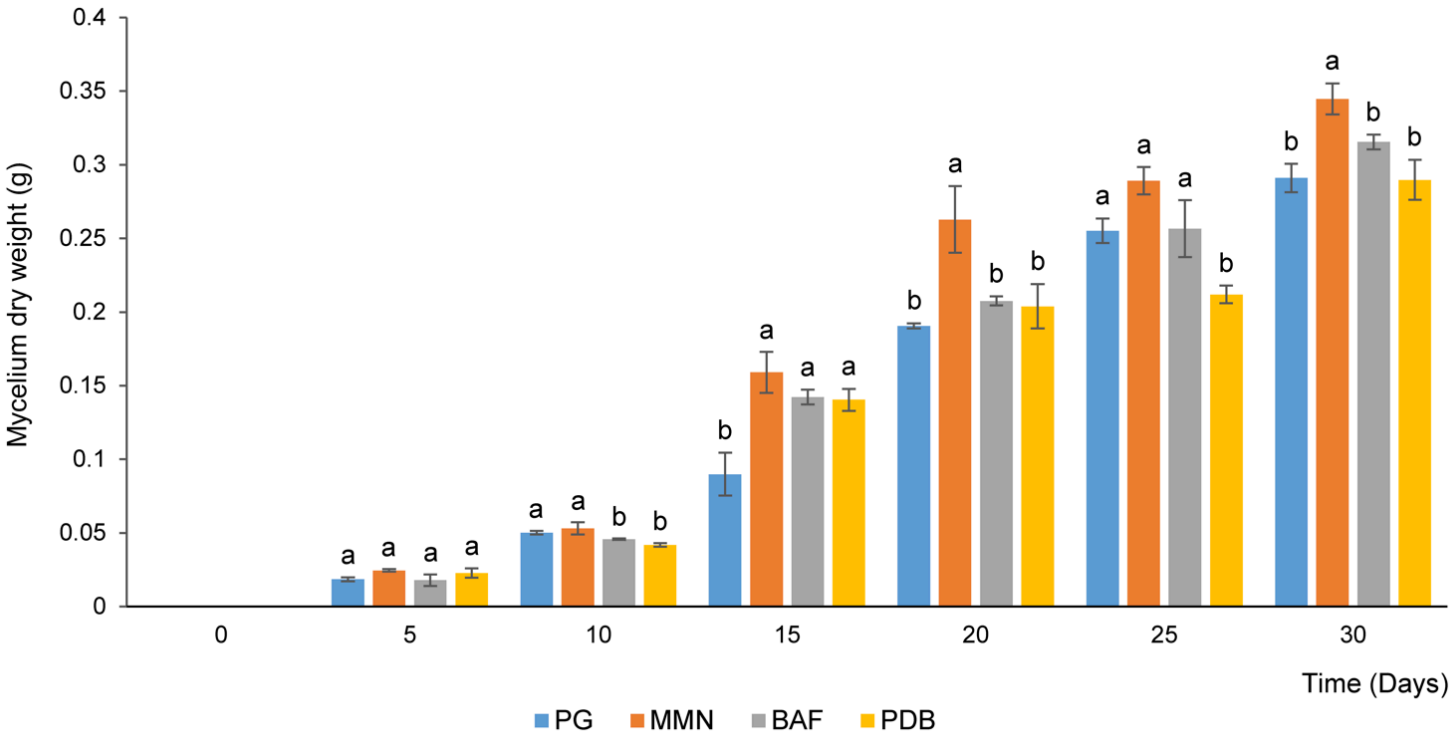

Figure 1

Dry weights of the strainK1 mycelium growing in different liquid cultural media for different timepoints up to 30 days. Cultures were harvested independently for each timepoint. Different letters showed significant differences ($P < 0.05$) in the dry weight of the same mycelial age. Abbreviations: PG (Pridham-Gottlieb medium), MMN (Modified Melin-Norkrans), BAF (Biotin-Aneurin-Folic Acid), and PDB (Potato Dextrose Broth)

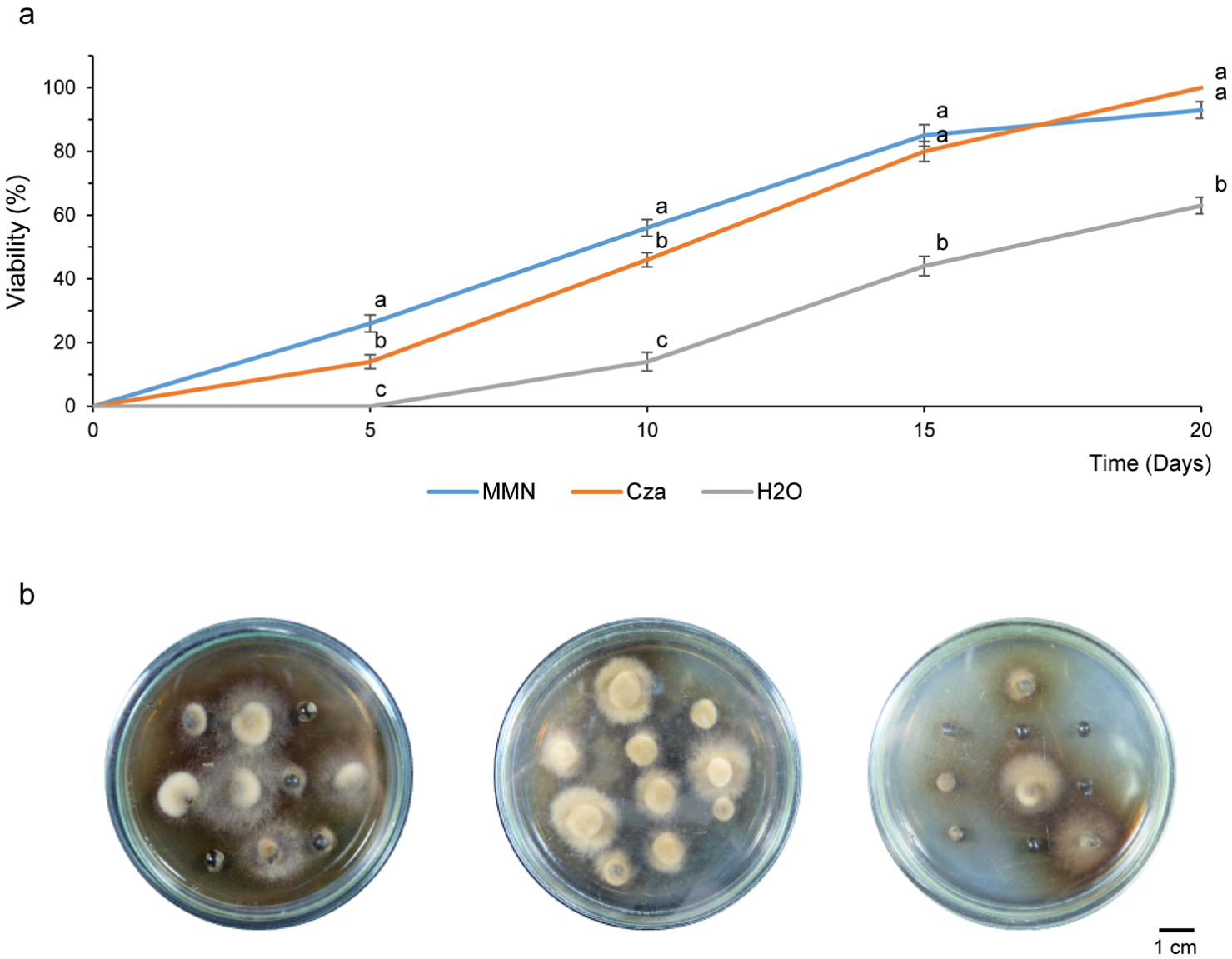

Figure 2

The effect of different alginate solutions on the viability of mycelia entrapped in alginate beads. (a) The percentage of viability of mycelia entrapped in alginate beads with different alginate solutions for 20 days. Different letters showed significant differences ($P < 0.05$) in the percentage of viability. (b) Features of mycelia of *A. odoratus* germinated from alginate beads made of different alginate solutions: MMN medium (left), Czapek medium (middle), and distilled water (right)

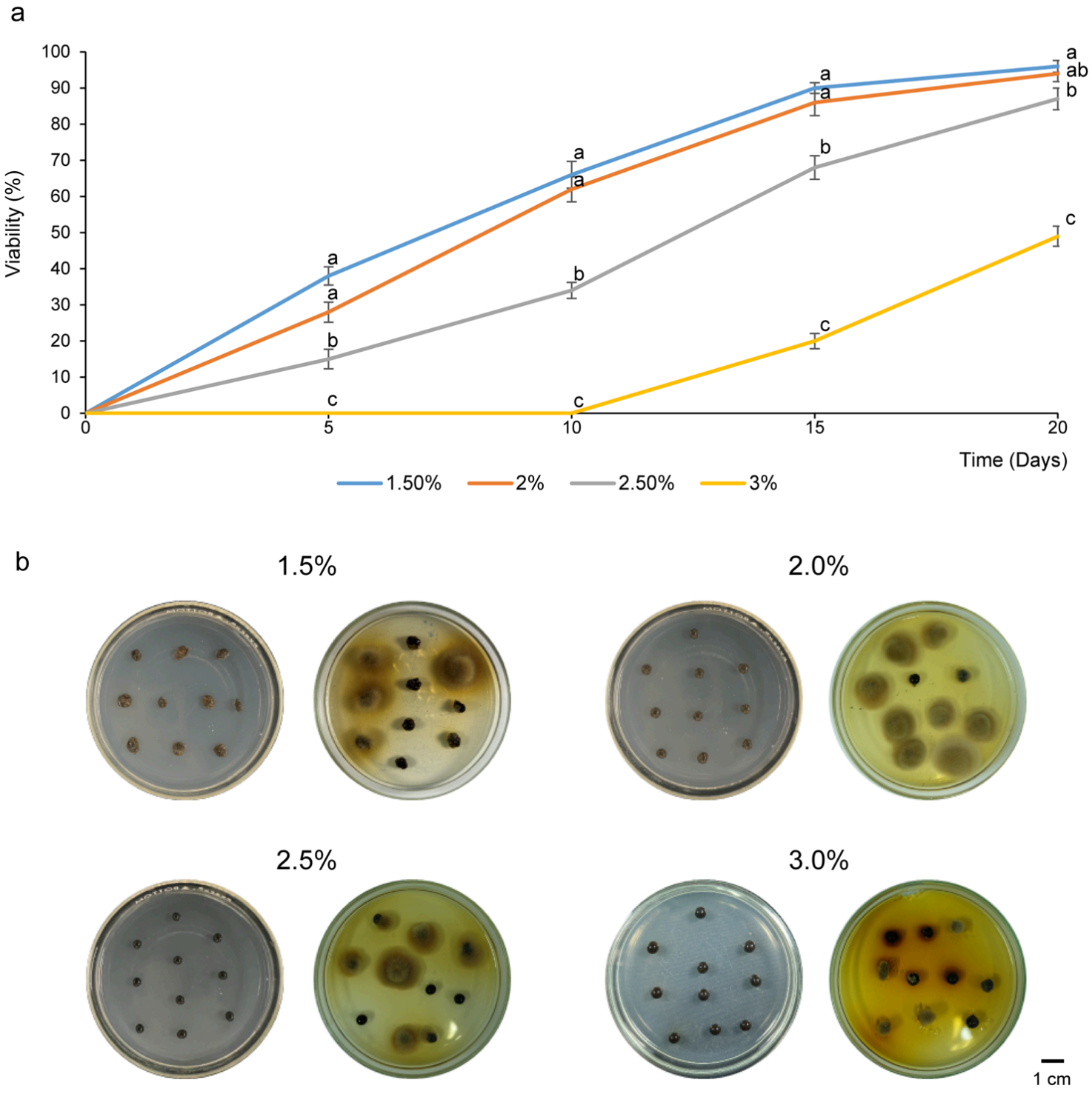

Figure 3

The effect of different concentrations of sodium alginate on the viability of mycelia entrapped in alginate beads. (a) Line graphs showing the percentage of viability of *A. odoratus* K1 mycelia entrapped in different concentrations of sodium alginate for 20 days. Different letters showed significant differences ($P < 0.05$) in the percentage of viability (error bar: SE). (b) Features of alginate beads and mycelial germination of *A. odoratus* entrapped in different sodium alginate concentrations

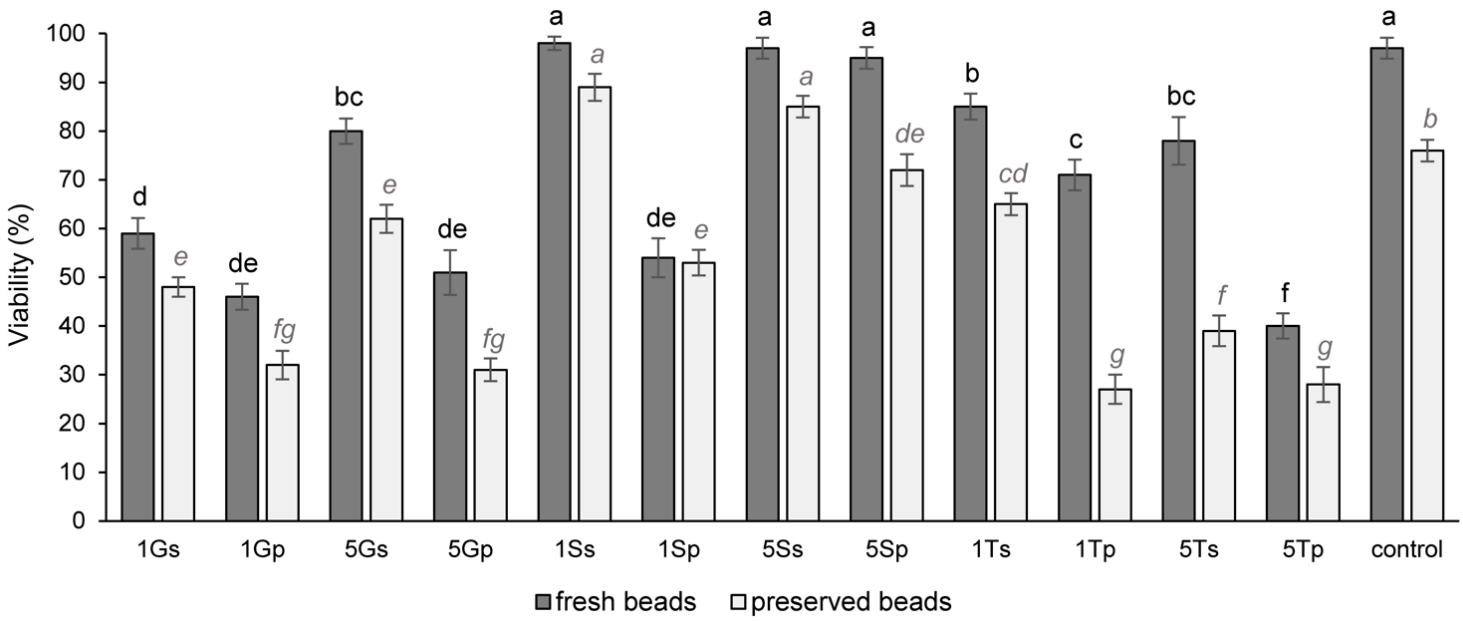

Figure 4

The effect of different sugar and protectant additives on the viability of mycelia entrapped in alginate beads. Bar plot showing the viability of mycelia entrapped in fresh alginate beads and after 15-day preservation at 25 °C. The alginate beads contained different sugar: glucose (G), sucrose (S), trehalose (T), with different concentrations: 1% (1), 5% (5), and added protectant additives: 5% sorbitol (s), 5% polyethylene glycol (p).

Different regular letters showed significant differences ($P < 0.05$) in the viability of mycelia entrapped in fresh alginate beads, and different italic letters showed significant differences ($P < 0.05$) in the viability after preservation for 15 days (Error bar: SE)

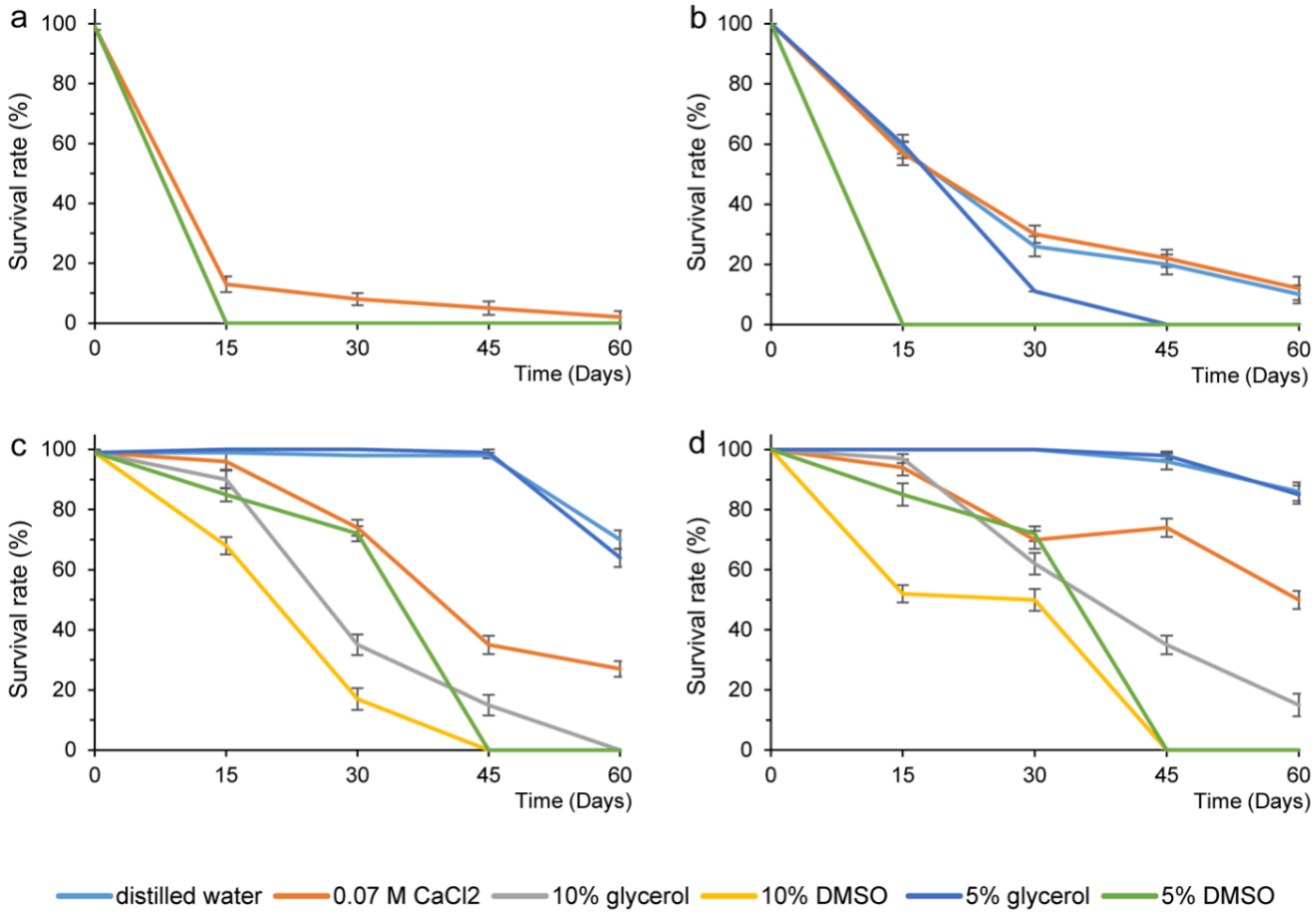

Figure 5

The effect of different preservation solutions and temperature on the survival rate of mycelia entrapped in alginate beads. Line graphs showing survival rate of *A. odoratus* strain K1 mycelia entrapped in selected alginate beads following preserved in different solutions and temperature: (a) alginate beads containing 1% sucrose and 5% sorbitol preserved at 4 °C, (b) alginate beads containing 5% sucrose and 5% sorbitol preserved at 4 °C, (c) alginate beads containing 1% sucrose and 5% sorbitol preserved at 25 °C, (d) alginate beads containing 5% sucrose and 5% sorbitol preserved at 25 °C

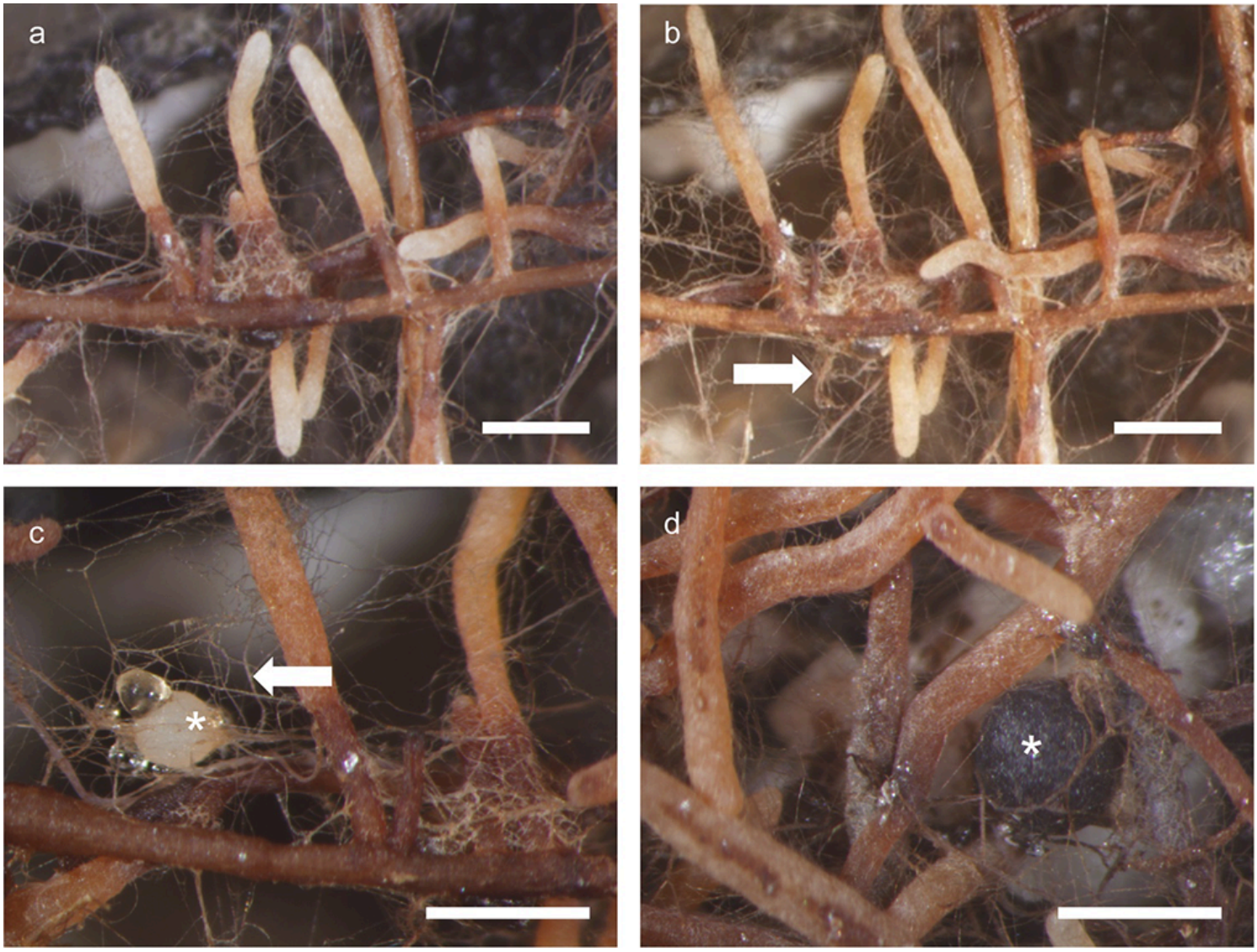

Figure 6

The development of *H. odorata* root colonized with *A. odoratus*, forming the mantle, extraradical mycelia (arrow), and sclerotium (asterisk) after inoculation with fresh beads for (a, c) 50 days and (b, d) 60 days. Scale bar = 1 mm

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplement.pdf