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REVIEW

Adventitious rooting of conifers: influence of biological factors

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Abstract Vegetative propagation of superior conifer trees can be achieved, e.g., through rooted cuttings or rooted microshoots, the latter predominantly through in vitro tissue culture. Both techniques are used to achieve rapid multiplication of trees with favorable genetic combinations and to capture a large proportion of the genetic diversity in a single generation cycle. However, adventitious rooting of shoots (cuttings) is often not efficient due to various problems, such as scarcity of roots and cessation of their growth, both of which limit the application of vegetative propagation in some conifer species. Many factors are involved in the adventitious rooting of shoots, including physical and chemical ones, such as plant growth regulators, carbohydrates, light quality, temperature and rooting substrates, or media [reviewed by Ragonezi et al. (Trees 24(6):975–992, 2010)]. The focus of this review is on biological factors, such as inoculations with *Agrobacterium rhizogenes*, plant-growth promoting rhizobacteria and other endophytes, and mycorrhizal fungi, which were found to stimulate adventitious rooting. These microorganisms could contribute not only to adventitious root development but also to help in protecting conifer plants against pathogenic microorganisms, facilitate acclimation and transplanting, and contribute to more sustainable, chemical-free forests.

Keywords Biotization · Mycorrhization · Plant-growth promoting bacteria · Gymnosperms

Abbreviations

BnR	Binucleate Rhizoctonia
DNA	Deoxyribonucleic acid
ECM	Ectomycorrhizal fungi or Ectomycorrhizas
ERM or EMF	Ericoid mycorrhizal fungi; Ericoid mycorrhizas
GA_3	Gibberellic acid or Gibberellin A_3
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
M	Molar
μM	Micromolar
MHB	Mycorrhization helper bacteria
Mm	Millimolar
MS	Murashige and Skoog (1962) culture medium
NAA	1-Naphthaleneacetic acid
PGPR	Plant-growth promoting rhizobacteria
PGR	Plant growth regulators
RSB	Root-stimulating bacteria
Ri	Root-inducing plasmid
t-DNA	Transfer DNA
Ti	Tumor-inducing plasmid
TIBA	2,3,5-Triiodobenzoic acid
VAM	Vesicular–arbuscular mycorrhizas

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Introduction

In the 1980s and 1990s, much research focused on understanding the relationship between rhizosphere soil microorganisms and plant species. The rhizosphere microorganisms, especially mycorrhizal fungi and bacteria, are able to increase the tolerance of plants to abiotic and biotic stresses, stimulate plant growth, and enhance rooting, among others. Majority of the higher plant species are associated with detrimental or beneficial mycorrhizal fungi and rhizospheric bacteria. Beneficial bacteria are divided in two major groups: plant promoting rhizobacteria (PGPR) and mycorrhization helper bacteria (MHB) (Hrynkiewicz and Baum 2012). Many species of both groups, and their interactions, can enhance growth, defend plants against pathogens, and help them to cope with environmental constrains, such as nitrogen depletion from the soil or excess of pollutants and heavy metals. The application of microbial inoculum was studied and tested with several agroforestry plant species in the past. Unfortunately, despite the gained knowledge of the effect of different helper microorganisms and the positive results obtained, little has effectively been transferred from Agricultural and Forestry Experimental Stations and other Government Institutions to commercial enterprises. Small to medium companies around the world produce and sell inoculum as biofertilizers (Carvajal-Muñoz and Carmona-Garcia 2012) for specific plant species, but their products are limited to mostly mycorrhizal inoculum (Berch et al. 1999). The main reasons for the lack of field applications on a large scale could be: the high variation in the required inoculum to improve survival and growth of plants in the field, the persistency of the inoculum in the soil, the inconsistency of the response due to genetic differences within and between the plant and fungus or bacterial species, cultural differences in nurseries, differences in the quality of seed or vegetatively propagated material, weather conditions during the planting, differences in forestry techniques, differences from site to site, duration of field trials, absence of suitable time- and cost-effective strategies for a site and species specific selection, differences in the soil preparation, application of the microbial inoculum, and the strong restriction of information on on-site efficiency of inoculated microbial strains (Hrynkiewicz and Baum 2012). Other important aspects concerning application of genetically engineered microorganisms are specific to each country's regulatory requirements for manufacturing, use, import, export, and storage of the manipulated microorganisms.

Notwithstanding the above concerns, no one can ignore the enormous benefits to forestry and agriculture that could stem from the substitution of the current chemical practices that ultimately contaminate air, soil, and water, with beneficial microorganisms that minimize environmental impacts and take advantage of the resources available in nature, which are less invasive or harmful. In this context, it is not surprising that the researchers explored the enormous beneficial potential of the soil microorganism to initiate and develop a robust adventitious root system to ensure the acclimatization and growth of plants after transplanting to field locations. Means for providing cuttings and microcuttings of the recalcitrant conifer species with a functional root system using beneficial microorganisms is the main objective of the present review.

In an earlier review (Ragonezi et al. 2010), we summarized available information related to physical and chemical factors applied in rooting of conifer cuttings and of in vitro propagated microshoots. However, with a few exceptions, the chemical treatments and/or physical conditions have not been encouraging, because even if adventitious root induction and expression were achieved, the roots frequently stopped growing as was observed in *Pinus pinea* microshoots by Oliveira et al. (2003). An alternative treatment for stimulation of adventitious rooting is biotization, which has been applied for rooting of both cuttings and in vitro propagated microshoots. Biotization under controlled conditions (mycorrhization or bacterization) can be achieved by several axenic and non-axenic techniques with all the benefits described above.

Agrobacterium rhizogenes

Agrobacterium rhizogenes is closely related to *A. tumefaciens*, the causative agent of crown gall disease. *A. rhizogenes*is is a gram negative soil bacterium, which is capable of infecting a plant through a wound causing hairy root disease, because the infected sites produce abundant adventitious roots (Moore et al. 1979; Tepfer 1983; Petit et al. 1987; Chandra 2012).Virulence in both bacterial species is conferred by large plasmids. Infected plant tissue synthesizes novel metabolites, such as opines that are not found in normal plant tissues. Opine synthesis persists when the infected plant tissue is cultivated in vitro in the absence of the pathogenic bacterium, which is a consequence of gene transfer and integration from the pathogen to the plant cell genome. A small specific part of the tumor inducing (Ti) plasmid of A. *tumefaciens*, termed T-DNA (transfer-DNA), is incorporated into host plant nuclear DNA and transcribed into mRNA. A specific region of T-DNA confers the ability to synthesize the characteristic opine. The discovery of opines in roots induced by *A. rhizogenes* suggested that they too might contain T-DNA derived from the virulence plasmid of the pathogen

(Chilton et al. 1982). The mechanism of the formation of adventitious roots is the integration of bacterial t-DNA, which consists of the Ri (root inducing) plasmid carrying a series of genes coding for indole-3-acetic acid (IAA), into the cell genome (Ercan et al. 1999; Li and Leung 2003; Britton et al. 2008). Synthesis of bacterial opines is thus an example of natural genetic engineering in which the vector is either the Ti or Ri plasmid. The symptoms of infection with *A. rhizogenes* are the increased sensitivity of cells to the effect of auxins, as well as the production of roots. Although Gelvin (1990) noted that the T-DNA of some Ri-plasmids of *A. rhizogenes* containing auxin biosynthetic genes, these loci were not always necessary for the hairy root formation. While *Larix decidua* transgenic plants were regenerated from hairy root explants (Huang et al. 1991) in other species, it has not been confirmed whether the adventitious roots were transgenic (Magnussen et al. 1994), thereby introducing a degree of ambiguity into the mechanism of hairy root formation. A plausible explanation in the latter case could be that T-DNA was expressed only transiently or that the non-transformed cells in the vicinity of those transformed regenerated roots. Trees with transgenic adventitious roots would most likely be subjected to the regulatory issues, which are outside the scope of this review, particularly that they bring a special set of challenges having no parallel among transgenic food crops. However, *Agrobacterium* species are natural genetic engineers (both wild strains and engineered), and according to the recent genome sequencing results, it appears that the cultivated clones of the crop plant, sweet potato, incorporated into their genome T-DNA sequences during evolutionary times (Kyndt et al. 2015). This potentially may change the perception of "unnatural" status of transgenic crops and perhaps trees.

The bibliography related to *A. rhizogenes* is extensive, and here, we cite a few selected articles and reviews (Chilton et al. 1982; Tepfer 1983; White et al. 1983, 1985; Cardarelli et al. 1985; Boulanger et al. 1986; Richaud et al. 1987; Cardarelli et al. 1987; Schmulling et al. 1988; Häggman and Aronen 2000; Meyer et al. 2000; Veena and Taylor 2007; Citovsky et al. 2007; Britton et al. 2008; Otten et al. 2008; Mohajjel-Shoja et al. 2011; Georgiev et al. 2012; Özyiğit 2012; Choudhury et al. 2014).

One of the first studies involving a conifer and *A. rhizogenes* was carried out by McAfee et al. (1993) who achieved improved ARF in *Pinus monticola* Dougl. microshoots obtained in vitro from mature embryos. Co-cultivation of microshoots with A4 or pRi transconjugant R1000 strains was carried out and compared with the controls and treatments with 1-naphthaleneacetic acid (NAA) and IAA. Co-cultivated shoots had both the number and quality of induced roots improved compared with the PGR treatments. The same positive results were obtained with co-cultivated *Pinus banksiana* Lamb. and *Larix laricina* (DuRoi) K. Koch derooted seedlings (McAfee et al. 1993).

In another study, *Picea abies*, *Pinus sylvestris,* and *Pinus contorta* 2-to-3-week-old seedlings were inoculated with the supervirulent strains of *A. rhizogenes* by Magnussen et al. (1994), whereas Bergmann et al. (1997) used *P. radiata* clonal plants micropropagated from seeds. The results from these studies were highly variable and dependent on the bacterial strain and the conifer species used as well as the transformation protocol. Detection of the expression of inserted genes has been problematic and could not be verified (Magnussen et al. 1994).

Agrobacterium rhizogenes LBA9402 strain was more effective compared with A4RSII for the induction of adventitious rooting on hypocotyls of *Pinus contorta* (Yibrah et al. 1996). In that study, the roots originated from tissues of endodermis. The authors concluded that the roots followed the same pattern of rhizogenesis as auxin-induced roots in the hypocotyl cuttings previously described by Grönroos and von Arnold (1987).

The advantages of *A. rhizogenes* inoculation/co-cultivation over auxin root induction in Scots pine cuttings (of fascicular bud origin) were also demonstrated by Aronen et al. (1996). Cuttings from two different seasons (spring and late summer) were incubated for 20 h in 0.5-mM indole-3-butyric acid (IBA) solution and afterward dipped in a bacterial suspension of A4, A4 (GUSint) or R1600 followed by immediate planting. The summer cuttings that were inoculated produced more roots compared with the IBA treatment alone (24.5 versus 16.2 %, respectively). More than 80 % of fascicular shoots rooted in the best inoculated genotypes; the best response after IBA treatment was only 30–40 %. The authors have suggested that the external factors, such as conditioning and treatments of ortets, and the excision time of the ramets had significant impact on root formation. Fascicular shoots in the spring lot were comparable with the natural shoots at the beginning of the growing season, while summer cuttings rooted better than those from the spring lot, which may be due to differences in the developmental stage of the propagules.

In *Pinus nigra*, 60–97 % of 3-to-8-week-old seedling explants formed roots following infection with *A. rhizogenes* wild strains 8196, 15834, or with the pRiA4abc transconjugant strain of *A. tumefaciens* (C58 chromosomal background) (Mihaljevic et al. 1996). In this case, the plant response to infection was dependent on the bacterial strain, the age of the explant and the period of co-cultivation.

One of the first reports on transgenic conifer tissue using *A. rhizogenes*-mediated gene transfer was published by Mihaljevic et al. (1999). Coast redwood [*Sequoia sempervirens* (D. Don.) Endl., clone CA_3] basal cut ends of 2-cm-long shoot tips derived from in vitro culture were

inoculated by immersion in a freshly grown bacterial culture of *A. rhizogenes* wild strain 8196 or with the pRi A4 transconjugant strain (C58 chromosomal background). The inoculated explants were placed on a moist sterile filter paper in a Petri-dish and co-cultivated for 12, 24, or 48 h. Roots were formed by 58–69 % of the shoots, but no hairy-root phenotype was found in these transformed plants. The authors described the phonotype of the transformed roots as similar to normal ones. Rooting frequency and the number of adventitious roots on the inoculated shoot-tips were slightly modified by the bacterial strain, duration of inoculation and explant support system.

Zaspel and Edwal (2001) inoculated cuttings of various conifer trees (*Larix*, *Taxus*, *Thuja* and *Picea*) with beneficial bacteria (*Bacillus*, *Pseudomonas,* and *Agrobacterium*) to promote adventitious root development. Improved rooting of two of the three tested clones of *L. decidua* cuttings was observed after inoculation with *A. rhizogenes* DSM 30148 strain. The rooting percentage was greatly influenced by the genotype, with the LH17 clone being the most responsive and with rooting at over 60 %.An even higher success was reported for *Thuja* sp. cuttings, which rooted at 100 % after the sole application of the same *A. rhizogenes* strain. The survival of the treated plants transferred to the greenhouse varied between 10 and 40 %.

Pinus maximartinezii Rzedowsky and *P. Pinceana* Gordon and Glend, two endemic species of pine from Mexico, were regenerated in vitro by organogenesis starting from mature embryos and the microshoots rooted after pulse treatments with an auxin or after inoculation with *A. rhizogenes*. The rooting percentage was low (13 and 7 %) for the auxin-treated shoots of *P. maximartinezii* and *P. pinceana*, respectively in comparison with 60 % (*P. maximartinezii*) and 67 % (*P. pinceana*) rooting obtained by inoculation with *A. rhizogenes* (Villalobos-Amador et al. 2002).

Li and Leung (2003) reported the effects of two strains of *A. rhizogenes* (A4T and LB9402) on root formation in radiata pine (*Pinus radiata* D. Don). The inoculations were made in various explants (hypocotyl explants, intact seedlings, derooted seedling cuttings, and adventitious shoots) with or without application of IBA. Considerable differences were found in the rooting response depending on the explant used and the strains of *A. rhizogenes*. Of all treatments, the best rooting response (approximately 75 %) was obtained from hypocotyl segments inoculated with LBA9402 prior to transfer to the semi-solid ½ MS (Murashige and Skoog 1962) medium supplemented with 20-g l^{-1} sucrose and 9-mg l^{-1} IBA. LBA9402 increased not only the percentage of rooting but also the adventitious root number in the shoot explants. Similarly, in *Pinus halepensis*, LBA9402 infected embryos, seedlings, and shoots produced calli and adventitious roots at the wound sites in 64 % of the seedlings and 71 % of seedling cuttings. More than 85 % of mature embryos exhibited susceptibility in the radicle to *A. rhizogenes* treatment as monitored by β-glucuronidase (GUS) assay. On the other hand, adventitious shoots that were induced on 2.5-year-old seedlings by pruning and spraying with 6-benzylaminopurine, infected by injecting the bacterial suspension into their basal side, and showed adventitious roots and root primordia in 74 and 40 % of 2- and 5-month-old shoots, respectively, two months later (Tzfira et al. 1996).

In vitro shoots of *Araucaria excelsa* Lamb.var. *glauca* (*Araucaria heterophylla*) were induced to root in the presence of different PGRs (IBA, NAA) and ancillary compounds to increase the rooting under in vitro conditions (Sarmast et al. 2012). Neither ancillary compounds, such as salicylic acid, putrescine, nor hydrogen peroxide affected the rooting of this recalcitrant species. The authors hypothesized that high tannin and resin levels in the tissues of this conifer species might be the reason for rooting failure. Nevertheless, shoots cultured on MS medium containing 7.5 μM of both IBA and NAA for 15 days followed by PGR-free half-strength MS medium, resulted in a 33 % increase in rooting with one or two roots. Alternatively, inoculation with *A. rhizogenes* K599 strain improved rooting percentage up to 40 % when combined with both IBA and NAA. No rooting was obtained when shoots were inoculated with *A. rhizogenes* without PGRs.

Given that the genetic improvement programs are slow in conifers and that the introduction of specific genes through crossbreeding has proved difficult, the use of *A. rhizogenes* can be a rapid and direct route for the introduction and expression of specific genes for difficult to root conifers (Huang et al. 1991).

Plant-growth promoting rhizobacteria (PGPR) and other endophytes

Plant-growth promoting rhizobacteria (PGPR) are free-living a symbiotic soil bacteria with the ability to colonize plant roots (Chanway 1997; Zahir et al. 2003; Lucy et al. 2004; for a review, see Saharan and Nehra 2011). At the same time that these microcolonies benefit from the nutrients secreted by plant root systems, they can also directly or indirectly stimulate plant growth. These organisms have contributed to plant survival and evolution; they can infect multiple genera, yet exhibit genotype specificity within species (Johnston-Monje and Raizada 2011). Common genera of PGPR are: *Pseudomonas*, *Bacillus*, *Azotobacter*, *Arthrobacter*, *Clostridium*, *Hydrogenophaga*, *Enterobacter*, *Serratia*, and *Azospirillum* (Benizri et al. 2001; Gray and Smith 2005). These PGPR are classified as biofertilizers, plant stimulators or

biological control agents according to the degree to which they can fix nitrogen, directly promote growth or protect plants against plant pathogens, respectively (Bloemberg and Lugtenberg 2001). PGPR can affect plant growth by biologically active substances (Spaepen et al. 2009). It is known that some PGPR produce/alter the concentration of plant hormones, such as IAA, gibberellic acid (GA3), cytokinins, and ethylene (Husen 2003; Nihorimbere et al. 2011). Indole-3-acetic acid (IAA) is the best-characterized auxin produced by many plant-associated bacteria, including PGPR (Spaepen et al. 2007). Exogenous IAA controls a wide variety of processes in plant development and plant growth: low concentrations of IAA can stimulate primary root elongation, whereas high IAA levels stimulate the formation of lateral roots, decrease primary root length, and increase root hair formation (for more information, see the review of Vacheron et al. 2013). It is known that IAA is synthesized by rhizobacteria from tryptophan (Kamilova et al. 2006), but other IAA biosynthetic pathways have been described depending on the metabolic intermediates of the PGPR involved (Spaepen et al. 2007). PGPR asymbiotically fix nitrogen; and act as antagonists against microbial plant pathogens either through production of siderophores, β-1,3-glucanase, chitanase, antibiotics, and cyanide or through solubilization of mineral phosphate and other nutrients (Mafia et al. 2009).

Several studies have been performed on gymnosperms with the majority focusing on species of *Pinus, Picea, Tsuga,* and *Pseudotsuga* genera (Chanway and Holl 1993a, b, 1994; Chanway 1997; Shishido and Chanway 2000). Isolates of *Arthrobacter citreus* and *Pseudomonas fluorescens* promoted significant increases in shoot height and dry weight in *Picea mariana* (Miller) Britton, Sterns and Poggenburg, and *Picea glauca* (Moench) Voss. Inoculation of *Pseudotsuga menziesii* (Mirbel) Franco seedlings with unidentified rhizobacterial isolates produced concomitant increases in plant height and diameter of up to 27 %. Finally, *Tsuga heterophylla* (Rafinesque) Sargent seedlings inoculated with *Bacillus polymyxa* achieved dry weight gains of over 30 % (Chanway 1997). Here, we will focus exclusively on the applications of these bacteria to induce/increase the root system, but clearly, much more work is still necessary in conifers/PGPR interaction.

In the EP0804081B1 patent, the property of the University of Tennessee Research Corporation, the inventors claimed the development of a method that promotes adventitious rooting of plants by co-culturing with non-pathogenic bacteria. The novel bacterium species was closely related to the subdivision of the *Proteobacteria* and more specifically to *Caulobacter subvibrioides.* The discovered bacterium was designated as root-stimulating bacteria (RSB). Several results were given of the efficiency of RSB treatments. Hypocotyl explants taken from 6-week-old slash pine (*Pinus elliottii*) seedlings produced at least one root in 93 % of the cultures containing living RSB, whereas none of the control explants produced roots during the 90-day experiment. The roots produced with RSB treatment were similar to normal seedling roots and were indistinguishable from roots produced spontaneously in control cultures. The RSB-stimulated roots in slash pine had single and branched primary roots. Some of the microplants developed secondary branching, which is necessary for the development of secondary fibrous root system. RSB was also tested on 12-week-old hypocotyl explants of eastern white pine (*Pinus strobus*). RSB treated plants displayed significant levels of rooting after 7 weeks in culture (42 %) compared with the controls, which showed no rooting. Also, hedgings (cuttings) taken from established stools of loblolly pine (*Pinus taeda*) dipped into living cultures of RSB and inserted into a peat:perlite (50:50, v:v) mixture, under mist in the greenhouse, produced 20 % rooted plants in one of the genotypes tested after 12 weeks compared with no roots in non-treated controls. These RSB were initially isolated as a contaminant from in vitro cultured slash pine (*Pinus elliottii* Engelm.) seedling explants because of its ability to stimulate a greater degree of rooting than were normally observed in the hypocotyl cuttings of slash pine seedlings (Burns and Schwarz 1996). Initial attempts to identify the bacterium failed as it would not grow on the media normally used in the diagnostic tests. Later, Sharma (2003) showed that this RSB most closely resembled bacteria of the *Sphingomonas* group and that the rooting ability was characteristic of some of the *Sphingomonas* and related bacteria.

Chanway and Holl (1993a, b) inoculated hybrid spruce (*Picea glauca* × *engelmannii*) with *Pseudomonas putida*. The hybrid spruce seeds and seedlings were collected from two sites in British Columbia, Canada; one site was near Mackenzie and the other near Salmon Arm. One strain of *Pseudomonas putida* was isolated from the rhizosphere from each site. These strains were deemed distinct from each other based on the analysis of bacterial fatty acids. Experiments were conducted to determine if *P. putida* affected spruce seedling emergence, and if the nature or magnitude of these effects were related to the geographic origin of the bacteria and seeds. Inoculation of spruce with *P. putida* that did not originate from the same site as the seed caused an increase in the number of seedlings that emerged, but this effect was not statistically significant. However, when the origin of the spruce seed was matched to that of the *P. putida* strain, a significant ($P < 0.05$) increase in the number and rate of seedling emergence was detected. These results are significant with respect to the regeneration strategy of spruce in natural forests. After 1 year following inoculation and in the field, the seedling

biomass or branch number increased up to 49 % confirming its root growth promoting influence on the Salmon Arm spruce ecotype. *Pseudomonas putida*, which originated from Salmon Arm spruce seedlings, increased seedling biomass or branch number in two field trials, but had an inhibitory effect in three other tests. In 1994, the same authors inoculated seeds and seedlings of two different Douglas-fir ecotypes in a controlled environment. They used the N74 strain of *Arthrobacter oxydans* and the K23 strain of *Pseudomonas aureofaciens* (isolated from naturally regenerating Douglas-fir seedlings collected from two different sites: Williams Lake and Chilliwack, respectively). *A. oxydans* strain N74 stimulated seedling branching and enhanced root and shoot dry weights of the Williams Lake ecotype but had no significant stimulatory effect on growth of the Chilliwack ecotype. On the other hand, *P. aureofaciens* (K23) significantly stimulated shoot branching and root dry weight of Chilliwack Douglas-fir but had no stimulatory effect on growth of that from Williams Lake (Chanway and Holl 1994).

Bent et al. (2001) studied the presence of other rhizobacteria in *Pinus contorta* and the related alterations in root hormone level and growth. Rhizospheric density and root hormone concentrations were determined after treatment with a single growth promoting rhizobacterium *Paenibacillus polymyxa* strains L6 and Pw-2 or in a combination of bacteria: *P. polymyxa* strain L6 + *Pseudomonas fluorescens* strain M20 or strain Pw-2 + *Pseudomonas fluorescens* M20 strain. There was no difference in the growth of pines inoculated with the L6 strain and those inoculated with the L6 + *Pseudomonas fluorescens* M20 strain. Results showed that seedlings inoculated with *P. polymyxa* Pw-2 strain had more lateral roots and a greater root mass after 12 weeks from inoculation than plants inoculated with *P. polymyxa* Pw-2 + *Pseudomonas fluorescens* M20. Also, the authors discovered that growth promotion mediated by *P. polymyxa* L6 and Pw-2 was not correlated with the average population density of each strain in the rhizosphere. However, the bacterial species-specific effects were observed in the root hormone levels: IAA concentration was elevated in roots inoculated with *P. polymyxa* L6 or Pw-2, while dihydrozeatin riboside concentration was elevated in roots inoculated with *P. fluorescens* M20.

Vonderwell et al. (2001) aimed at elucidating of the mechanism by which some bacterial strains increase root growth response in forest seedlings grown in nurseries. Seeds of loblolly pine (*Pinus taeda* L.) were inoculated at the sowing in either hand-mixed peat:vermiculite:perlite (2:1:1, v:v:v) potting substrate or in commercial Promix®, peat:vermiculite (1:1, v:v) with an equal amount of either *Bacillus subtilis* strains LS211 and INR7, or sterile distilled water used as a control. Seedling biomass, root length, root surface area, average root diameter, and root volume were measured at 6 and 12 weeks after sowing. Growth promotion was variable and dependent on the substrates and time elapsed since sowing. In a second experiment, root respiration rate and the total root IAA content were quantified at 6 and 12 weeks in seedlings grown in Promix® substrate. In this experiment, it was found that INR7 decreased the whole root system respiration by 22 % and increased root biomass and root length compared with control at the sixth week. The bacterial LS211 strain had no effect on root respiration. Furthermore, INR7 increased the total root IAA concentration by 1.7 timesover controls at the sixth week, whereas LS211 had no effect. Although the cause and effect could not be established, these studies suggested that root growth promotion was influenced by the growth medium and that IAA concentration and root respiration rates were two physiological mechanisms correlated with rhizobacterial activity and growth promotion.

Mycorrhization

In nature, innumerable plant species form symbiotic associations with higher fungi that live inside and around their roots. These beneficial associations or mycorrhizal symbiosis are basically defined by the host/partner combination and the morphology of the symbiotic structures. Depending on the way in which fungi interact with a host plant roots, particularly with respect to the nature of the interface (intracellular or extracellular) that forms between a host plant and a fungus, the mycorrhizas are subdivided into three categories: vesicular arbuscular mycorrhizas (VAM), ectomycorrhizas (ECM), and ericoid mycorrhizas (ERM) (for more detail, see Brundrett 2004).

Arbuscular mycorrhizas occur in herbaceous genera (approximately 80 % of all plant species), while ectomycorrhizas are most common in tree species (including species in *Betulaceae*, *Pinaceae*, *Fagaceae*, *Dipterocarpaceae*, *Leguminaceae,* and *Myrtaceae* families). Ericoid mycorrhizas are confined to the genera within the *Ericales*, including *Ericaceae* and *Vacciniodeae,* in the Northern Hemisphere and *Epacridaceae* in the Southern Hemisphere. Different mycorrhizal types differ not only in their host preference; in the structures, they form during association with the host root, but also in the ways by which they enhance host plant growth (Finlay 2008). Nowadays, ectomycorrhizae that appeared about 200 million years ago (Cairney 2000) represent less than 5 % of all mycorrhizal associations with vascular plants, but are omnipresent in the *Pinaceae* family, and are the dominant type in habitats where climate is strongly seasonal and soil nutrient availability is poor (Malloch et al. 1980).

Ectomycorrhizal symbiosis is structurally characterized by the presence of a dense mass of fungal hyphae forming

tissue and a pseudoparenchymatous tissue sheathing the root, called mantle. The external mantle is connected inside the root to the Hartig net (apoplastic hyphae) and with a network of extra radical hyphae proliferating into the soil (Graham and Miller 2005). Extramatrical hyphae, the mantle, and the intraradicular hyphae network are active metabolic entities that provide essential nutrients (nitrogen and phosphate) to the host plant and a stable carbohydrate-rich niche in the roots for the fungal partner, thus making the relationship a mutualistic symbiosis (Allen 1991; Read 1999; Martin et al. 2001; Taylor and Alexander 2005). Several studies have shown the benefits of using ECM fungi *Amanita*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*, *Rhizopogon*, *Scleroderma,* and *Suillus* in conifer micropropagation (Gay 1990; Wallander 2000; Niemi et al. 2000; Taylor et al. 2004; Niemi et al. 2004, 2005; Adriaensen et al. 2006; Ragonezi et al. 2012; Heinonsalo et al. 2015). ECM inoculations can enhance root formation and/or subsequent root branching of cuttings in vivo and in seedlings (Normand et al. 1996; Karabaghli et al. 1998; Niemi et al. 2000, 2002). Also, fungal inoculations can increase the plant's ability to overcome the stress related to nursery and growth after transplantation (Fini et al. 2011). Successful colonization of roots and further changes "to construct" an ectomycorrhizal root is mediated by biochemical signals (Seddas et al. 2009). These rhizospheric signals include auxins, flavonoids, alkaloids, cytokinins (Martin et al. 2001), and also plant phenolic compounds, such as *p*-coumaric acid, *o*-coumaric acid (Ragonezi et al. 2013), coumarin, naringenin, and other flavonoids (Lynn and Chang 1990; Felten et al. 2009; Mandal et al. 2010; Amalesh et al. 2011; Hassan and Mathesius 2012; Plett and Martin 2012).

Here, we explore the use of ECM to induce/overcome rooting problems of conifer cuttings and in vitro regenerated microshoots. David et al. (1983) induced rooting of *Pinus pinaster* from in vitro cloned shoots with auxin treatment (NAA 10^{-6}M for 18 days) and demonstrated that rooting ability persisted over five successive induction cycles within the 9-month period. However, the auxin-induced root initials did not elongate. When the shoots with initiated stunted roots were co-cultivated with *Pisolithus tinctorius* or *Hebeloma cylindrosporium*, roots resumed growth, and at the same time, short lateral root formation was stimulated. The fungi improved the quality of the root system, which was a pre-requisite for the successful transplantation of plants from the test tubes to the field.

The effect of the ectomycorrhizal fungus *Hebeloma hiemale* and of its culture filtrate on in vitro rooting of *Pinus halepensis* derooted shoot hypocotyls was studied by Gay (1990) in an attempt to determine if ectomycorrhizal fungi could enhance adventitious root formation in Gymnosperms. In the presence of 0.1-mM tryptophan (IAA precursor), *H. hiemale* strongly enhanced rooting of the hypocotyls in the absence of PGRs. The rooting of the inoculated hypocotyls was 96.6 %, whereas it was only 7.6 % in the absence of the fungus. *H. hiemale* did not produce IAA in the culture filtrate when tryptophan was not added to the medium and did not stimulate rooting of the hypocotyls. In contrast, a culture filtrate obtained in the presence of tryptophan contained IAA and the ethyl acetate extract from this filtrate stimulated 100 % rooting. Based on these results, the author concluded that IAA production was the cause of the rhizogenic activity of *H. hiemale*, which was able to metabolize the supplied tryptophan into IAA.

With the objective to increase root growth of conifer seedlings at outplanting, Scagel (1994) compared the effect of biologically produced IAA (or ethylene) from ECM symbiosis with the exogenous application of PGRs to the roots. The goal was to increase the endogenous IAA content to the level above the threshold required to generate new roots. The experiments showed a wide range of in vitro IAA and ethylene production capacity in the tested ECM. The hypothesis that ECM fungi would differentially affect the endogenous IAA content in roots in the symbiotic state and according to their different intrinsic capacity to produce either IAA or ethylene in vitro was not consistently validated.

Normand et al. (1996) inoculated microcuttings of *Pinus pinaster* and *P. sylvestris* clones derived from in vitro culture with wild-type or IAA-overproducing mutant strains of the ECM fungus *Hebeloma cylindrosporium.* The results demonstrated that two clones of *P. pinaster* and one clone of *P. sylvestris* did not root in the absence of the auxin, but they produced roots in the presence of each of the fungal strains tested. Both the wild type and the IAA overproducing mutant were effective in the promotion of rooting. When inoculated plantlets were transferred ex vitrum, they were surrounded by the hyphae network, which later formed well-defined mycorrhizal structures.

Ectomycorrhizal fungus *Laccaria bicolor* strain S238 N was used to inoculate Norway spruce (*Picea abies*) seedlings under the axenic conditions (Karabaghli-Degron et al. 1998). The question asked was whether IAA produced by *L. bicolor* S238 N strain could be responsible for the rhizogenic stimulation in *Picea abies.* The presence of the fungus slowed the tap-root elongation during the first 15 days of co-culture but afterward stimulated it by 136 %. In addition, S238 N strain enhanced in vitro lateral root formation by 4.3-fold. When the fungus was separated from the roots by a cellophane membrane, the compounds released by the fungus were also effective. One of the compounds was identified as IAA in a pure culture of the fungus. When 2,3,5-triiodobenzoic acid (TIBA), an auxin inhibitor, was added to the culture medium, the lateral root

formation of inoculated seedlings was significantly inhibited. While TIBA had no significant effect on IAA release by *L. bicolor* S238 N, it counteracted the stimulation of lateral rhizogenesis by an exogenous supply of IAA. These results suggested that TIBA inhibited the transport of fungal IAA in the root.

In another study, the ability of the ectomycorrhizal fungi *Pisolithus tinctorius* and *Paxillus involutus* that produce IAA and affect the formation and growth of roots in Scots pine (*Pinus sylvestris* L.) in vitro hypocotyl cuttings was elucidated by Niemi et al. (2002). Inoculations with either fungus were more effective in enhancing root formation than IBA treatment applied to the base of hypocotyls. Both fungi produced IAA in the absence of exogenous tryptophan, but the mycelium and culture filtrate of *P. tinctorius* contained higher concentrations of free and conjugated IAA than the mycelium and culture filtrate of *P. involutus*. Inoculation with either fungus or short-term application of culture filtrate of either fungus to the base of hypocotyl cuttings enhanced root formation. Fungal IAA production was not directly correlated with root formation, because inoculation with *P. involutus*, the weaker producer of IAA, promoted a better rooting response than inoculation with *P. tinctorius*, suggesting that, in addition to IAA, other fungal components play an essential role in root formation. One of these factors may be diamine putrescine, whose *P. involutus* strain was able to produce and release at high concentrations. Rooting of *Pinus pinea* L. microshoots derived from mature seed cotyledons was achieved after induction with a combination of auxin and hypertonic shock, but the root growth in vitro was not sustained. Some fungi isolated from ectomycorrhizas found in the *P. pinaea* stand were, therefore, co-cultured with the plantlets (Oliveira et al. 2003). About half of the fungal isolates tested helped the plants to resume root growth. These results demonstrated the need for further investigation of the effect of other fungi and fungal associations to stimulate rooting in conifers.

Ragonezi et al. (2012) determined that in vitro co-culture of *Pinus pinea* plantlets with *Pisolithus arhizus* helped to overcome the cessation of adventitious root growth and resulted in a root system that was better adapted to post-transplantation stress. None of the inoculated plantlets died in spite of using exclusively sterile vermiculite in the early phase of acclimatization during which a vast mycorrhizal symbiosis was established. Moreover, a fewer roots were lost during transplantation, which was facilitated by the morphological modifications of the inoculated roots, such as the presence of the hyphae around the roots and the internal Hartig net, which increased root thickness and contributed to a more robust root system.

Bacteria/mycorrhiza interaction

The bacterial flora associated with mycorrhizae and non-mycorrhizal roots was examined in seedlings of *Picea engelmannii*, *P. pungens*, *Pinus aristata*, *P. flexilis*, and *Pseudotsuga menziesii* by Oswald and Ferchau (1968). Later, Frey-Klett et al. (2007) explored the natural associations that occurred between mycorrhizal fungi and microbial communities, which regulated the mycorrhizal symbiosis. The authors distinguished between the helper bacteria, which assist mycorrhiza formation, and those that interact positively with the functioning of the symbiosis. Many studies also suggested that bacterial–fungal interactions play important roles during mycorrhiza formation and affect plant health (Schrey et al. 2012).

Rifampicin-resistant derivatives of plant growth promoting *Bacillus polymyxa* strains L6, Pw-2, and S20 were evaluated in the interaction of bacteria ± mycorrhizal co-inoculation on pine and spruce seedling growth (Shishido et al. 1996). The objective was to determine whether the mechanism by which bacteria stimulated seedling growth depended on the presence of ectomycorrhizas. Bacterial inoculation did not influence the mycorrhizal status of seedlings, but all three *Bacillus* strains stimulated growth of both conifer species. Root biomass, in particular, was significantly enhanced by up to 18 % compared with uninoculated controls. Mycorrhizal fungi improved the growth of spruce seedlings, but plant growth promotion by *B. polymyxa* was similar for mycorrhizal and non-mycorrhizal seedlings of both species. The results suggested that *B. polymyxa* strains L6-16R, Pw-2R, and S20-R enhanced conifer seedling growth through a mechanism unrelated to mycorrhizal fungi.

Karabaghli et al. (1998) used the ectomycorrhizal fungus *Laccaria bicolor* S238 N and the bacterium *Pseudomonas fluorescens* BBc6 separately and in combination to induce in vitro rooting of de-rooted shoot hypocotyls of *Picea abies*. *L. bicolor* increased the percentage of hypocotyls forming roots if tryptophan was added to the co-culture. The fungal inoculation also enhanced adventitious root elongation and branching as well as the aerial growth of the cuttings. *P. fluorescens* also enhanced the number of roots per rooted hypocotyl. Similar results were obtained by adding exogenous IAA to the rooting medium. *P. fluorescens* BBc6 had no effect on root elongation and branching. The production of IAA by pure cultures of *L. bicolor* S238 N and *P. fluorescens* BBc6 was stimulated in the presence of tryptophan; thus, the authors concluded that the effect of the fungus in stimulating adventitious root formation and subsequent elongation and branching can be attributed, at least partially, to the synthesis of IAA by the fungus.

The finding that *P. fluorescens* BBc6 had no effect on root elongation and branching, although it produced IAA, suggested that IAA was not the only factor involved in the stimulation of these processes or that the bacterium produced other compounds that counteracted the stimulatory effects of IAA on root elongation and branching. Indeed, Bent et al. (2001) demonstrated that roots of *Pinus contorta* inoculated with *P. fluorescens* M20 had an increase in dihydrozeatin riboside concentration.

Adventitious rooting of *Pinus sylvestris* L. hypocotyl cuttings was promoted by either the binucleate *Rhizoctonia* (BnR) and to a lesser extent by ectomycorrhizal fungi *Suillus bovinus* or *Laccaria bicolor* as reported by Kaparakis and Sen (2006). Four BnR isolates induced differentiation of root meristems and significantly induced adventitious rooting in young derooted seedlings. Rooting rates were significantly higher in BnR treatments than in pretreatments with IBA (200 μM) or with co-cultivation with ECM fungi. Mechanisms involved in root meristem differentiation, e.g., auxin production, wound response, and oligosaccharide signals, were also discussed with respect to the host-fungal signaling mechanisms. In a study with *P. sylvestris* L. seedlings, the BnR inoculations stimulated longer roots and reduced root width; however, the root infection was <6 % (Grönberg et al. 2006). At harvest, 240 days post inoculation, no significant plant and root growth differences were identified, although the number of short roots was significantly increased. BnR infection detected in roots was characterized by the presence of intercellular fungal hyphae and subtending intracellular monilioid fungal cells located in the outer cortical cells of long roots.

Conclusions

This review is the first attempt to compile most of the information about the use of microbial organisms to induce, resume, or increase the root systems of conifer cuttings and microshoots. The examples included in this review could help researchers, foresters, and nursery–growers in the selection of specific microorganisms or the combination of microorganisms with other physical and/or chemical factors to overcome the recalcitrance of conifers to produce an adequate adventitious root system.

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