https://doi.org/10.17221/52/2026-JFS

Laboratory synthesis of ectomycorrhizae between ectomycorrhizal fungi and *Pinus* hosts

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Citation: Li J., Shi X., Li J. (2026): Laboratory synthesis of ectomycorrhizae between ectomycorrhizal fungi and *Pinus* hosts. J. For. Sci., 72: 400–421.

Abstract: Ectomycorrhizal (ECM) fungi are integral components of forest ecosystems, playing crucial roles in ecological succession and biodiversity maintenance. Fossil evidence suggests that Pinaceae represent one of the earliest ectomycorrhiza-forming host lineages, and that the symbiotic relationship between *Pinus* species and ECM fungi is characterised by strong host specificity. Ectomycorrhizal fungi functionally substitute for root hairs, expanding the absorptive surface area of host root systems, thereby enhancing nutrient uptake, improving stress tolerance, and boosting the survival of outplanted seedlings. In recent years, ECM has garnered widespread attention and been widely applied in fields including forest seedling cultivation, stress-site afforestation, plant disease management, and edible mycorrhizal fungi utilisation. The ecological and practical importance of ectomycorrhizae has driven current research to focus on mechanisms of symbiosis establishment, ecological functions, and application strategies. However, progress in these research areas is highly dependent on the improvement and optimisation of experimental systems and methodologies for the synthesis of ectomycorrhizae. This review focuses on common methods for the laboratory synthesis of ectomycorrhizae (Figure 1), systematically summarising the fundamental principles, operational procedures, advantages, and limitations of key techniques, including two classic culture systems (sterile growth pouch system and agar plate symbiosis system) and two main inoculation approaches (mycelial plug inoculation and spore inoculation). It aims to provide a methodological reference for fundamental research on ectomycorrhizae and for their application in *Pinus* seedling cultivation and ecological restoration.

Keywords: ecological restoration; inoculation methods; host specificity; nutrient uptake; symbiosis establishment

Supported by the Modern Industry School of Edible Fungi, Southwest Forestry University, Kunming City, China (Grant No. SYJ2510).

https://doi.org/10.17221/52/2026-JFS

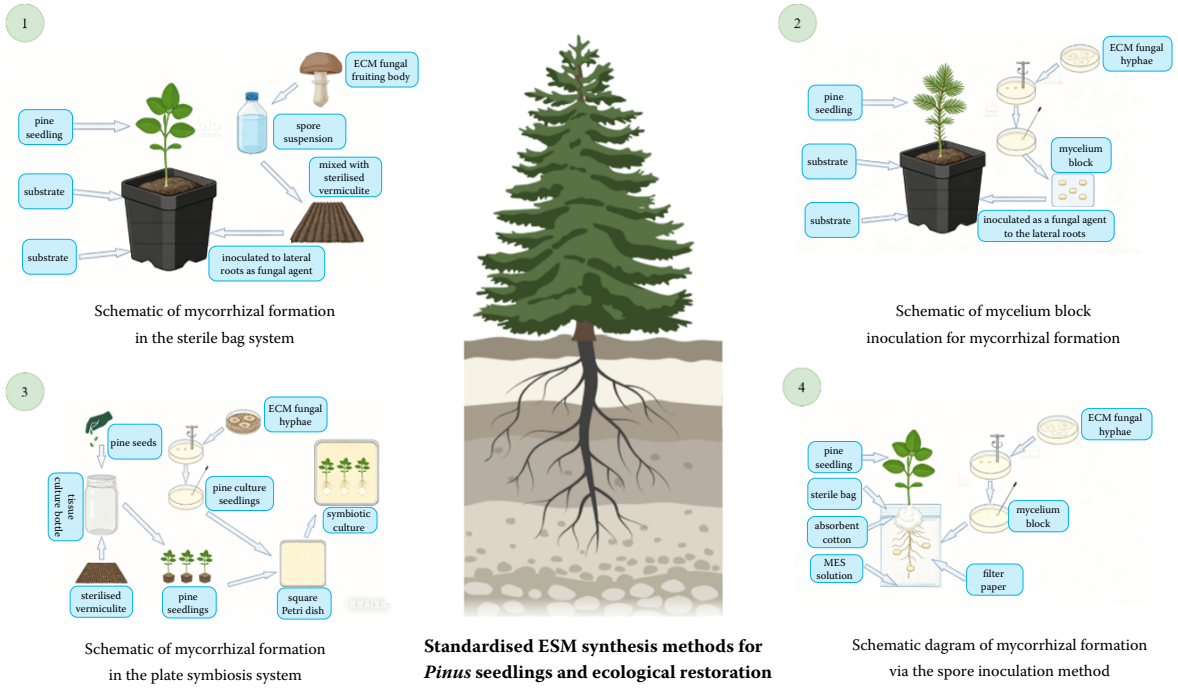

Figure 1. Standardised ectomycorrhizae (ECM) synthesis methods for *Pinus* seedlings and ecological restoration

Ectomycorrhizal (ECM) fungi represent a functionally critical guild in forest ecosystems, fulfilling key roles in driving ecological succession and sustaining biodiversity (Bao et al. 2020). China harbours abundant ECM fungal resources, with particularly high species richness in Yunnan, Sichuan, and Guizhou provinces, making these regions ideal sites for research on ECM fungal diversity and applications. Non-mycorrhizal plant families include the Brassicaceae (e.g. *Arabidopsis thaliana*), Chenopodiaceae, and Proteaceae, as these lineages have lost key genes required for symbiosis signalling (e.g. *SymRK* and *CCaMK*) (Cosme et al. 2018). *Pinus* species are obligately ectomycorrhizal, as they have retained and evolved these symbiotic genetic pathways, and their root systems rely on fungal symbionts for efficient nutrient and water uptake, especially in nutrient-poor soils (Agerer 2006). Fossil evidence suggests that the Pinaceae represent one of the earliest ectomycorrhiza-forming host lineages (Tedersoo et al. 2012). The symbiotic relationship between *Pinus* species and ectomycorrhizal fungi is characterised by varying degrees of host specificity, ranging from high specialisation to broad host generalisation. This variation arises from coevolutionary processes between host trees and fungal symbionts over long evolutionary timescales (Zhao 1990). Notably, host specificity in ECM symbioses should be understood as a continuum rather than a binary trait. At one end of this spectrum, genera such as *Suillus* exhibit relatively narrow host ranges, showing a strong preference for members of the Pinaceae (Molina, Trappe 1994). At the other end, fungal genera such as *Pisolithus*, *Laccaria*, and *Hebeloma* are widely recognised as broad-host-range generalists, capable of forming symbioses with a wide diversity of host trees across multiple families (Brundrett et al. 1996; Martin et al. 2008). This spectrum of specificity has profound implications for both fundamental research and practical applications: specific host-fungus combinations are valuable for studying co-evolutionary mechanisms, while generalist fungi offer greater flexibility for forestry inoculation programs. Phylogenetic and ecological studies indicate that ECM fungi evolved from saprophytic ancestors and play a crucial role in enhancing plants' resistance to abiotic stress and pathogen attack. Host plant demand for mineral nutrients is a key driver of the establishment of ectomycorrhizal symbioses (Martin et al. 2017). The distribution of ectomycorrhizal fungi among different host species within the Pinaceae is also shaped by the fungal host specificity, resulting in pronounced differences in the structure of ECM fungal communities associated with Pinaceae hosts

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at both spatial and host taxonomic levels (Cheng, Liangdong 2014; Yang et al. 2019).

No definitive consensus has been reached on the developmental staging of the ECM symbiosis establishment. Currently, the most widely accepted framework is the three-stage model proposed by Felten et al. (2009), which includes the exchange of signal molecules prior to physical contact, the colonisation of host roots by ECM fungi, and the establishment of functional ECM coupled with nutrient exchange. The interactions between the two symbionts prior to physical contact include plant recognition of and response to fungal signals (manifested as lateral root initiation and root elongation) and fungal recognition of and response to plant signals (manifested as spore germination, hyphal branching, and directed growth toward roots). The colonisation stage involves hyphal contact with root surfaces and intercellular hyphal extension between epidermal and cortical cells. The establishment of functional ECM signifies the onset of nutrient exchange between the ECM fungus and the host: on one hand, extraradical hyphae forage the soil for nutrients such as N, P, and K, and transport them to the plant through the mantle and Hartig net; on the other hand, the ECM fungi obtain carbohydrates from the host to support their own growth requirements. Ectomycorrhizae are mutualistic symbioses formed by the ECM fungal mycelia colonising the non-suberised feeder roots of forest trees; the mycelium does not penetrate root cells, but is mainly distributed on the root surface and within the intercellular spaces of the root cells (Finlay 2008). In mycorrhizal symbioses, fungal hyphae can mobilise and acquire soil nutrients that are otherwise unavailable to plant roots, thereby enabling mycorrhizal plants to acquire mineral nutrients more efficiently than non-mycorrhizal plants. The host plant provides the fungal symbiont with photosynthates; the primary carbon compound transferred is sucrose, which is rapidly converted by the fungus into trehalose and polyols. These fungal-specific storage compounds cannot be reabsorbed by the host plant, matching the metabolic preferences of the fungal symbiont and ensuring unidirectional carbon flow. Through sustained molecular crosstalk between the two genomes, the symbiosis ultimately achieves a high degree of functional and morphological integration of the two partners (Mello, Balestrini 2018). In ECM symbiotic systems, the fungus utilises host photosynthates as a carbon source, while significantly enhancing the host's mineral nutrient acquisition and, to some extent, preventing soil-borne pathogens from invading the root system, thereby promoting tree growth and improving stress tolerance (Finlay 2008). Ectomycorrhizae functionally replace the absorptive role of root hairs, expanding the absorptive surface area of the root system and promoting host growth by producing plant hormones, extracellular enzymes, antimicrobial metabolites, and volatile organic compounds; simultaneously, they form a protective barrier against certain soil-borne pathogens, helping to improve seedling survival during afforestation and forest regeneration (Luan 1994).

Laboratory-based ectomycorrhizal synthesis has a well-established history, with early successful cases dating back to the 1930s (Birch 1937). Since then, a variety of experimental systems have been developed to study the establishment of ECM symbioses under controlled conditions, collectively providing a robust methodological framework for *in vitro* ectomycorrhizal synthesis (Brundrett et al. 1996), establishing many foundational techniques that remain widely used today. More recent advances have centred on optimising culture conditions and expanding the repertoire of culturable ECM fungal species (Murat 2015; Sýkorová et al. 2016). Despite these methodological advances, the vast majority of relevant studies remain restricted to controlled laboratory settings, and the translation of *in vitro* findings into field applications remains a major bottleneck for applied ECM research.

Given the broad ecological functions and applied potential of ECM fungi and their symbiotic associations, early research in this field was largely dedicated to confirming the symbiotic nature of the interaction and developing foundational inoculation techniques. In recent years, the research frontier has gradually shifted toward three core directions: mechanisms underlying symbiosis establishment, ecological functioning, and large-scale application strategies. Further advancement of these research threads depends heavily on the refinement and standardisation of laboratory-based inoculation methodologies. It is broadly accepted that the success of symbiosis establishment is assessed primarily by two key indicators: the extent of ECM fungal colonisation in host root systems and the formation of characteristic ECM morphological structures (Murat 2015; Andrés-Alpuente

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et al. 2014). Substantial variation in mycorrhisation efficiency is observed across published studies, and this discrepancy is largely driven by three key experimental variables: host tree species identity, fungal inoculum taxa, and the inoculation methodology adopted (Garcia-Barreda et al. 2017), underscoring the pressing need for a standardised, systematic technical framework to guide method selection and condition optimisation. Currently, the most widely adopted approaches for *in vitro* ectomycorrhizal synthesis include sterile bag cultivation systems (Wang et al. 2020c), mycorrhizal synthesis using plate-based symbiosis systems (Feng 2020), mycelium block inoculation (Guo 2012; Yuan 2024), and spore inoculation (Huang et al. 2022a, b; Deng et al. 2025). A systematic synthesis of the core technical details, scope of applicability, and inherent limitations of these methods will aid future researchers in selecting experimental systems aligned with specific research goals, avoiding methodological biases, and advancing the standardisation of ectomycorrhizal approaches across both basic and applied research. Building on this imperative, the present review systematically summarises the four widely used *in vitro* ectomycorrhizal synthesis methods outlined above, offering a methodological reference for studies investigating ECM symbiosis development and practical application in *Pinus* species. Rather than presenting primary experimental findings, this review systematically synthesises and critically appraises established methodological protocols, serving as a consolidated technical reference for researchers in the field.

ECTOMYCORRHIZAL (ECM) FUNGI

Ectomycorrhizal fungi represent one of the most ecologically critical groups of symbiotic fungi in forest ecosystems. Upon colonising host feeder roots, they assemble a compact hyphal mantle on the root surface and form a Hartig net within the intercellular spaces of the root cortex (Zhou, Qi 1993). Mature ectomycorrhizae exhibit distinct morphotypes, most commonly dichotomously branched forms, coralloid clusters, and simple rod-shaped structures. On colonised feeder roots, root hairs are typically greatly reduced or entirely absent, and the root surface is enclosed by a compact fungal mantle. These diagnostic morphological features make ectomycorrhizae readily distinguishable with the naked eye or a hand lens (Tong 2006). Host ranges of ECM fungi vary across taxa. While some genera exhibit strong host specificity (e.g. *Suillus* associates predominantly with Pinaceae), others are widely recognised generalists (e.g. *Pisolithus tinctorius* forms symbioses with over 50 host genera across multiple families, including Pinaceae, Fagaceae, and Myrtaceae; *Laccaria laccata* associates with a similarly broad spectrum of hosts). This continuum of host specificity is a critical consideration when selecting fungal strains for inoculation programs, as generalist species offer greater versatility for nursery production, whereas specialist species may deliver superior performance when paired with their preferred hosts under specific site conditions.

ECM fungi exhibit rich species diversity (Feng et al. 2025). Current taxonomic surveys indicate that within the Basidiomycota and Ascomycota, nearly 18 orders, approximately 280 genera, and over 20 000 fungal species are known to form ectomycorrhizal symbioses with 250–300 genera and 6 000–7 000 species of vascular plants (Feng et al. 2019). ECM fungal resources in China are particularly abundant, with over 800 documented species belonging to 40 families and 80 genera (Guo 2012; Zhang et al. 2018), widely distributed across major mixed coniferous–broadleaf forests and commercial forest types. These primarily include the genera *Russula*, *Lactarius*, *Amanita*, and *Boletus* within the Agaricales; the genera *Scleroderma* and *Pisolithus* within the Gasteromycetidae; and representatives of the Ascomycota such as *Tuber* and *Cenococcum* (Chen 1989; Yu, Liu 2002; Zhou et al. 1983), offering considerable research and application potential. Through symbiotic relationships with host trees, these fungi play a vital role in promoting tree growth, enhancing stress tolerance, and providing non-timber forest products, making them a key structural and functional component of forest ecosystems.

The hosts of ECM fungi are primarily trees and shrubs of gymnosperms and angiosperms, including numerous species from the pine family (Pinaceae), cypress family (Cupressaceae), Salicaceae, Leguminosae, Fagaceae, Betulaceae, and many other woody plant families (Si et al. 2017; Yang et al. 2019). Tree species in China capable of forming ECM symbiotic associations include *Eucalyptus camaldulensis* Dehnh, *Pinus massoniana* Siebold & Zucc, *Pinus sylvestris* var. mongolica Litv., *Populus tomentosa* Carrière, *Picea*

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asperata Mast, and *Betula platyphylla* Sukaczev, along with a range of regionally important afforestation tree species (Wang et al. 2020b; Zhao et al. 2020).

Many edible ectomycorrhizal fungi play a key role not only in soil organic matter decomposition and nutrient cycling within forest ecosystems, but also in promoting plant growth and enhancing afforestation seedling survival. In forestry production practices, inoculation with ectomycorrhizal fungi has demonstrated significant effects on improving seedling survival rates and stress tolerance (Zhao 2023). Furthermore, inoculation with ECM fungi promotes nutrient uptake and accumulation in *Pinus massoniana* Siebold & Zucc and *Pinus sylvestris* var. mongolica Litv. seedlings, thereby benefiting seedling growth (Tu et al. 2022; Wang et al. 2022a). Results from the application of ECM fungi in pine seedling production and afforestation indicate that ECM fungal inoculation can increase seed germination rates, promote seedling growth, and significantly improve afforestation survival rates in the field (Hu 1992; Li et al. 1994). ECM fungi establish a mutualistic relationship with plant root systems (Finlay 2008). Once established, ECM symbiosis enhances nutrient and water acquisition in host plants, improving their growth performance. This symbiotic association also confers upon hosts the capacity to persist under degraded and extreme environmental conditions (Huang, Wang 2013; Sharma et al. 2021; Fu et al. 2024).

ECM fungi primarily reproduce through spore dispersal and vegetative growth of the mycelium (Yu, Liu 2002). However, laboratory observations have revealed that the spores of certain ECM fungal species exhibit low germinability or even complete germination failure, making mycelium-based culture the predominant approach for experimental propagation. Most agaric ECM fungi produce fruiting bodies covered by universal or partial veils, and their hyphal tissues differ substantially in regenerative capacity depending on their location. Hyphae located at the cap–stipe junction possess low tissue differentiation and thus demonstrate strong regenerative potential for *in vitro* culture, whereas the highly differentiated internal stipe tissues exhibit poor mycelial regeneration. Accordingly, mycelial tissues from the cap–stipe junction and the outer stipe surface are generally preferred for fungal strain isolation. In contrast, air-exposed gill tissues are commonly contaminated with microorganisms and are unsuitable for isolation, with only immature, veil-protected young gills serving as an exception (Figure 2).

Methodological note: The tissue isolation method adopted in this study targets the internal context tissue of fruiting bodies. This tissue resides between the outer pileus and the inner hymenophore tubes, providing a relatively sterile microenvironment for fungal isolation. Following surface sterilisation with 75% ethanol, the outer tissue layers were aseptically removed in a laminar flow hood. Internal tissue blocks of approximately 0.5 cm^3 were excised and inoculated onto PDA (Potato Dextrose Agar) medium for subsequent mycelial purification and cultivation.

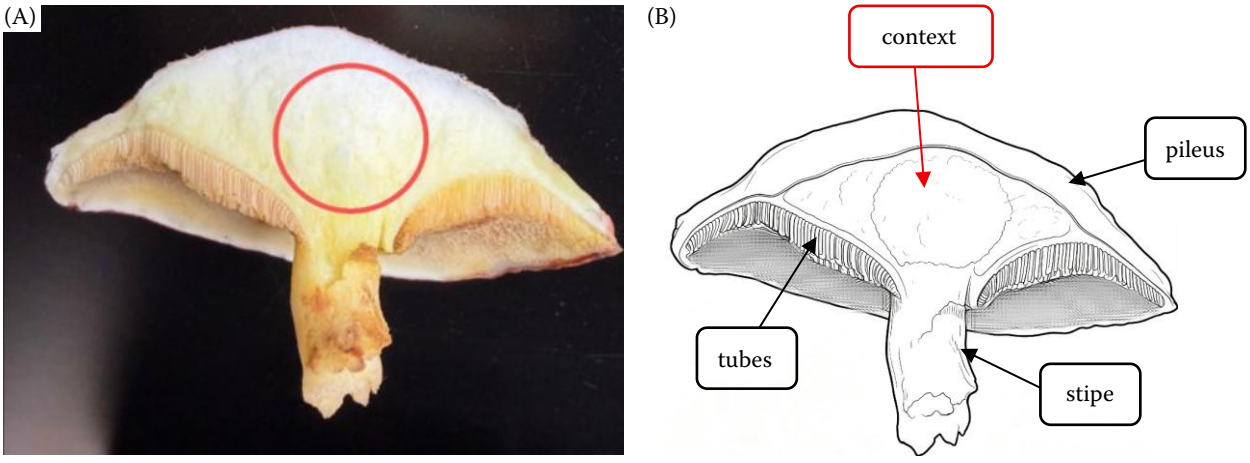

Figure 2. Longitudinal section of the fruiting body of *Suillus granulatus* and tissue isolation method: (A) Longitudinal section of the *S. granulatus* fruiting body, with the internal context tissue (pileus) indicated; (B) Schematic diagram of the internal structure of the fruiting body (showing the pileus, context, tubes, and stipe)

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ECTOMYCORRHIZAE (ECM)

ECM symbiotic systems display diverse morphological characteristics. The predominant morphotypes include monopodial (club-shaped, Figure 3A), dichotomous (Figure 3B), coral-shaped (Figure 3C), and pinnate forms (Agerer 1991). Among these morphotypes, monopodial mycorrhizae are unbranched and present short rod-like structures. Dichotomous mycorrhizae produce two equal or subequal branches at the root tip, with their mantle bases fused into a unified structure. Coral-shaped mycorrhizae arise from repeated continuous bifurcation of the apical tissue. Pinnate mycorrhizae are characterised by numerous short lateral branches arranged nearly parallel along the main axis; these branches gradually shorten toward the apex, forming a typical pinnate architecture (Agerer 2001, 2006).

ECM generally consists of several parts, including the mantle, Hartig net, external hyphae, and rhizomorph. The mycelium of ECM fungi first penetrates the root cap and upper epidermis, accumulates and proliferates around the root system, and wraps around young roots in overlapping layers. It eventually differentiates into a mantle, while many hyphae within the inner layer of the mantle can penetrate the root epidermis to enter the cortex tissue, extending through the intercellular spaces and highly branching to form a labyrinthine hyphal network – known as the Hartig network (Wang et al. 2020a). The Hartig net structure is a key characteristic of the ECM and serves as the interface for nutrient exchange between the host plant and the symbiotic fungus (Gong et al. 1997). Meanwhile, the superficial mantle hyphae extend outward to develop extraradical hyphae. These hyphae proliferate extensively throughout the soil, effectively expanding the host's absorptive surface area. They capture water and nutrients from distant soil regions and transport these resources to host roots. In addition, dispersed extraradical hyphae gradually aggregate and intertwine to form compact rhizomorphs that can penetrate deep into the soil profile. As key functional structures, rhizomorphs act as a structural framework for long-distance nutrient and water transport; they also exhibit reproductive capabilities (Figure 4).

The ecological functions of ECM symbiosis are mainly reflected in promoting host plant growth, maintaining forest ecosystem stability, and facilitating ecological restoration. ECM fungi can partially substitute for root hairs to expand the root absorptive area of host plants. Furthermore, they synthesise diverse bioactive substances, including plant hormones, enzymes, antibiotics, and volatile compounds, to accelerate host growth. In addition, ECM structures serve as a physical and biological barrier against soil-borne root pathogens, strengthen host stress tolerance, and ultimately improve the survival rate of afforested tree seedlings (Luan 1994). ECM fungi play a crucial role in nutrient cycling (e.g. C, N, P, etc.) (Kadowaki et al. 2018). Their rapidly growing hyphae (Chen et al. 2016) can promote litter decomposition, and the mantle can also participate in nutrient and water uptake (Hacquard et al. 2013). Accumulating empirical evidence demonstrates that ECM symbiosis delivers substantial growth-promoting benefits for host woody plants (Zhang et al. 2018), mineral nutrient uptake (Sun et al. 2023), and stress resistance (Wang et al. 2022a; Chen et al. 2023). Furthermore, ECM symbiosis possesses considerable potential for ecological restoration and can

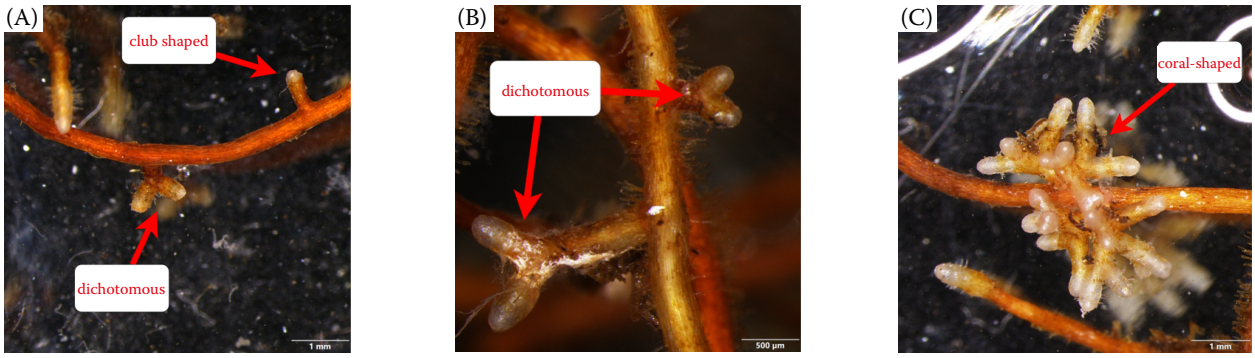

Figure 3. Morphological characteristics of ectomycorrhizae (ECM)

A, B and C show the ectomycorrhizae formed by *Suillus pinetorum* H.Engel & Klofac and *Pinus armandii* Franch.

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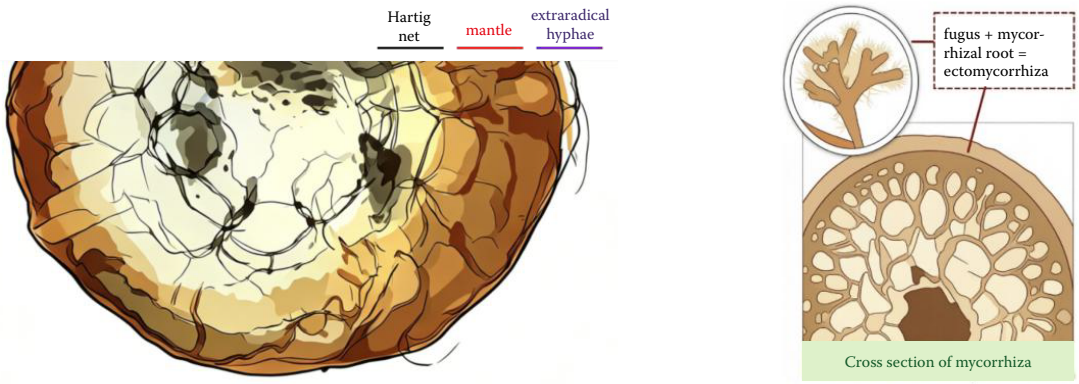

Figure 4. Microstructural characteristics of ectomycorrhizae (ECM)

effectively alleviate the adverse impacts of acid rain and heavy metal pollution. Existing studies have shown that inoculation with ECM fungi not only effectively mitigates the effects of acid rain on soil organic carbon (Ye et al. 2019) but also improves the physicochemical properties of contaminated soil and alleviates heavy metal toxicity (Ye et al. 2019; Sun 2021).

Given the diverse ecological functions of ECM symbiosis, mycorrhizal seedling inoculation technology has been widely applied in forestry practices worldwide since the 1980s. Early adopters, including the United States, Canada, and Japan have extensively utilised ECM-colonised seedlings for large-scale afforestation. Furthermore, several countries and regions have promoted mycorrhizal technology for ecological restoration of arid, desertified, and degraded lands, including Siberian regions of Russia, Morocco and South Africa in Africa, as well as Israel and Iran in the Middle East. Research on mycorrhizae in China began in 1955, when the Chinese Academy of Forestry Sciences first conducted experiments inoculating *Pinus tabulaeformis* with mycorrhizal fungi. Subsequently, mycorrhizal fungi from *Pinus sylvestris* var. and *Pinus mongolica* were isolated, cultured, and used for afforestation; however, research on ECM then stagnated for a period of 20 years. It was not until the mid-1970s that domestic mycorrhizal research regained momentum. Key breakthroughs were achieved by the Guangdong Provincial Forestry Research Institute, which conducted systematic inoculation experiments on Pinaceae plants and obtained promising preliminary results, laying a foundation for subsequent ECM research in China (Luan et al. 2013). Although mycorrhizae research in China started late, it has developed rapidly (An 2014). In recent years, ECM symbiosis has attracted extensive research attention and shown promising application potential in multiple fields, including forest seedling cultivation, afforestation under adverse site conditions, plant disease prevention and control, and the industrial utilisation of edible mycorrhizal fungi. Exogenous ECM inoculation serves as a core technical approach for exploring symbiotic mechanisms and breeding high-quality mycorrhizal seedlings, primarily by improving host nutrient acquisition capacity and stress resistance. However, mycorrhizal colonisation efficiency and subsequent growth benefits vary substantially across studies, owing to differences in host tree species, fungal inoculum types, and inoculation protocols. Therefore, establishing a systematic and standardised technical framework for method selection and condition regulation is urgently required to support standardised and efficient ECM application in forestry practice.

Beyond their well-documented roles in facilitating plant growth and enhancing stress tolerance, ECM fungi synthesise a wide range of metabolites that are essential for the establishment and normal functioning of symbiotic associations (Shaw et al. 1982). Among these, phytohormones, such as indole-3-acetic acid (IAA), abscisic acid (ABA), and cytokinins (CKs), regulate host root development and mycorrhizal morphogenesis (Shaw et al. 1982). Enzymes, including chitinases, glucanases, and phosphatases, are involved in defence against soil-borne pathogens and the mineralisation of organic phosphorus (Hacquard et al. 2013). Antibiotics and antifungal compounds suppress soil-borne pathogenic fungi (e.g. *Fusarium* and *Phytophthora*), thereby enhancing host disease resistance (Li et al. 2019). Volatile organic com-

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pounds (VOCs), such as terpenes and esters, mediate mycorrhizal recognition, attract beneficial microorganisms, or repel herbivores (Martin et al. 2008). Furthermore, fungal secondary metabolites, including phenolics and flavonoids, participate in early symbiotic signal exchange, thereby modulating fungal colonisation behaviour and host immune responses (Finlay 2008; Hacquard et al. 2013). In summary, these metabolites constitute the core chemical dialog between symbiotic partners and underpin the comprehensive ecological functions of ECM symbiosis (Shaw et al. 1982; Martin et al. 2008; Wang et al. 2022b).

METHODS FOR ECTOMYCORRHIZAL INOCULATION

Mycorrhizal synthesis in sterile bag systems

The sterile bag culture system represents a standardised experimental platform that enables the establishment of high-quality, highly reproducible ECM symbiosis between fungi and host seedlings under strictly controlled axenic conditions. It is commonly used to investigate symbiosis formation mechanisms (Shaw et al. 1982), validate gene function (Martin et al. 2008), and conduct quantitative physiological studies (Jones et al. 1991) (Figure 5).

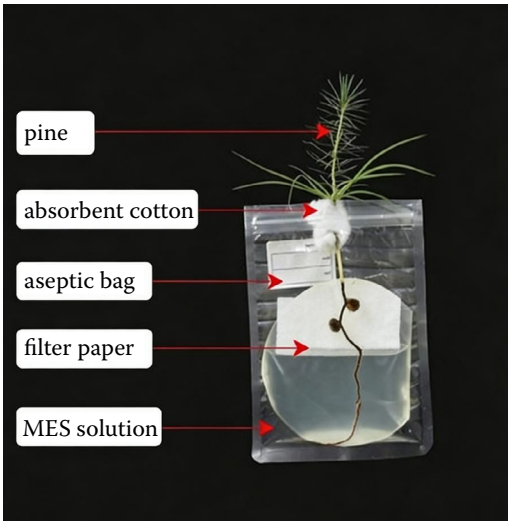

Figure 5. Schematic of mycorrhizal formation in the sterile bag system

Cotton wool serves multiple functions in this system, including physical support, seedling fixation, sterile isolation, and spatial compartmentalisation; the packing density and moisture content of cotton wool are critical factors that determine the success rate of mycorrhizal formation

Filter paper functions to construct a stable gas–liquid interface and capillary water-retention system, while facilitating root orientation and standardised sampling procedures. Filter paper thickness significantly influences mycorrhizal induction efficiency, with a thickness range of 0.36–0.40 mm recommended for preliminary experimental exploration. In this study, a single layer of filter paper (approximately 0.18 mm in thickness) was set as the negative control. This structural parameter was further optimised via reverse engineering based on four-week observations of seedling lateral root density, mycelial coverage, and mantle formation status.

MES buffer [2-(N-morpholino) ethanesulfonic acid] is a zwitterionic buffer commonly used to maintain a stable, slightly acidic pH (5.5–6.7) in plant and fungal culture media. In this system, it maintains rhizosphere pH, stabilises metal ions in MMN (Melin–Norkrans Medium) nutrient solution, preserves solution clarity, and minimises UV interference.

Inoculation process. Pine seeds were vernalised and disinfected, then sown individually in sterile substrate to germinate and grow. The optimal time for inoculation was when 3–5 lateral roots had developed (Guo et al. 1989). The inoculation procedure was performed as follows. Pine seedlings with well-developed lateral roots were selected for inoculation. Ectomycorrhizal fungal cultures grown in medium for 1–2 months were collected for subsequent inoculation treatment (Guo et al. 2006), and select mycelium in good condition for inoculation. Seedling roots were rinsed with sterile water to completely remove residual cultivation substrate. Fungal mycelial blocks were cut into uniform cubes of approximately 1 cm^3. The processed pine seedlings were placed in PVC self-sealing bags pre-lined with sterile filter paper. The lateral roots were gently spread using sterile forceps, and three to five mycelial blocks were carefully positioned adjacent to the lateral roots for inoculation. In this procedure, 25 mL of MES buffer with a concentration of 1.25–2.50 $mmol \cdot L^{-1}$ was applied, following the experimental protocol reported by Wang et al. (2020c). This concentration range was synthesised from multiple previous studies and is recommended as a reliable reference for preliminary ECM inoculation experiments. Place all self-sealing bags in an opaque container and culture them in a climate chamber under the following conditions: 18 h of light and a temperature between 20 °C and 30 °C (Bi et al. 1989)

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with variations among strains. Subsequently, incubation temperature was set according to the experimental fungal materials. A temperature of 25 °C was adopted as the optimal condition, as it represents the optimal temperature for most ectomycorrhizal fungi and serves as the most reliable reference for mycorrhizal induction experiments. The seedlings were first incubated in complete darkness at 18 °C for 6 h, followed by cultivation under a photosynthetically active radiation of 100 μmol·m^{-2}·s^{-1} (Wang et al. 2020c).

Mycorrhizal formation in a sterile pouch system. After approximately six weeks of greenhouse cultivation, molecular identification of ectomycorrhizae was performed to verify the successful establishment of target ECM symbiosis. The identification procedures included mycorrhizal sampling, genomic DNA extraction, PCR (Polymerase Chain Reaction) amplification, agarose gel electrophoresis, ITS (Internal Transcribed Spacer) sequencing, and phylogenetic tree construction. Structural and functional characteristics of the mycorrhizal symbiotic system were evaluated through comprehensive ECM analysis, covering mycorrhizal morphotype, biomass, mantle thickness, and mycorrhizal diameter. In addition, seedling growth indicators, including plant height, stem biomass, root morphological traits, and nutrient uptake capacity, were determined to assess the growth-promoting effects of ECM fungi on host plants. These comprehensive analyses further clarify the interactive mechanisms between ectomycorrhizal fungi and their host seedlings.

Advantages of the sterile pouch system inoculation method. This system provides well-controlled conditions for standardised ectomycorrhizal formation and permits real-time monitoring of mycorrhizal developmental processes (Yang et al. 2021). Initial mycorrhizal structures form approximately two weeks after inoculation. This rapid and abundant mycorrhizal maturation enables precise quantification of mycorrhizal formation timing in this system.

Disadvantages of the sterile pouch inoculation method. Since *Pinus* seedlings are grown in outdoor greenhouses, their growth environment is not completely sterile, leading to severe contamination after inoculation with mycelium blocks and requiring large-scale inoculation; it is also difficult to simulate the actual physical and chemical conditions of soil (Diaz et al. 1996), etc.

Mycorrhizal synthesis in the plate symbiosis system

The plate symbiosis system is a simplified mycorrhizal model established on a two-dimensional agar surface. It enables the induction and continuous observation of early-stage ectomycorrhizal symbiosis under strictly controlled axenic conditions, rendering it highly suitable for cytological analysis and early symbiotic signal transduction research (Plett et al. 2015) (Figure 6).

Inoculation process. Following and modifying the method of Feng et al. (2019), and after appropriate modifications, pine seeds were sterilised, vernalised, and germinated. After surface sterilisation, seeds were sown in tissue culture flasks (8 cm in diameter and 12 cm in height) filled with

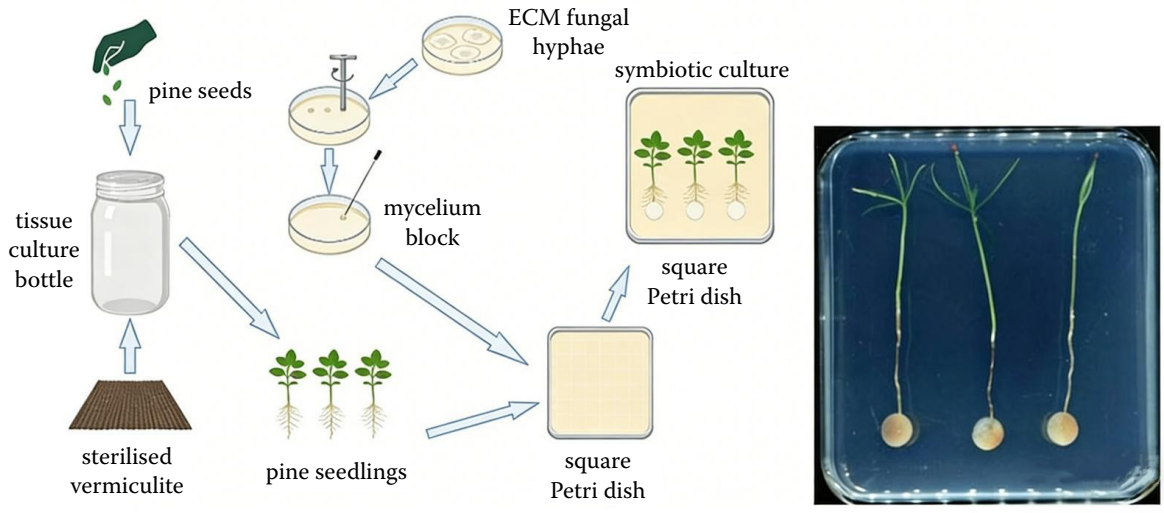

Figure 6. Schematic of mycorrhizal formation in the plate symbiosis system

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sterile moist vermiculite. The seeds were first dark-cultured at 25 °C for three days and subsequently transferred to a controlled growth chamber. The chamber was set to a 14 h photoperiod with a light intensity of 120 μmol·m^{-2}·s^{-1} and a 10 h dark period at 24 °C. According to our practical cultivation experience, a temperature range of 20–30 °C is optimal for seedling growth. Each treatment consisted of ten culture dishes, with fifteen seeds inoculated per dish. After approximately 21 days of cultivation, seedlings with uniform growth status were selected for subsequent inoculation experiments.

The detailed inoculation protocol was implemented as follows. Sterile pine seedlings were transplanted onto square Petri dishes containing 30 mL of modified DCR medium (Douglas-fir cotyledon revised medium). As a classic tissue culture medium originally designed for conifer micropropagation, DCR medium has been widely applied in mycorrhizal research, and its formula and concentration can be adjusted appropriately according to experimental requirements. Each dish held three seedlings. Activated ECM fungal blocks with a diameter of 1.0 cm were inoculated onto the root tips of pine seedlings, while non-inoculated seedlings were set as the blank control. Each treatment was established with ten replicate dishes. The root growth zone at the bottom of the Petri dishes was wrapped with aluminium foil, and all dishes were placed vertically in an artificial climate chamber for cultivation. The culture conditions were set as a 14 h light period with a light intensity of 160 μmol·m^{-2}·s^{-1} and a temperature range of 20–30 °C, and a 10 h dark period at 24 °C.

Due to limited space on the Petri dishes, the pine seedlings were transplanted after 28 days into seedling pots filled with sterilised vermiculite (7 cm in diameter, 8 cm) for continued cultivation (14 h of light, temperature between 20 °C and 30 °C; darkness for 10 h, temperature of 24 °C;) watered with 50 mL every 5 days and 20 mL of Hoagland nutrient solution every 10 days [complete plant nutrient solution; full strength Hoagland (1×) inhibits ectomycorrhiza formation; a 1/5 dilution 20% (Walker et al. 2003) is recommended as the basal nutrient solution for mycorrhizal plants].

Symbiosis system mycorrhiza formation. Five *Pinus* seedlings were randomly selected on days 4, 7, 14, 21, 28, and 60 post-inoculation to observe the formation of mycorrhizal symbionts (Feng et al. 2019). The morphological characteristics of the symbiotic establishment process were investigated through microscopic observation of the formed ectomycorrhizae (optical microscopy and scanning electron microscopy).

Advantages of the plate symbiosis system inoculation method. Currently, most conventional techniques for mycorrhizal seedling propagation in forestry remain relatively rudimentary. These approaches generally rely on simple seed sterilisation treatments using disinfectants such as potassium permanganate, mercuric chloride, and sodium hypochlorite, followed by seed germination under open experimental conditions. Subsequently, ECM fungal inoculants are applied to seeds or seedlings via conventional methods including soaking, mixing, dipping, spraying, and root drenching in open environments (Bao et al. 2014).

Such conventional inoculation approaches are highly susceptible to exogenous microbial contamination during mycorrhizal formation, which inevitably reduces seedling quality and hinders precise, dynamic monitoring of mycorrhizal development. In contrast, the Petri dish inoculation system enables the establishment of axenic symbiosis between ECM fungi and host seedlings under strictly sterile conditions. It yields contamination-free mycorrhizal seedlings, provides accurate and reliable data on the entire ECM formation process, and further facilitates in-depth exploration of underlying symbiotic mechanisms (Feng et al. 2019).

Disadvantages of the plate symbiosis system inoculation method. Although the plate-based symbiotic system is convenient for observation, its highly artificial environment may restrict root development and induce stress, thereby affecting the authenticity of mycorrhizal formation (Brundrett et al. 1996).

Mycelium block inoculation method

The mycelial block inoculation method employs pure cultured ectomycorrhizal mycelium as the inoculum. Direct physical contact between fungal mycelia and host roots enables efficient establishment of ECM symbiosis. Accordingly, this approach has become a reliable inoculation technique that effectively improves inoculation efficiency and stability in mycorrhizal research (Sýkorová et al. 2016) (Figure 7).

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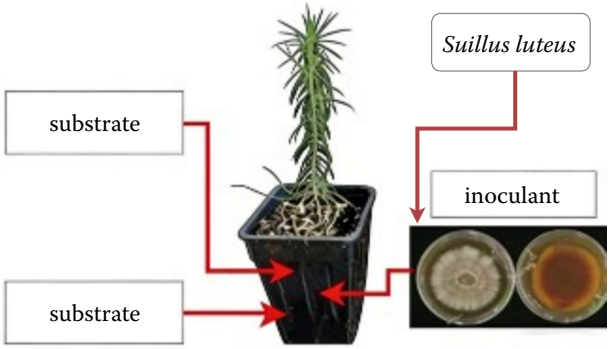

Figure 7. Schematic of mycelium block inoculation for mycorrhizal formation

Inoculation process. Pine seeds were sequentially sterilised, vernalised, and germinated to obtain uniform seedlings. Experiments were initiated after the seedlings developed 3–5 lateral roots. Under axenic culture conditions, including test tubes, round or square Petri dishes, conical flasks, and tissue culture flasks, ectomycorrhizal fungal mycelial blocks or liquid inoculants were placed in the substrate adjacent to the seedling root systems and lateral roots to establish symbiotic colonisation.

The mycelial plug inoculation method has been widely employed in ectomycorrhizal research since the early synthetic studies of Melin (1925) and later systematised by Brundrett et al. (1996), who established standardised protocols for fungal isolation, inoculum production, and seedling inoculation under controlled conditions. This method has been successfully applied to a broad range of ECM fungal taxa and host species, including both temperate and tropical pines (Molina, Trappe 1994; Duñabeitia et al. 1996; Sýkorová et al. 2016) further demonstrated its applicability in ecological restoration contexts, using mycelial inoculum for the reforestation of fly ash deposits with promising results. However, caution is warranted when extrapolating laboratory results to field settings, as the physiological state of the mycelium – particularly following prolonged subculturing – can substantially affect colonisation success (Thomson et al. 1993).

Mycelium block inoculation and mycorrhiza formation. Six months after inoculation, morphological and molecular identification of ectomycorrhizae was conducted [the formation of ectomycorrhizae was examined; plant root systems were observed under a stereomicroscope; after confirming the presence of mycorrhizae, the macroscopic morphology and anatomical characteristics were observed following the method of Agerer (2006), recorded, and photographed]. Fresh mycorrhizal samples were collected with sterile forceps and placed on microscope slides, followed by rinsing with distilled water. The processed samples were divided into two portions. One portion was preserved in sterile water for subsequent DNA extraction and mycorrhizal sectioning, in which cross-sections were prepared using a cryostat microtome. The other portion was used for morphological observation of the mycelial mantle and extraradical hyphae under an optical microscope. Combined ectomycorrhizal structural analysis and seedling growth analysis were further performed to characterise mycorrhizal morphology, screen optimal fungus–host combinations, and elucidate the reciprocal interactive mechanisms between ECM fungi and host seedlings.

Advantages of the mycelium block inoculation method. The mycelium block method offers significant advantages in terms of colonisation efficiency, controllable dosage, strain purity, and reproducibility (Brundrett et al. 1996). For fungi that depend on heterothallic mating (approximately 65% of basidiomycetes), if the mycelium block originates only from monokaryotic mycelium, it cannot form a fertile fruiting body; both compatible mating type A and B mycelial cells need to be inoculated.

Disadvantages of the mycelium block inoculation method. Mycelia subjected to repeated subculturing over extended periods on nutrient-rich media [e.g. PDA (Potato Dextrose Agar, a general-purpose routine medium for fungi) or MMN] may experience a 'blunting' or 'diminish.' This is referred to as the 'domestication effect' or 'attenuation of infectivity' (Brundrett et al. 1996). As a result, such mycelia may lose their ability to recognise hosts, compete for colonisation, and interact with soil microorganisms in complex soil environments, leading to actual colonisation and growth-promoting effects in the field that fall short of expectations.

Spore inoculation method

The spore inoculation method employs reproductive spores of ECM fungi as inoculants. Spores germinate in the rhizosphere of host roots to successfully establish ectomycorrhizal symbiosis. This technique serves as a vital technical approach for assessing the symbiotic potential of recalcitrant or uncultivable fungal species and supports large-scale inoculation applications in mycorrhizal research (Perry et al. 1989) (Figure 8). The spore

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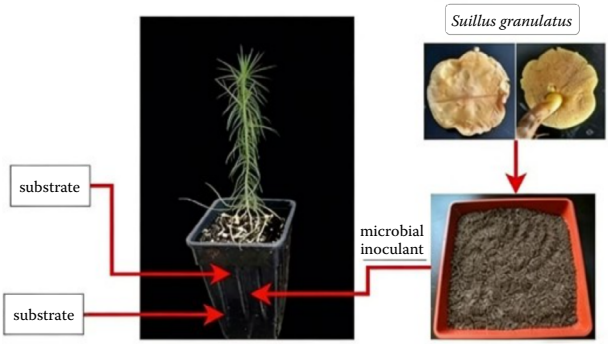

Figure 8. Schematic diagram of mycorrhizal formation via the spore inoculation method

inoculation method has been a cornerstone of ectomycorrhizal inoculation trials since the pioneering work of Marx and Bryan (1970), who successfully synthesised *Pisolithus tinctorius* mycorrhizae on *Pinus taeda* using basidiospore inoculation. Its utility in large-scale forestry applications was subsequently demonstrated by Perry et al. (1989), who showed that spore-based inoculants could effectively mediate interspecific competition among conifer seedlings in field settings. Comparative studies have indicated that spore inoculation can achieve success rates comparable to or exceeding those of mycelial inoculants under certain conditions (Bougher et al. 1990; Diaz et al. 1996), although germination variability and slow colonisation speed remain persistent limitations.

Inoculation process. Pine seeds were surface-sterilised, vernalised, and germinated to develop uniform seedlings. Experimental inoculation was initiated after the seedlings produced 3–5 lateral roots. The spore layer was carefully scraped from the fruiting bodies of ectomycorrhizal fungi and fully homogenised using a grinder to prepare a spore suspension. Spore concentration was subsequently quantified with a hemocytometer, with a preliminary optimal inoculation dosage ranging from 1×10^4 to 1×10^7 spores per seedling. The cultivation substrate was prepared according to the prescribed proportion and subjected to two rounds of sterilisation before use. For pot inoculation, each pot was first filled with one-third of the sterilised substrate. The spore suspension was evenly applied around the loose seedling root system, and the pot was subsequently supplemented with additional substrate until the medium level reached 2 cm below the pot rim.

Mycorrhizal formation via spore inoculation. The inspection method is the same as that described in the section 'Mycelium block inoculation and mycorrhiza formation' above.

Advantages of the spore inoculation method. The spore inoculation method is suitable for difficult-to-culture fungi, is cost-effective, simple to operate, and preserves genetic diversity (Diaz et al. 1996); it facilitates long-term storage and transportation, has a low technical barrier, and is effective (Trappe 1979). The method allows for the establishment of a durable soil inoculum bank and direct field inoculation (Molina, Trappe 1994). These characteristics give it an irreplaceable and pivotal role in the research and application of ectomycorrhizal fungi.

Disadvantages of the spore inoculation method. Disadvantages of the spore inoculation method include low and unstable germination and colonisation rates, difficulty in standardising inoculation doses, slow colonisation speed, and potential contamination (Diaz et al. 1996), among others.

The four mycorrhizal synthesis methods described above are commonly used for laboratory-based synthesis of ectomycorrhizal fungi; the appropriate method should be selected based on specific research objectives (Table 1).

The sterile bag and plate symbiosis systems represent classic *in vitro* axenic culture systems. Both systems exhibit high mycorrhizal colonisation efficiency when the host seedlings and fungal strains maintain vigorous physiological activity (Fu et al. 2009). Regarding the stability of mycorrhizal structures, the sterile bag system utilises a three-dimensional solid substrate that more closely mimics natural soil conditions, resulting in more stable and durable symbiotic structures; in contrast, while the plate system offers high experimental uniformity and controllability, its two-dimensional culture environment limits the development of symbiotic structures, leading to relatively fragile mycorrhizae (Xiong 2021). In terms of species applicability, the sterile bag system exhibits a broader application spectrum and is particularly suitable for most woody plants and slow-growing fungal strains. In contrast, restricted by its two-dimensional spatial structure, the plate symbiosis system has a relatively limited application scope and is primarily applicable to herbaceous plants and fast-colonising symbiotic pairs capable of matching the rate of root growth (Huang et al. 2020).

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Table 1. Comparison of four common mycorrhizal synthesis methods

Method	Advantages	Disadvantages	Typical results/ applicable scenarios
Sterile pouch system mycorrhizal synthesis	controlled conditions for ectomycorrhizal formation	outdoor greenhouse environment not fully sterile, leading to potential contamination	suitable for investigating symbiosis formation mechanisms, validating gene functions, and conducting quantitative physiological studies; provides rapid and reproducible mycorrhiza formation
	real-time observation of mycorrhizal development	requires large-scale inoculation	
	rapid formation (~2 weeks), facilitating quantification of mycorrhizal formation time	difficult to simulate actual soil physical and chemical conditions	
Plate symbiosis system mycorrhizal synthesis	establishes a symbiotic system under sterile conditions	highly artificial environment may restrict root development and induce stress	suitable for cytological and early signal transduction studies; ideal for observing the morphological characteristics of the symbiotic establishment process
	produces sterile seedlings, providing reliable information on the ECM establishment process	reduces the authenticity of mycorrhizal formation	
	facilitates elucidation of symbiotic mechanisms	–	
Mycelium block inoculation method	high colonisation efficiency	long-term subculturing on nutrient-rich media may cause 'domestication effect' or attenuated infectivity	suitable for experimental systems requiring high efficiency in establishing symbiosis and precise dosing; broadly adaptable to most fungal-plant combinations
	controllable inoculation dosage	cannot form fertile fruiting bodies using only monokaryotic mycelium	
	high strain purity	requires co-inoculation of both compatible mating type A and B mycelial cells	
	good reproducibility	–	
Spore inoculation method	suitable for difficult-to-culture fungi	low and unstable germination/ colonisation rates	suitable for evaluating the symbiotic potential of difficult-to-culture fungi, large-scale production, long-term storage, and large-scale field inoculation trials
	cost-effective and easy to operate	difficult to standardise inoculation doses	
	preserves genetic diversity	slow colonisation speed	
	facilitates long-term storage and transportation	risk of contamination	
	low technical barrier, enabling direct field inoculation	–	

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Both the mycelium block inoculation method and the spore inoculation method are performed under non-strictly sterile conditions (Huang et al. 2022a). The two inoculation methods differ substantially in colonisation efficiency. The mycelial block inoculation method achieves faster infection and higher mycorrhizal colonisation rates, making it well-suited for experimental systems that require rapid and efficient establishment of ECM symbiosis. In comparison, spore inoculation excels in large-scale application and long-term preservation. Given the dormant nature of fungal spores, this method facilitates convenient storage and transportation of inoculum materials (Zhu 2019). Regarding mycorrhizal stability, the mycelium block inoculation method exhibits higher stability during the early stages of infection, characterised by 'rapid and stable colonisation'; although the spore inoculation method has lower initial stability (due to the risk of spore germination), once a symbiotic relationship is successfully established, the resulting mycorrhiza may demonstrate superior long-term maintenance and stress resistance, particularly showing good application prospects in large-scale production scenarios simulating natural conditions (Lee, Eom 2024). The two inoculation methods also differ greatly in species adaptability. The mycelial block inoculation method presents broad-spectrum applicability and performs well for most fungal–host combinations. In contrast, spore inoculation shows strict species specificity and is only applicable to specific fungal taxa with high germinability, making it more suitable for large-scale industrial propagation.

To provide a more intuitive and comprehensive reference for researchers selecting appropriate ECM fungal–host combinations and synthesis methods, we compiled a summary of representative laboratorybased mycorrhizal synthesis studies involving *Pinus* species (Table 2). This table includes ECM fungal species, corresponding host plants, the inoculation methods used, successful mycorrhiza formation, and key references. The compiled cases cover a broad phylogenetic range of ECM fungi, including *Lactarius*, *Suillus*, *Pisolithus*, *Rhizopogon*, *Tuber*, and others, and demonstrate successful symbiosis establishment under various laboratory conditions, such as the sterile pouch system, plate coculture, mycelial plug inoculation, and spore inoculation. Notably, previous studies have documented the formation of fruiting bodies following the transplantation of mycorrhizal seedlings into open potted conditions. This systematic compilation of existing methodologies provides a practical reference for future experimental design and further identifies key knowledge gaps that warrant subsequent comprehensive exploration and verification.

From laboratory to forestry: Practical considerations for inoculation methods

The four laboratory-based ectomycorrhizal synthesis methods exhibit distinct differences in applicability, cost efficiency, and practical performance when translated into forestry production. Clarifying these differential characteristics enables researchers to rationally select suitable approaches according to specific experimental purposes and research demands.

Role of mechanism-oriented methods. The sterile pouch system and the plate symbiosis system are both *in vitro* sterile co-culture systems whose primary value lies in providing highly controllable experimental platforms for elucidating symbiotic mechanisms.

The sterile pouch system can initiate mycorrhizal formation within approximately two weeks, rendering it suitable for investigating symbiotic signal transduction, verifying gene functions, and conducting quantitative physiological analyses. However, this system is limited by potential contamination risks and its inability to simulate natural soil physicochemical properties. The plate symbiosis system allows continuous visualisation of early symbiotic processes on a two-dimensional agar interface, making it ideal for cytological observation and signal transduction research. Nevertheless, its highly artificial cultivation environment may reduce the ecological authenticity of mycorrhizal formation and development.

Based on the reviewed evidence, we suggest that the fundamental value of these two systems lies not in their direct application to nursery production, but rather in screening optimal fungal-host combinations for practical use and in providing a theoretical basis for understanding symbiotic mechanisms (Molina, Trappe 1994; Brundrett et al. 1996).

Critical comparison of the four methods. Based on the studies reviewed above, the four inoculation methods differ markedly in success rate, applicability, and reproducibility. From a practical perspective, mycelial plug inoculation gener-

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Table 2. Summary of artificially synthesised ectomycorrhizal fungi and corresponding host plants

ECM fungal species	Host plant	Synthesis method	Mycorrhiza formation	References
Amanita caesarea	*Pinus gerardiana*	aseptic seedling culture in vermiculite-peat substrate, *in vitro* incubation for 5 months	yes	Sehgal and Sagar 2023
Boletus griseus	*Pinus tabuliformis*	pure culture synthesis	yes	Zhao and Guo 1989
Boletus pinophilus	*Pinus radiata*	vegetative mycelium + spore inoculation in open containers	yes	Duñabeitia et al. 1996
Hebeloma longicaudum	*Pinus radiata*	pure culture synthesis	yes	Duñabeitia et al. 1996
Laccaria laccata	*Pinus patula*, *P. pseudostrobus*, *P. oocarpa*, *P. elliottii*	*in vitro* synthesis (mycorrhiza formed at 6 weeks)	yes	Reddy and Natarajan 1996
Laccaria laccata	*Pinus patula*	*in vitro* synthesis + nursery trial	yes	Reddy and Natarajan 1997
Laccaria amethystina	*Pinus densiflora*	*in vitro* synthesis followed by transplanting to open pots	yes (8–9 months)	Yamada et al. 2001
Laccaria deliciosus	*Pinus sylvestris*	sterile pouch system	yes	Wang et al. 2020c
Lactarius deliciosus	*Pinus pinea*, *P. radiata*, *P. armandii*	mycelial plug inoculation	yes	Deng et al. 2025
Lactarius hygrophanus	*Pinus densiflora*	aseptic inoculation of pure culture mycelium	yes (10 months)	Chung et al. 2002
Lactarius indigo	*Pinus oocarpa* var. *oocarpa*	mycelial inoculation in peat-vermiculite plastic containers (first synthesis)	yes	Flores et al. 2005
Leccinum aurantiacum	*Pinus gerardiana*	aseptic seedling culture in vermiculite-peat substrate, *in vitro* incubation for 5 months	yes	Sehgal and Sagar 2023
Paxillus involutus	*Pinus resinosa*	*in vitro* inoculation in test tubes	yes	Duchesne et al.1988
Paxillus involutus	*Pinus densiflora*	aseptic inoculation of pure culture mycelium	yes (10 months)	Chung et al. 2002
Pisolithus tinctorius	6 *Pinus* spp.	*in vitro* synthesis	yes	Reddy and Natarajan 1996
Pisolithus tinctorius	*Pinus taeda*	basidiospore inoculation	yes (2 months)	Marx and Bryan 1970
Pisolithus tinctorius	*P. tabuliformis*, *P. massoniana*, *P. sonda*, *P. elliottii*	tissue culture mycorrhizal synthesis (paper-sandwich method)	yes	Chen 1989
Rhizopogon luteolus	*Pinus patula*, *P. pseudostrobus*, *P. oocarpa*, *P. elliottii*	*in vitro* synthesis	yes	Reddy and Natarajan 1996
Rhizopogon roseolus	*Pinus thunbergii*	aseptic synthesis (initial mycorrhiza observed 2 weeks after mycelial plug inoculation)	yes	Shimomura et al. 2010
Rhizopogon rubescens	*Pinus densiflora*	*in vitro* synthesis followed by transplanting to open pots	yes (8–9 months)	Yamada et al. 2001

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Table 2. To be continued

ECM fungal species	Host plant	Synthesis method	Mycorrhiza formation	References
Rhizopogon songmaodan	*Pinus armandii*, *P. yunnanensis*	greenhouse spore inoculation	yes	Wang et al. 2020c
Rhizopogon truncatus	*Pinus ponderosa*	greenhouse spore suspension inoculation	yes	Massicotte et al. 1999
Rhizopogon spp. (7 species)	*P. resinosa*, *P. strobus*	vegetative inoculum	yes	Molina and Trappe 1994
Russula fragilis	*Pinus densiflora*	aseptic inoculation of pure culture mycelium	yes (10 months)	Feng 2021; Chung et al. 2002; Feng et al. 2019
Scleroderma citrinum	*Pinus patula*, *P. pseudostrobus*, *P. oocarpa*, *P. elliottii*	*in vitro* synthesis (mycorrhiza formed at 6 weeks)	yes	Reddy and Natarajan 1996
Scleroderma polyhrizum	*Pinus radiata*	vegetative mycelium + spore inoculation in open containers	yes	Duñabeitia et al. 1996
Suillus americanus	*Pinus resinosa*, *P. strobus*	*in vitro* synthesis	yes	Palm and Stewart 1984
Suillus bovinus	*Pinus densiflora*	aseptic inoculation of pure culture mycelium	yes (10 months)	Chung et al. 2002
Suillus brevipes	*Pinus resinosa*, *P. strobus*	*in vitro* synthesis	yes	Palm and Stewart 1984
Suillus luteus	*Pinus resinosa*	*in vitro* synthesis	yes	Palm and Stewart 1984
Suillus luteus	*Pinus radiata*	vegetative mycelium + spore inoculation	yes	Birch 1937
Suillus luteus	*Pinus armandii*	spore inoculation	yes	Huang et al. 2022b
Suillus neoalbidipes	*Pinus resinosa*	*in vitro* synthesis	yes	Palm and Stewart 1984
Suillus placidus	*Pinus strobus*	*in vitro* synthesis	yes	Palm and Stewart 1984
Suillus punctipes	*Pinus strobus*	field ecological survey (naturally occurring mycorrhizae)	yes	Smith and Thiers 1964
Suillus variegatus	*Pinus sylvestris*	*in vitro* synthesis	yes (2 months)	Duddridge and Read 1984
Thelephora terrestris	*Pinus patula*, *P. pseudostrobus*, *P. oocarpa*, *P. elliottii*	*in vitro* synthesis	yes	Reddy and Natarajan 1996
Tricholoma flavovirens	*Pinus densiflora*	*in vitro* synthesis followed by transplanting to open pots	yes	Yamada et al. 2001
Tricholoma matsutake	*Pinus densiflora*	aseptic inoculation of pure culture mycelium	yes (8–9 months)	Yamada et al. 2006
Cenococcum geophilum	*Pinus resinosa*	axenic synthesis in Petri dishes	yes	Peter et al. 2016
Hebeloma cylindrosporum	*Pinus pinaster*	*in vitro* co-culture system	yes	Marmeisse et al. 2004
Laccaria bicolor	*Pinus banksiana*	sterile pouch system	yes	Wong et al. 1990
Paxillus involutus	*Pinus sylvestris*	mycelial plug inoculation	yes	Turnau et al. 1993; Rudawska et al. 1997

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ally achieves the highest and most stable success rate, as it employs actively growing pure mycelium in direct contact with host roots. The sterile pouch system appears to rank second in terms of reliability, with its success rate influenced by environmental cleanliness and substrate formulation. The plate symbiosis system, while valuable for observation, tends to yield relatively lower mycorrhizal formation rates due to its two-dimensional constraints. The spore inoculation method exhibits the greatest variability, as spore germination rates differ substantially among fungal species and even among batches (Bougher et al. 1990; Diaz et al. 1996), making reproducibility difficult to guarantee. The spore inoculation method uses reproductive spores as inoculum and is particularly suitable for difficult-to-culture fungi. Its advantages include low cost, preservation of genetic diversity, and convenience for long-term storage. Large-scale field trials have demonstrated that the overall success rate of spore suspension inoculation (49%) is higher than that of mycelial slurry inoculation (35%), with lower production costs (Bougher et al. 1990). Its main disadvantages are low and unstable germination rates, difficulty in standardising inoculation dosage, and slow colonisation speed.

In terms of fungal ecological adaptability, based on the evidence reviewed, host-specific taxa (e.g. *Suillus*) and slow-growing fungi appear to be better suited to the sterile pouch or mycelial plug inoculation methods, which allow precise control of symbiotic conditions. Generalist fungi (e.g. *Laccaria*, *Pisolithus*) seem less sensitive to method selection (Molina, Trappe 1994; Martin et al. 2008). From a practical perspective, for difficult-to-culture fungi, spore inoculation may be the most feasible approach currently available for assessing their symbiotic potential (Trappe 1979; Bougher et al. 1990).

Three major sources of experimental variability can be identified: physiological status of the fungal strain (culture age, subculture frequency, and medium composition; prolonged subculturing can lead to 'attenuation of infectivity,' (Thomson et al. 1993); genetic background and physiological age of the host (different provenances and seedling developmental stages significantly affect mycorrhisation efficiency, with inoculation at the 3–5 lateral root stage being optimal (Guo et al. 1989); and culture substrate and nutritional conditions (carbon-to-nitrogen ratio, phosphorus concentration, pH, and buffer systems all significantly influence mycorrhizal formation; full-strength Hoagland solution inhibits mycorrhisation, whereas 1/5 dilution is most suitable (Walker et al. 2003). From a practical perspective, we suggest that researchers systematically document and report these parameters to enhance the comparability and reproducibility of experimental results.

From laboratory indicators to field performance. Based on the reviewed evidence, the root system architecture (branching pattern, lateral root number, mantle thickness) and shoot-to-root ratio of mycorrhizal seedlings synthesised under laboratory conditions may serve as important indicators for predicting field afforestation performance (Molina, Trappe 1994; Brundrett et al. 1996).

Ectomycorrhizal fungi functionally replace the absorptive role of root hairs through the formation of the mantle and Hartig net (Molina, Trappe 1994). Mycorrhizal *Pinus* seedlings have been reported to exhibit enhanced root plasticity under drought stress, and the development of mantle thickness and extraradical hyphae may influence the extent of nutrient foraging (Brundrett et al. 1996). Based on the reviewed evidence, seedlings with a higher root-to-shoot ratio and adequate mycorrhizal colonisation generally possess greater stress resistance. Field trials have shown that the survival rate of mycorrhizal seedlings in afforestation can reach 92.6–98.7%, representing an increase of 6.2–9.0% over non-inoculated controls (Molina, Trappe 1994; Brundrett et al. 1996).

In summary, based on the reviewed evidence, the sterile pouch system and plate symbiosis system provide platforms for mechanistic studies and strain screening; the mycelial plug inoculation method offers the highest efficiency but requires attention to the risk of infectivity attenuation (Thomson et al. 1993); and the spore inoculation method demonstrates clear advantages in cost and scalability, although germination instability remains a critical bottleneck (Bougher et al. 1990). From a practical perspective, we suggest that future research should shift its focus from 'whether ectomycorrhizal symbiosis can be established' to 'whether the established symbiosis can function in the field,' thereby advancing ectomycorrhizal research from a 'method-oriented' to an 'outcome-oriented' paradigm.

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CONCLUSION AND OUTLOOK

Pinus species are typical obligately ectomycorrhizal trees; under natural conditions, seedlings without suitable ECM fungal symbionts often exhibit poor survival and growth (Agerer 2006; Tedersoo et al. 2012). In forestry practice, ECM fungal inoculation can significantly improve afforestation survival rates, particularly on poor sites, while reducing fertiliser and pesticide inputs and promoting forest health (Luan 1994; Molina, Trappe 1994; Brundrett et al. 1996).

This review systematically synthesises laboratory-based methods for establishing symbiosis between *Pinus* hosts and ECM fungi. The four inoculation techniques differ markedly in their performance across three key dimensions: success rate and reproducibility, fungal ecological adaptability, and practical applicability. Mycelial plug inoculation generally achieves the highest and most stable success rate, as it employs actively growing pure mycelium with controllable physiological status, making it the preferred choice for mechanistic studies requiring reliable symbiosis establishment. The sterile pouch system ranks second, offering controlled conditions but constrained by contamination risks. The plate symbiosis system, while valuable for real-time observation of early symbiotic events, suffers from lower formation rates due to its two-dimensional constraints. Spore inoculation exhibits the greatest variability, with success rates highly dependent on fungal species and spore batch quality, yet it remains indispensable for evaluating the symbiotic potential of difficult-to-culture fungi. These trade-offs underscore that, from a practical perspective, method selection should be guided by specific research objectives rather than a one-size-fits-all approach.

Beyond laboratory efficiency, the transition from controlled synthesis to field application faces two major challenges: the 'domestication effect' of mycelial inocula, where prolonged subculturing reduces colonisation capacity and growth-promoting efficacy (Thomson et al. 1993), and the germination instability of spore inocula, which compromises reproducibility (Bougher et al. 1990). Based on the reviewed evidence, we suggest that addressing these challenges requires integrating laboratory screening with field validation and establishing standard protocols for inoculum production and quality control.

Based on the reviewed evidence, we tentatively suggest that future research may advance along three complementary directions. First, multi-omics integration (transcriptomics, metabolomics, and proteomics) could be employed to compare symbiosis signalling pathways activated under different inoculation systems – for example, elucidating why spore inoculation, despite slower initial colonisation, may lead to more durable symbioses than mycelial inoculation in certain fungal–host combinations (Plett, Martin 2015). Second, CRISPR-based functional genomics in model ECM fungi such as *Laccaria bicolor* and *Hebeloma cylindrosporum* may enable validation of key symbiotic genes identified through omics approaches, particularly those involved in host recognition and nutrient exchange under specific experimental conditions (Marmeisse et al. 2004; Martin et al. 2008). Third, from a practical perspective, cost-benefit analyses of mycorrhizal seedling production should be prioritised to bridge the gap between laboratory-scale synthesis and large-scale nursery application. This may include optimising inoculum production costs, developing mixed inoculation strategies combining multiple fungal strains, and establishing quality standards for commercial mycorrhizal products (Molina, Trappe 1994; Bougher et al. 1990).

From a practical perspective, with continued advances in molecular biology, high-throughput phenotyping, and process automation, we suggest that a more standardised and scalable system for ECM symbiosis establishment can be anticipated. Such developments may facilitate the efficient production of mycorrhizal *Pinus* seedlings and enhance their role in the ecological restoration of degraded sites and climate-adaptive forestry.

Acknowledgment: We thank the reviewers for their constructive comments on an earlier version of the manuscript.

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Received: June 29, 2026
Accepted: August 17, 2026