Chapter 7
Beneficial Roles of Microorganisms in Woody Plant Micropropagation

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Abstract Plants coexist with microorganisms throughout all stages of their life, and micropropagated plants are no exception. Currently, micropropagation laboratories are increasingly seeking to understand and harness the benefits of plant–microbe interactions, which range from mutualism to antagonism, to improve culture performance. In this chapter, we will first take a brief look at the types of microorganisms that live inside plants and how they interact with them in vivo and then discuss some examples of their beneficial use during different stages of in vitro cultivation of woody plants. We can conclude that including microorganisms in micropropagation can improve plant growth, root quality, and successful acclimatization, along with other important characteristics, such as preparing trees to withstand ex vitro challenges like abiotic stress or tolerance to pathogens under field conditions. The application of biotechnology to plant–microorganism interactions has other ecological and economic advantages, such as improving crop sustainability, the bioremediation capacity of plants, along with others such as increasing truffle production and producing bioactive substances of interest to other fields. Although its application is not without challenges and risks, there are still opportunities and benefits to explore and exploit.

Keywords Bacteria · Endophytes · Fungi · Mycorrhiza · Pathogen control

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1 Introduction

A common feeling among researchers working in micropropagation is the discouragement when we find fungi, bacteria, or yeast growing in our plant cultures. We experience a mixture of disbelief, fear of losing the precious plant material, and a certain feeling of having been treated unfairly by life because—not so infrequently—the contaminated propagules were precisely the ones that were growing best. This last observation was made by Edwin B. Herman more than 30 years ago, when he pointed out that "in some cases, the presence of microorganisms has resulted in unexpected improvements in the cultures, including stimulation of organogenesis and regeneration of sturdier plantlets with better root systems and improved transplant tolerance" (Herman, 1990). Only 8 years later, Jerzy Nowak (1998) reviewed pioneering in vitro co-cultivation experiments involving more than 20 plant species and microorganisms such as bacteria, endomycorrhizal fungi, and extracts of cyanobacteria and fungi. The results of these "biotizations" ranged from higher multiplication, rooting, and acclimatization rates to greater tolerance to pathogens or less hyperhydricity. Treated plants included trees such as pine (Burns and Schwarz, 1996), black locust (Balla et al., 1997), and chestnut (Wilhelm et al., 1997). About twenty years later, interest in plant–microorganism interaction had grown significantly. In 2020, the number of publications using microbes to stimulate plant growth exceeded 22,000. However, only around 300 of those studies dealt with in vitro culture (Cantabella, 2021). Although the number of studies has increased over the past five years, it remains low relative to the broader plant tissue culture literature, suggesting that in vitro researchers still view microbes as a major obstacle to successful micropropagation and/or lack the resources needed to investigate the microbiome. Distrust can arise from lack of knowledge or the—not so unfounded—fear that in case of a mistake, things could get out of our control. In fact, bacteria and fungi are used more frequently during the ex vitro acclimatization phase, when growth is resumed under natural conditions and the risk for the rest of our in vitro germplasm is reduced (Orlikowska et al., 2017).

Agrobacterium-mediated genetic transformation was one of the first cases of intentional use of bacteria during in vitro culture. Genetic transformation reduces the time to introduce novel traits in a plant, this is especially significant for trees with long generation periods (Palomo-Ríos et al., 2021). Safety concerns hampered the deployment of transgenic trees in Europe, but this technology has been widely applied as a tool for genetic analysis of trees (Vettori et al., 2016). Due to space limitations, the use of *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*) and *R. rhizogenes* (formerly *A. rhizogenes*) for tree transformation will not be addressed in this chapter, but updates can be found in Osakabe et al. (2016), Bruegmann et al. (2023) and Maharjan et al. (2025).

The purpose of this chapter is to show some success stories using microorganisms in in vitro culture, and to reflect on whether they can be an opportunity rather than a threat. Wherever possible, we will focus on trees and other woody plants. Tree-associated microbiomes are likely to be more complex than those of herbaceous

plants, owing to the larger size of trees, the presence of woody tissues, and their substantially longer life histories. We will pay particular attention to the contributions that microorganisms can make to addressing species specific problems, as well to technical innovations that facilitate the coexistence of plants and microorganisms in vitro and prepare micropropagated plants for subsequent development under field conditions with a greater likelihood of success.

Reviews about beneficial microbes include Hardoim et al. (2015), Zhyr et al. (2025), Das et al. (2025) and Torto et al. (2026), with the following focusing on plant tissue culture: Orlikowska et al. (2017), Quambusch and Winkelmann (2018), Kanani et al. (2020), Soumare et al. (2021), Cantabella et al. (2022a), Dória et al. (2025), and Guru et al. (2026).

2 Functions of Plant Endophytes

Plants harbor a wide variety of endophytes, including bacteria, fungi, archaea, and even unicellular eukaryotes, such as algae and amoebae. However, defining the term "endophyte" remains a complex task. Since the early nineteenth century, when the term *entophytae* was first coined to describe a group of partially parasitic fungi inhabiting plant tissues, the definition has undergone significant refinement. In Hardoim et al. (2015), we can find the history of these definitions as well as a comprehensive description of the type of microbes that coexist with plants, the relations they establish and how they evolve depending on the internal and external environment. Currently, one of the most accepted definitions refers to those organisms that live throughout the whole, or a part of the life cycle within living plant tissue, without causing symptoms of disease (Wilson, 1995). Its accuracy, however, is limited by the fact that some microbes can live as latent pathogens inside the plants, becoming pathogenic under specific conditions like abiotic changes, the presence of other microbes or quorum sensing. In addition, endophyte effects can vary depending on the stage of plant life cycle (Hardoim et al., 2015).

In vivo, endophytes receive nutrients and a protective niche from the host plant; in return, they typically confer a range of beneficial effects. They may provide protection against invading pathogens, nematodes, and arthropod herbivores, either by direct interaction between the endophyte and pathogen (endophyte feeding on the pathogen or vice versa), competitive exclusion by differential ability to utilize the substrates, inhibition of pathogen by extracellular chemicals produced by the endophyte, such as defensive metabolites or allelochemicals, or by inducing systemic resistance (Guardado-Fierros et al., 2025; Torto et al., 2026). Endophytes can benefit the host by enhancing nutrient uptake, by fixing atmospheric nitrogen, solubilizing phosphorus and iron, and help plants to accumulate, mobilize or degrade soil pollutants (Domka et al., 2019; Kidd et al., 2021; Striganavičiūtė et al., 2025). In addition, endophytes may produce plant growth-promoting hormones like auxins and cytokinins, as well as stress-relief compounds that help plants withstand environmental stresses, such as drought and salinity (Das et al., 2025).

One of the mechanisms of action of endophytes does not require direct contact between the beneficial microbes and the plant pathogen, nor does it rely on the uptake of diffusible metabolites. Instead, this process is mediated by low molecular weight volatile organic compounds (VOCs). These compounds are synthesized by both plants and microorganisms to facilitate intra- and interspecific communication (Poveda 2021). These VOCs inhibit growth and development of pathogens, induce the activation of plant defenses, or promote plant growth and development, either directly or by inducing changes in plant volatilomes. A comprehensive and freely accessible database of the chemical composition of microbial VOCs has recently been published (Kemmler et al., 2025). VOCs may find application in agriculture, medicine and biotechnology (including plant micropropagation). They can be produced by the so-called unculturable microorganisms, which are just microbes that we have not learned to cultivate yet (Quambusch and Winkelmann, 2018). Understanding their chemical nature will allow progress in their application and the development of appropriate cultivation techniques (Vartoukian et al., 2010; Kanani et al., 2020). In addition, advances in chemical synthesis may allow its use in plant micropropagation without the associated risks of microbial overgrowth.

3 Types of Endophytes

Based on the criterion of abundance, most endophytes can be categorized as bacteria or fungi. *Bacterial endophytes* include Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria (Bulgarelli et al., 2013). One of the best-known plant-proteobacterial symbiosis is formed by leguminous plants and rhizobia, which allows the fixation of atmospheric nitrogen (Harman and Uphoff, 2019). This obligate rhizobial symbiosis represents only one extensively studied example, as numerous other facultative beneficial interactions between plants and endophytic bacteria have been documented to date (Ibáñez et al., 2017; Frank, 2018; Pirttilä, 2018). Moreover, some bacteria live symbiotically with plant endophytic fungi, with certain endofungal bacteria colonizing plants in a similar manner to their fungal hosts (Das et al., 2025).

Fungal endophytes colonize the internal tissues of plant stems, leaves, and roots during part or all of their life cycle, effectively enhancing host resilience against various abiotic and biotic environmental stressors. A large group of fungal endophytes belong to mycorrhiza, a symbiotic association between fungi and the roots of vascular plants. In most cases, mycorrhizal fungi cannot produce their own organic matter and rely entirely on the host plant for organic carbon in the form of photosynthetic carbohydrates. In return, fungi support the plant by enhancing its nutrient and water uptake, improving plant growth and resilience to abiotic and biotic stresses (Declerck et al., 2005). Based on the morphological characteristics of the fungal structures and their associations with host plants, mycorrhizas have been classified as ectomycorrhizas colonizing the surface and the extracellular spaces of the plant and endomycorrhizas, penetrating the root cortical cells of plants. Some fungi show

characteristics of both types and are called ectendomycorrhiza (Yu et al., 2001; Gutiérrez et al., 2003). Mycorrhizas can be classified into other categories, like arbutoid, ericoid, orchid, monotropoid, and arbuscular mycorrhizal fungi (AMF, formerly "vesicular-arbuscular mycorrhizae"). AMF are endomycorrhizae that associate with about 80% of plants (Radi et al., 2025a). Inoculation of micropropagated plants with mycorrhiza improves root development, enhances photosynthetic efficiency, water conductivity, and nutrient uptake (especially phosphorus), leading to benefits for micropropagation, such as increased survival and a shorter acclimatization period. Effects in horticultural and forest woody plants include the mitigation of biotic and abiotic stresses, such as drought, toxic metals, saline soil, root pathogens, high soil temperatures, and pH disturbance (Kapoor et al., 2008).

Ectomycorrhizal fungi (ECM) include Basidiomycetes like *Laccaria*, *Lactarius* and *Pisolithus*, frequently used as inoculants for broadleaf forest trees as well as commercially important conifers, such as *Abies*, *Larix, Picea, Pinus,* and *Pseudotsuga*. Associations between ECM and plants can be facilitated by soil bacteria like *Pseudomonas*, *Rhodococcus*, *Streptomycetes*, *Burkholderia*, and *Bacillus*—known as mycorrhiza helper bacteria—which colonize the surface of fungal hyphae, stimulate hyphal growth, and can live within hyphae as endobacteria (Watkinson, 2016).

Other types of Basidiomycetes with potential interest in micropropagation are *Serendipita indica* (also known as *Piriformospora indica*) and *Sebacina vermifera* (order Sebacinales). They establish symbiotic relationships by colonizing root epidermal cells, cortical cells, and intracellular space of a wide range of plants. They can grow in the absence of a host, which facilitates their use in vitro even if it makes their classification as endophytes or mycorrhizas a matter of controversy (Bokati and Craven 2016). *Serendipita* benefits for plants in field conditions have been recently reviewed (Wan et al., 2024; Liu et al., 2026), as well as its potential for micropropagation (Tarte et al., 2022). *Serendipita* spp. promoted growth and stress tolerance of woody plants such as walnut, peach, and camellia in greenhouse and field conditions (Liu et al., 2021; Liang et al., 2023; Fu et al., 2024).

Non-mycorrhizal endophytic fungi include a type of Ascomycetes called "Dark septate endophytes," characterized by melanized hyphae that form networks within plant roots. Their ecological value lies in their association with trees that live in a wide range of environments, including extreme habitats such as deserts and high-altitude regions (Netherway et al., 2024). *Trichoderma* spp. are other endophytic Ascomycetes frequently used as biocontrol agents. These microorganisms confer protection by priming the host's innate defense systems and through direct antagonistic mechanisms, including mycoparasitism, antibiosis, and the synthesis of bioactive secondary metabolites directed against phytopathogens (Harman & Uphoff, 2019; Guzmán-Guzmán et al., 2023).

4 Application of Microorganisms in Micropropagation

Research on microbial applications in micropropagation has traditionally focused on enhancing root development, plantlet growth, and acclimatization, particularly in preparing plants to survive ex vitro stressors like drought or pathogens. However, microbes are increasingly being utilized in earlier stages, such as shoot multiplication, somatic embryogenesis, and initial in vitro establishment. Because many studies in this chapter address multiple traits across different developmental phases, they do not always fit into rigid categories. Therefore, we have organized the following reports based on the primary objectives of each study to better illustrate the diverse benefits of these microbial interactions.

4.1 Microbial Applications During In Vitro Establishment

In general, in vitro establishment is the stage where researchers focus on eliminating microorganisms (pathogens and saprophytes) rather than utilizing them. However, there are reports about potential beneficial effects of microbial inoculation during in vitro establishment. Here we present three studies with trees of Rosaceae, Bignoniaceae, and Myrtaceae families, in which early application promoted growth (Cantabella et al., 2020), facilitated establishment (Salotti et al., 2023), and showed promising results for biocontrol (Kholostiakov et al., 2025).

Cantabella et al. (2020) compared an endophytic bacterial strain (*Pseudomonas oryzihabitans* PGP01) and two fungal strains (*Cladosporium* sp. PGP02 and *Phoma* sp. PGP03) on the in vitro germination of immature embryos obtained by artificial crossings of nectarine, which may exhibit erratic acclimatization due to genotypic effects. The microbes were applied prior to in vitro stratification, which was performed in three steps at different temperatures. Under cold conditions, the two fungi showed excessive growth on the explants, which impaired germination. In contrast, *P. oryzihabitans* PGP01 maintained a reduced development during the cold phase and resumed growth afterwards. Fungi-treated explants did not germinate well, whereas all control and bacteria-treated embryos germinated. The main advantage of *P. oryzihabitans* PGP01 was higher root quality, shoot length, and survival in acclimatization conditions compared to the control.

Salotti et al. (2023) used growth-promoting bacteria to control fungal contamination of *Jacaranda mimosifolia* cultures. Seeds were conventionally disinfected and inoculated with *Methylobacterium* and *Stenotrophomonas* strains at the time of planting in tubes for seed germination. Bacteria-treated explants showed a significant reduction of fungal contamination during establishment, as well as increased multiplication, rooting, and acclimatization. Proposed mechanisms include antibiotic production, secretion of hydrolytic enzymes, or volatile organic compounds.

Kholostiakov et al. (2025) investigated the effects of beneficial seed-borne bacterial isolates from *Metrosideros excelsa* trees, currently being threatened by

the fungus *Austropuccinia psidii*, causal agent of the myrtle rust. The effect of microbes varied from antagonism to pathogen growth promotion. Microbes with antagonist properties were inoculated during in vitro seed germination trials in the absence of the pathogen. In these conditions, the *Bacillus* strain that showed more antagonism to *A. psidii* did not benefit in vitro plant growth, while other strains with intermediate biocontrol capacity, like a *Kocuria* bacterial strain and a *Penicillium* fungal strain significantly enhanced shoot and root biomass. Further research with inoculated plants challenged with the pathogen would reveal which strains confer more tolerance to the fungus and better long-term growth, as well as their potential for being used in natural conditions.

4.2 Microbial Applications During Shoot Multiplication and Somatic Embryo Production

Outcomes after microbial inoculation of woody plants during in vitro multiplication include higher shoot quality, multiplication coefficients, somatic embryo development, and enhanced pathogen tolerance. In this section we present results on bacterial and ECM inoculation on plants cultured by axillary shoots or by somatic embryogenesis, as well as examples of the use of microbial volatile organic compounds (VOCs). Overall, these studies highlight the importance of native endophytic communities, since the beneficial effects are frequently related to the use of microbes already present in vivo in the target species or that have been isolated from their in vitro cultures. Interestingly, whereas it is not surprising that there is a great variation in the effects of different microbes, it is not uncommon for strains of the same species to cause opposite results in the target plants.

Examples based on axillary shoots include pine, rose, thyme, jojoba, grapevine, eucalyptus, apple, and aspen. Pirttilä et al. (2004) observed that removing the bacterial endophytes from *Pinus sylvestris* buds altered in vitro morphology, which could be restored by adding endophytic products to the plant medium. Dolatabadi et al. (2011) observed enhanced growth, multiplication capacity, and root quality of thyme inoculated with strains of *Serendipita indica* and *Sebacina vermifera* fungi.

Salomon et al. (2014) inoculated *Vitis vinifera* microshoots with strains of *Bacillus licheniformis* and *Pseudomonas fluorescens* isolated from the rhizosphere of field growing plants. The *B. licheniformis* strain produced longer shoots and roots, as well as bigger leaves than controls. Both bacteria increased abscisic acid concentration which was inversely correlated with the rate of water loss from the plant. These results suggest that in addition to promoting growth, the microbes protected the plants against biotic and abiotic stress. Following a similar approach, Tamošiūnė et al. (2018) used several *Bacillus* and *Pseudomonas fluorescens* strains isolated from apple orchards for in vitro development of Gala apple shoots. Four of these endophytes (three *Bacillus* and one *P. fluorescens* strains) produced more shoots and

biomass compared to the non-inoculated control. In contrast, the remaining strains (*Bacillus* sp., *P. fluorescens,* and *P. orientalis*) inhibited biomass accumulation and shoot proliferation, indicating genotypic effects still to be elucidated.

The following studies also show diverging results depending on the microbe: Zawadzka et al. (2013) inoculated several ornamental plants with strains of *Curtobacterium pusillum*, *Methylobacterium extorquens* and *Paenibacillus glucanolyticus*, but only *C. pusillum* increased the number and length of rose shoots and roots. *Corymbia citriodora* is a timber tree in the Myrtaceae. De Andrade et al. (2025) inoculated *C. citriodora* in vitro explants with isolates of *Pantoea vagans*, *Bacillus* sp. and *Exiguobacterium sibiricum,* all of them with capacity for auxin synthesis. *E. sibiricum* increased multiplication rates, *Bacillus* produced rooting percentages similar to synthetic auxin treatments and *P. vagans* did not show beneficial effects during the in vitro stage.

The potential for using the same bacteria for plant propagation and biocontrol was proposed in the following studies. Ait Barka et al. (2002) inoculated Chardonnay grapevine shoots with PsJN strain of *Pseudomonas* (renamed *Paraburkholderia phytofirmans* PsJN), observing increased growth and tolerance to *Botrytis cinerea*. Multiplication and rooting increased after jojoba inoculation with strains of *Azospirillum brasilense*, *Methylobacterium aminovoran,* and *Rhodococcus pyridinivorans*, with the activities of key plant defense enzymes suggesting a potential for pathogen biocontrol, especially for *Rhodococcus* (Perez-Rosales et al., 2018). Striganavičiūtė et al. (2021) studied how bacteria isolated from hybrid aspen cultures influenced pathogen tolerance. Aspens are often attacked by *Phellinus tremulae*, a rot-causing fungus. The inoculation with *Pseudomonas* sp. or *Paenibacillus* sp. isolated from in vitro cultures induced distinct responses in genotypes with different susceptibility to the fungus. The most susceptible ones responded with reduced shoot length and increased phenol production, while the more tolerant clones showed positive growth responses that increased after subsequent pathogen inoculation.

Examples based on *somatic embryos* include application of *ectomycorrhiza* during conifer somatic embryo production and plant conversion. Somatic embryo production goes through the stages of initiation, proliferation, maturation, and germination/plant conversion. For progressing from proliferation to maturation, embryos have to stop producing secondary embryos. This is difficult in several species and causes asynchronous development of mature embryos. Niemi et al. (2007) reported a positive effect of a *Pisolithus tinctorius* strain on the maturation of *Pinus sylvestris* somatic embryos. Krajňáková et al. (2012) cultured embryogenic *Abies cephalonica* cell lines with *Laccaria bicolor*, *L. laccata,* and *P. tinctorius* strains. These fungi reduced proliferation, which in turn increased embryo maturation. This response could be related to the plant growth regulator production by the fungus (Niemi et al., 2004). However, the responses depended on the interaction between embryogenic cell lines and fungal strains, and in some cases successful maturation was not directly correlated with proliferation inhibition. This suggests that processes other than plant growth regulator production may be influencing the response.

Effect of Microbial Volatile Organic Compounds (VOCs)
As commented in Sect. 2, microbial VOCs positively impact plant growth and development. Strains of *Bacillus*, *Pseudomonas*, *Trichoderma*, non-pathogenic *Fusarium oxysporum,* and *Serendipita* spp. are among the microorganisms producing beneficial VOCs. In woody plants, Guevara-Avendaño et al. (2019) found that *Bacillus* and *Pseudomonas* VOCs reduced the mycelial density of common avocado pathogens such as *Fusarium solani, Colletotrichum gloeosporioides,* and *Phytophthora cinnamomi.* In conventional systems, plants and VOC-producing microbes have to be carefully placed in different compartments of the same container to prevent direct contact between fungal hyphae and plant tissues, as described in Venneman et al. (2020) and Miñambres et al. (2025) with *Serendipita indica* co-cultivated with *Arabidopsis* or *Populus tremuloides*, respectively. An interesting alternative has been proposed by Fraj and Werbrouck (2023): the fungus was cultured in a separated bottle equipped with filters and connected to a temporary immersion system. The air flow directed the fungal volatiles through the outlet of the bottle to the plants, which were placed inside a SETIS™ bioreactor. This system ensured the plants received volatiles but not fungal cells and made it possible to control the frequency of volatile exposure. This strategy can be adapted to simplify the experimental design of studies with other microorganisms producing VOCs.

4.3 Microbial Applications to Improve Rooting, Acclimatization and Long-Term Plant Survival

Frequent problems associated with recalcitrant woody plants are low rooting percentages and in vitro formed root systems not well adapted for acclimatization and field deployment. The application of microorganisms during rooting causes positive impacts on the formation of functional root systems and on survival rates in nurseries. In addition, it can prepare plants to cope with the conditions they will encounter after planting in the field, such as poor soil nutrition, water stress, pathogens, and pests. Although biotization of micropropagated plants can be performed at the moment of ex vitro transfer, there are reports of inoculation during the in vitro stage. Here we will focus on in vitro studies where endophytes promote successful rooting and protect plants against current pathogen threats, with examples based on fruit and forest trees inoculated with bacteria and fungi, including endo and ectomycorrhiza. We will briefly describe technical advances that facilitate the co-cultivation of plants and these microorganisms. The three following subsections describe experiments performed with bacteria and non-mycorrhizal fungi (Sect. 4.3.1), ectomycorrhiza (Sect. 4.3.2), and arbuscular mycorrhiza (Sect. 4.3.3).

4.3.1 Rooting and Acclimatization After Inoculation with Bacteria and Non-mycorrhizal Fungi

Microbe-mediated success has often been linked to endophyte ability to produce auxins. However, studies described in this section suggest that other substances or mechanisms may also be involved. Other aspects considered here include the importance of a balanced microbiome and the variation in responses of different microorganisms depending on the plant genotype.

Quambusch et al. (2014) demonstrated that genotypic differences in plant propagation ability can be related to the endophytes present in the in vitro cultures. They used six *Prunus avium* genotypes selected for wood quality that differed greatly in their in vitro rooting and acclimatization ability, and characterized the in vitro associated endophytes through sequencing of 16S rRNA amplicons derived from plant DNA extracts. All genotypes harbored *Mycobacterium* sp.; however, *Rhodopseudomonas* sp. or *Microbacterium* sp. were dominant in the two easily propagated genotypes. Inoculating the genotypes with lower rooting capacity with *Rhodopseudomonas* sp. *and Microbacterium* sp. strains significantly improved their rooting performance.

Cantabella et al. (2021) studied how strains of *Pseudomonas oryzihabitans, Cladosporium ramotenellum,* and *Phoma* spp. influenced the in vitro rooting of easy and hard-to-root *Prunus* and *Pyrus* rootstocks. Vermiculite was used as a substrate, to simulate a soil system in vitro. When applied to the hard-to-root *Pyrus* rootstock after auxin treatment, both *C. ramotenellum* and *Phoma* increased the rooting percentage from 56 to 100%, as well as root number and quality. Better results were consistently obtained across all three rootstocks and for each of the three microorganisms when no exogenous auxin was applied, although genotypic variations in quality and biomass production were observed. Since all three microbes are able to produce auxin, the authors suggested this could be the cause of microbial root induction. In a subsequent study, the *Prunus* rootstock was cultivated in the temporary immersion bioreactor GreenTray®. The conditions inside the bioreactor—without agar and with air exchanges produced by forced ventilation—are more similar to the in vivo environment than closed tubes with agar. The liquid medium can be easily modified or systematically sampled to monitor biochemical changes caused by the interaction of plants and microbes, creating opportunities for the study of symbiotic relationships. Cantabella et al. (2022b) demonstrated the usefulness of bioreactors for studying the interaction between trees and beneficial and non-beneficial endophytes.

Yarte et al. (2022) described the effect of *Pseudomonas* sp. *Paenibacillus* sp. *Methylobacterium* sp. *Rhizobium* sp. and two *Bacillus* strains on the rooting of pink lapacho untreated or treated with exogenous auxin. Since most of the treatments were successful, and only one of the *Bacillus* strains produced less roots than controls, the authors suggested that inoculation with selected strains may replace totally or partially the auxin induction requirements of the plant. Other experiments indicate that the root promoting effect and plant quality goes beyond the auxin

content of the microbial extracts, and may be related to the presence of other compounds in the microbial secretions. This has been observed with strains of *Pantoea agglomerans* and other rhizobacteria, either through co-cultivation of the microorganism and the plant or through the use of cell-free extracts containing only microbial metabolites. Luziatelli et al. (2020a, b) reported that metabolites obtained from *P. agglomerans* C1 improved rooting and acclimatization of *Prunus* rootstocks, hazelnut and pear, even with extracts containing three times less bacterial auxin than treatments with commercial auxin. Souza et al. (2022) reported similar results with rhizobacteria extracts applied to apple rootstocks. In the same trend, Vinskienė et al. (2025) observed a positive effect on apple rooting using bacterial strains that do not produce significant amounts of indole-related compounds, indicating that the promoting effect of the inoculum did not involve root growth stimulation by direct auxin production.

An interesting aspect of these studies is the interaction of the inoculum with the receiving endophytic community. Vergani et al. (2024) inoculated grapevine shoots with two beneficial *Kosakonia* and *Rhizobium* strains and subjected them to nutritional deficiency conditions. While both strains colonized the plant endosphere, only the *Rhizobium* strain increased root biomass. The study of the composition of the endophytic native community indicated that it was differently modulated by both strains. *Rhizobium* caused a minor impact compared to *Kosakonia*, which showed an opportunistic colonization pattern. Overall, the results highlight the importance of preserving the native endophytic community structure and functions during plant microbiome experiments.

4.3.2 Rooting and Acclimatization After Ectomycorrhizal Inoculation

Studies of plant inoculation with *ectomycorrhiza* during rooting started more than 40 years ago, probably because the requirements for co-cultivation of ECM and plants can be met relatively easily in vitro, especially when compared with those of arbuscular mycorrhiza. Most of the studies referring ECM application occurred during or after the rooting step, and were focused on enhancing root quality, plantlet development and acclimatization success, frequently with the bonus of increased tolerance to biotic and abiotic stress.

ECM structures can be formed in vitro in a wide range of substrates including agar, although options with higher permeability—such as perlite, sphagnum peat, vermiculite and soilrite—are often more effective (Zhu et al., 2010). Factors that are important for in vivo inoculation (Quoreshi, 2000; Fei et al., 2024) are also relevant for in vitro experiments (Pohjanen et al., 2014; Chen et al., 2023). For example, nutrient application during the first steps of symbiosis establishment should be adjusted to avoid toxic effects which could inhibit fungal growth. Once the fungus has adapted, supplementing nutrients progressively can enhance plant growth (Quoreshi, 2000). Several other decisive factors influence the success of these microbial applications. First, the choice of fungal symbiont, the quality of the inoculum, and the timing of application, specifically the physiological status of

both the fungus and the plant, are critical. Second, technical elements such as the substrate used, the inoculation technique, and potential interactions with other endophytes must be considered. Finally, for long-term success, the specific biotic and abiotic characteristics of the soil at field deployment sites play a vital role.

In vitro fungal inoculation has resulted in an effective strategy for improving root quality, acclimatization and hardening of micropropagated plants (Martins, 2008), especially with recalcitrant forest trees that frequently do not form good root systems during in vitro rooting, such as some Fagaceae and conifers (Zhu et al., 2010). Some examples of experiments developed with axillary shoots and germinated somatic embryos of angiosperm and gymnosperm trees are cited below.

Grellier et al. (1984), Strullu et al. (1986), and Szuba et al. (2017) observed increased stem and root growth after inoculation of birch, chestnut and poplar shoots (respectively) with *Paxillus involutus*, an ECM fungus that enhanced lead accumulation in poplar (Szuba et al., 2017), as well as drought tolerance in chestnut (Mateus et al., 2024). Chestnut shoots established successful symbiosis with other ECMs as *Pisolithus tinctorius* and *P. arhizus* (Martins et al., 1996; Carvalho et al., 2013). Inoculation of somatic embryos from an adult elite tree of another Fagaceae, *Quercus suber*, with *P. tinctorius* and *Scleroderma polyrhizum* resulted in higher development of secondary roots and better ex vitro growth (Díez et al., 2000). The potential of *P. tinctorius* to alleviate stress conditions in forestry was also demonstrated in eucalyptus seedlings, which showed increased tolerance to salinity in greenhouse trials (Zayed et al., 2025).

Despite these encouraging results, it is necessary to assess the evolution of symbiosis after in vitro and greenhouse steps. *Arbutus unedo* shoots were inoculated during rooting with several species of ectomycorrhiza, such as *P. arhizus* and *Lactarius deliciosus* (Gomes et al., 2013, 2016), with improved plant growth and survival during the first months after transplanting. Notably, a long-term field study revealed that the inoculated fungus was no longer detectable 20 months after deployment. This finding highlights the significant challenge of inoculum persistence, particularly when introduced species must compete with established native soil microbial communities (Gomes et al., 2013).

Economically important conifers, including *Abies*, *Larix, Picea, Pinus,* and *Pseudotsuga* form ECM in field conditions, and inoculation before ex vitro planting enhances performance. Niemi et al. (2004) and Niemi and Scagel (2007) reported enhanced rooting and acclimatization of *Pinus sylvestris* shoots after ECM inoculation. Similarly, Oliveira et al. (2003) inoculated *Pinus pinea* rooted shoots with fungi isolated from ectomycorrhizas, obtaining more growth and vigor than control plants.

Micropropagation by somatic embryos of conifer trees is commercially used in countries like New Zealand, Canada, Sweden, and Finland (Aronen et al., 2025a, 2025b). Conifer production would benefit significantly from further optimization of protocols for root development, acclimatization, and survival under field conditions (Välimäki et al., 2021; Nielsen et al., 2022; Castander-Olarieta et al., 2025), for which mycorrhizal associations could be used. It is notable that inoculation with *Pisolithus orientalis* doubled survival of *Pinus massoniana* plantlets derived from somatic embryos (Chen et al., 2023) and conferred significant protection against pine

wilt disease, caused by the nematode *Bursaphelenchus xylophilus*. This pest represents a serious threat to pine forests worldwide and has already caused extensive mortality in China, Taiwan, Portugal, Spain, and the United States (Back et al., 2024). Besides pines, *Picea abies* (Norway spruce) is one of the main trees in breeding programs in Nordic countries (Jansson et al., 2017). This species is commercially propagated by seeds and by somatic embryos initiated from zygotic embryos obtained from selected crosses aimed to increase growth and wood quality, together with resistance towards pathogens and pests (Tikkinen et al., 2021). One of the current challenges is the annosus root rot, caused by basidiomycete members of the *Heterobasidion annosum* sensu lato species complex. This destructive disease has ecological and economic consequences in the Northern hemisphere, threatening the previously expected increase in productivity associated with rising temperatures and the planting of elite forest regeneration material (Jansson et al., 2017).

Strategies to counteract pathogen attack and improve vigor of germinated somatic embryos include inoculation with bacterial and fungal endophytes. Seed endophytes may offer the additional advantage of exerting effects on progeny plants when they are transferred to the next generation through vertical transfer. Endophytes such as *Meliniomyces bicolor* (ECM), *Phialocephala fortinii* (dark septate endophytic fungus) and *Paraphaeosphaeria neglecta* (endophytic fungus) showed potential to inhibit root rot fungi symptoms in inoculation assays with seedlings cultured in vitro (Velmala et al., 2018) and ex vitro (Durodola et al., 2023; Himanen et al., 2024). The protocols defined for in vitro inoculation of Norway spruce seedlings with other ECM such as *Piloderma croceu, Hebeloma crustuliniforme, H. cylindrosporum* and *Laccaria bicolor* (Nylund & Unestam, 1982; Brunner, 1991; Karabaghli et al., 1998) could be adapted to inoculate somatic embryos with the candidate microorganisms against annosus root rot.

4.3.3 Rooting and Acclimatization After Arbuscular Mycorrhiza Inoculation

Despite the potential benefits of establishing endomycorrhiza symbiosis during micropropagation, reports on AMF symbiosis with woody plants during in vitro stage are still scarce. Defining the appropriate conditions for AMF symbiosis during micropropagation required a significant research effort, mainly due to the incompatibility of the culture conditions of in vitro plants (partially heterotrophic) with those required for establishing the symbiosis (autotrophic). Kapoor et al. (2008) and Radi et al. (2025a) reviewed scientific contributions since the late 1960s to the present, concluding that nowadays, the culture system called Mycelium Donor Plant (MDP) is the most commonly used for in vitro mycorrhization. MDP is an adaptation of the autotrophic method of Voets et al. (2005, 2009), and the main steps are briefly described below:

1. Mycorrhizae are cultivated with roots of tomato, red clover, strawberry, onion, chicory, or a carrot hairy root line to produce fungal spores.

2. Rooted shoots of a donor plant—like *Medicago trunculata*—establish a symbiosis with these spores and form a mycelium network in autotrophic conditions (without sugars or vitamins).
3. Final step with rooted shoots of the target plant: These shoots can be produced and rooted in conventional nutrient medium with vitamins and sucrose, but to develop the symbiosis they are transferred to a new container or plate without these nutrients. Roots are placed close to the mycelium network formed in the donor plant roots (step 2), whereas the stem and leaves are placed outside the hyphal compartment, in contact with air that may be enriched with CO_2.

The MDP system has been applied to woody plants like rubber tree, pear, and argan. In the three cases the AMF was *Rhizophagus irregularis* (previously known as *Glomus intraradices*). Improved rooting efficiency and plantlet vigor, enhanced nutrient uptake, and better establishment under ex vitro conditions were obtained (Sosa-Rodriguez et al., 2013; Lotfi et al., 2019; Radi et al., 2025b). In argan, the mycorrhiza was also inoculated in unrooted shoots, which resulted in increased rooting percentages, as well as in significantly higher survival rates during acclimatization. In teak and mahogany, target plants were directly inoculated with root organ cultures, without intermediate donor plants (Chaiyasen et al., 2017; Romero-Ceciliano et al., 2025).

The next example describes the cultivation of desert truffles using biotechnological approaches. These are edible fungi—mainly of *Terfezia*, *Picoa,* and *Tirmania* genera—that establish ectoendomycorrhizal symbioses with Cistaceae plants. Truffles are economically interesting for their nutritional and gastronomic values. In addition, they have an ecological value because the *Helianthemum* spp. plants with which they establish symbiosis are well adapted to semi-arid and arid environments (Morte et al., 2020), representing a cultivation opportunity for agro-systems increasingly affected by climate change. Conventional propagation methods using seedlings and fungal spores could not meet the market demands; biotechnology made it possible to overcome these limitations, with new plant propagation strategies with inoculum production and use of plant growth-promoting rhizobacteria (Morte et al., 2012; Navarro-Ródenas et al., 2016). Micropropagation of the host plants resulted in higher proliferation and survival, which increased the number of plants available for establishing symbiosis. In addition, Morte and Andrino (2014) developed a photoautotrophic system for co-cultivating *H. almeriense* and *Terfezia claveryi*. Briefly, they replaced agar with sterilized perlite, used a sugar-free medium, increased light intensity, provided a relatively low humidity environment and prevented CO_2 depletion inside the containers by using plastic covers that allowed gas exchange (Morte & Andrino, 2014). Combining a helper bacteria (*Pseudomonas mandelii #29*) with drought stress caused synergistic increases in fungal colonization and nutrient uptake (Guarnizo et al., 2023). These strategies fostered plant and fungus development, allowed for physiological, biochemical, and molecular studies and the establishment of field plantations with positive impact on the socioeconomic development of rural areas (Morte et al., 2020). The establishment of symbiosis through biotechnology also increased tolerance to water deficit conditions. Trees typically

from dry areas, like *Quercus suber* and *Q. coccifera,* can be inoculated with *Terfezia boudieri* in nurseries (Slama et al., 2021), using the extension of the strategies described for *Helianthemum* plants applied to these trees could strengthen the resilience of forest ecosystems to climate change.

5 Challenges, Perspectives, and Conclusions

Despite the benefits of in vitro inoculation with microorganisms, there are still areas where further technical progress is needed, as well as biological interactions not yet fully understood. In vitro methods raise concerns about potential negative effects on environment and health, but they also offer advantages that have not yet been fully exploited.

Risks start when microorganisms and plants are still in the laboratory or nursery, due to potential cross-contamination. When laboratories propagate different species, microorganisms that can be beneficial for some plants may not be suitable for others. Avoiding cross-contamination requires careful aseptic control of production lines, equipment, growing media and plant containers. Luziatelli et al. (2020a) recommend using cell-free extracts containing only microbial metabolites, which reduces contamination risks and is more compatible with automation systems. Another challenge for in vitro biotized plants is the assessment of the stability of the association after several subcultures, as well as the persistence after field deployment. This is especially important if the benefit is not expected to end with acclimatization but is related to field production or to tolerance to abiotic or biotic stress. Soils comprise a biotically and chemically diverse and complex environment, that differs substantially from the substrates used in greenhouses and nurseries. In natural soils, the inoculated microbes will face competition from those naturally present in the planting areas. Monitoring evolution of plant microbiomes after several months in the field is hampered by morphological and biochemical similarities between microorganisms, and morphological identification should be complemented with molecular methods (Romano et al., 2020).

While some researchers, farmers, and foresters express concern about whether the benefits of endophyte inoculation might not persist under field conditions, others fear that microorganisms will persist and spread, with the risk of disrupting native microbial communities. If microbes spread outside their natural areas, they may hinder the development of native microbiome in those sites, challenging biodiversity (Zhyr et al., 2025), and even compromising human health. The One Health approach of the European Union considers the well-being of people, animals, plants, and the environments they share as a whole. In this context, using microbes as biostimulants, biofertilizers, or biocontrol inoculants, manure application, and the reuse of treated wastewater for crop irrigation creates an entry point into agri-food ecosystems for external microbial taxa and/or functional traits whose impact has not been fully assessed yet (Patania et al., 2025).

Relatively few studies deal with the interactions between different symbiotic organisms within the host plant (Pohjanen et al., 2014), which should be the first step before addressing their relationships with ecosystems. Vinskienė et al. (2024, 2025) studied cherry and apple cultures after dormant bud cryopreservation. Plant regeneration was reduced, which was caused by alterations of the microbial community composition. Inoculation with bacterial isolates from the donor trees restored the endophytic community and increased recovery. Bae et al. (2026) used metagenomics to identify the dominant endophytes of in vitro grapevine shoots, and applied targeted antibiotics against them. The subsequent enrichment with other bacterial strains—potentially more protective—increased regeneration. These examples suggest that the microbial community structure can be relevant for more aspects of plant development than previously considered, making it necessary to study microbial characterization and adequate preservation of balanced communities. SynComs, defined as simplified microbiomes composed of artificially selected and assembled microbial strains that exhibit emergent, community-level properties following current ecological and evolutionary theories, could be useful in the effective management of plant endophytic microbial communities. They could provide model systems to investigate plant–microbiome and microbe–microbe interaction, as well as be used as bioinoculants to modulate or engineer the plant microbiome toward desired functions. To create tree-specific SynComs, it is necessary to develop model tree systems and obtain tree-specific culture collections. In addition to characterizing the hidden biodiversity harbored by trees, it is necessary to optimize methods for inoculating these communities into trees, which could include micropropagation and bioreactors (Cambon et al., 2025).

Further applications of plant endophytes are related to their ability to produce bioactive compounds, as well as to act as elicitors of plant cells and tissues that synthesize secondary metabolites. Both features make them useful as microbial factories. Current challenges are related to the insufficient expression of relevant genes or the inability of isolated organisms to remain metabolically active in artificial environments. The application of epigenetic approaches can effectively modulate the transcription of biosynthetic genes in endophytes. By doing so, researchers can enhance biotechnological production while gaining a deeper understanding of the roles these microbes play within natural plant–microbe ecosystems (Liu et al., 2025).

In conclusion, there is still much to learn about the hidden world of microbes in plants, but since it is impossible to completely eliminate them from in vitro cultures, the wisest course of action is to be aware of their potential and make the most of them.

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