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RESEARCH ARTICLE

# Evaluation of Ectomycorrhizal Fungi as Potential Bio-control Agents against Selected Plant Pathogenic Fungi

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## Abstract

Ectomycorrhizal (ECM) fungal associations are prevalent among forest tree species particularly in the families of Betulaceae, Fagaceae, Myrtaceae, Pinaceae, Salicaceae etc. belonging to higher Basidiomycetes. Antagonistic potential of eight ECM fungal isolates viz., *Alnicola* sp., *Laccaria fraterna*, *Lycoperdon perlatum, Pisolithus albus, Russula parazurea Scleroderma citrinum*, *Suillus brevipes* and *Suillus subluteus* were tested against selected plant pathogenic fungi like *Alternaria solani*, *Botrytis* sp., *Fusarium oxysporum*, *Lasiodiplodia theobromae, Phytophthora* sp., *Pythium* sp., *Rhizoctonia solani*, *Sclerotium rolfsii and Subramaniospora vesiculosa* (= *Trichosporium vesiculosum*) under *in vitro*. Production of antifungal enzyme chitinase in chitin agar medium and Indole Acetic Acid (IAA) in tryptophan containing medium by these ECM fungi was also determined and quantitatively estimated. All the eight ECM fungal isolates showed inhibitory effect towards selected plant pathogens. Among different ECM fungi tested, *Suillus brevipes* showed the maximum percentage of inhibition (60.31%) followed by *S. subluteus* (49.46%) against the plant pathogens whereas *P. albus* exhibited least inhibition (33.27%). The growth of *Phytophthora* sp. was maximally inhibited (52.1%) followed by *S. vesiculosa* (49.82%) and *S. rolfsii*. was least inhibited (36.4%). These results were further established when *S. brevipes* produced the maximum chitinase (111.6 µg/mL) followed by *S. subluteus* (101.7 µg/mL) with the least production by *P. albus* (32.38 µg/mL). Contrary to this, IAA production was observed highest in *L. fraterna* (28.18 µg/mL) followed by *P. albus* (25.32 µg/mL), while *S. brevipes* produced only 16.75 µg/mL of IAA and the IAA production was lowest in *Russula* sp. (2.46 µg/mL).

**Keywords:** Antagonism, antifungal enzymes, chitinase, ECM fungi, indole acetic acid, plant pathogens.

## Introduction

Man's attempts at controlling plant disease go back at least to 700 B.C. At present, chemical fungicides and pesticides are applied in order to bring control over infections, but these usages of chemicals have not only killed the targeted ones but also our useful, friendly microbes, thus destroying the soil fertility and environment. The recent development in organic farming, has bought with it, the usage of bio-fertilizers and bio-pesticides. Feeder root fungal pathogens such as species of *Fusarium, Phytophthora, Pythium*, *Rhizoctonia* etc. infect immature and meristematic cortical tissues of roots and cause necrosis. Ever since the discovery of mycorrhizae, they have been utilized in many fields like forestry as a bio-fertilizer for higher plants, in nutrient absorption and phosphorous solubilisation, antagonistic activity and for its plant growth hormone secretion. Ectomycorrhizal symbiosis is a mutually beneficial union between fungi and the roots of vascular and non-vascular plants. The host of an ectomycorrhizal fungus is usually a gymnosperm (pine). In general, the fungi involved in ectomycorrhizae (ECM) come under basidiomycetes from the families, Amanitaceae, Boletaceae, Cortinariaceae, Russulaceae, Tricholomataceae, Rhizopogonaceae and Sclerodermataceae.

Plants don't do well in the absence of a mycorrhiza, even when they are growing in apparently fertile soil. In tropical forests, for example, almost all of the tree species depend on mycorrhizae to supply them with nutrients from the typically infertile soils on which these ecosystems develop. Moreover, the mycorrhizal fungi are critical to retaining nutrients within the forest biomass, and in preventing these chemicals from being washed away by the abundant tropical rains. The ECM habit increases the surface area of the root system and hence affords better intake of nutrients such as nitrogen, phosphorus and potassium from the surrounding soil (Subba Rao, 2007). Research has also proved that many of the mycorrhizal fungi are good producers of plant growth hormones especially auxins (indole-3-acetic acid) during their association with plants and under *in vitro* and they greatly influence the growth and development of these plants by changing the physiological and biochemical processes going on in the roots (Slankis, 1973). Plants with mycorrhizal association will have good shoot and root development compared to the non-inoculated (control) (Wallander *et al.,* 1992). ECM fungi also help in bringing resistance to plants from several diseases. The phenomenon called antagonism is most prevalent among most of the mycorrhizal fungi.

In spite of two partners being involved in the association, only one species is benefited at the cost of the other and hence one species is harmed. Well formed mycorrhizal roots are resistant to infection and non-mycorrhizal feeder roots are prone to fungal necrosis even when adjacent roots have become mycorrhizal (Natarajan and Govindasamy, 1990). ECM fungi are also known to possess chitinolytic activities. Chitinases are digestive enzymes that break down glycosidic bonds in chitin (Hodge *et al.,* 1995). Reports on the antagonistic effect of ectomycorrhizal fungi against plant pathogens are limited. Therefore, a study was conducted to determine antagonistic effect of different ECM fungal isolates from forest trees against selected plant pathogenic fungi infecting forest trees. Production of chitinase and plant growth hormone Indole Acetic Acid (IAA) by ECM fungi was also studied.

## Materials and methods

*Preparation of fungal inocula:* Eight different ECM fungi and nine different plant pathogenic fungal cultures were obtained from the germplasm bank at Forest Pathology lab, Forest Protection Division, IFGTB, Coimbatore, Tamil Nadu, India (Table 1). All the ECM fungal cultures were maintained as pure cultures in Modified Melin Norkan's (MMN) agar medium (Marx, 1969). Potato Dextrose Agar (PDA) medium (Hi media) was used to maintain plant pathogenic fungal isolates (Cappuccino and Sherman, 2002).

Table 1. Ectomycorrhizal and plant pathogenic fungi used in the study.

| S. No. | Ectomycorrhizal fungi | Plant pathogenic fungi |
|---|---|---|
| 1. | *Alnicola* sp. | *Alternaria solani* |
| 2. | *Laccaria fraterna* | *Botrytis* sp. |
| 3. | *Lycoperdon perlatum* | *Lasiodiplodia theobromae* |
| 4. | *Pisolithus albus* | *Fusarium oxysporum* |
| 5. | *Russula parazurea* | *Phytophthora* sp. |
| 6. | *Scleroderma citrinum* | *Pythium* sp. |
| 7. | *Suillus brevipes* | *Rhizoctonia solani* |
| 8. | *Suillus subluteus* | *Sclerotium rolfsii.* |
| 9. | | *Subramaniospora vesiculosa (=Trichosporium vesiculosum)* |

*Antagonistic potential of ECM Fungi:* ECM and plant pathogenic fungal cultures were grown in separate sterile petri plates containing PDA (Hi media) medium for 5-7 days and they served as inoculum source for the experiment. The antagonistic activity of ECM fungi was studied using dual culture technique (Natarajan and Govindasamy, 1990). Mycelial discs (5 mm diameter) of ECM and plant pathogenic fungi were cut using sterile cork borers and placed at two opposite ends of fresh sterile PDA plates. The plates were incubated at 28±2°C for 5-7 days and three replicates were maintained. Separate control plates were also maintained for both ECM and plant pathogenic fungi to verify the percentage of inhibition. The ability of the organism either to inhibit or colonize the growth of the pathogen was taken as hyper parasitism.

The radial growth of the pathogen was measured periodically and potent inhibition was calculated using the following formula:

$$\text{Inhibition \%} = \frac{C - T}{C} \times 100$$

C = radial growth of the pathogen in the control plate.
T = radial growth of the pathogen in the treated plate.

The data on % of inhibition was collected and statistically analyzed by ANOVA using SPSS 8.0 software and the means were separated by Duncan's Multiple Range Test (DMRT).

*Chitinase activity of ECM fungi:* Colloidal chitin was prepared from purified chitin (Hi media) according to the method of Roberts and Selitrennikoff (1988). Individual sterile medium plates containing sterile chitin agar amended with 0.5% colloidal chitin (Hodge *et al.,* 1995) were inoculated with actively growing mycelium of different ECM fungal isolates. The plates were incubated at 27±1°C for 5 days. Clearance of medium from translucent nature indicates extracellular chitinase activity of test organism. Chitinase activity was estimated according to the method of Miller (1959). Sterile chitin broth medium amended with 0.5% colloidal chitin was prepared and was inoculated with two mycelial discs (5 mm) of ECM fungal isolates and kept for incubation at 27±1°C for 5-10 days. Triplicates were maintained for all the ECM fungal isolates. A control flask was also maintained. On 10$^{th}$ day, the culture filtrate was obtained by filtration and centrifuged at 3000 rpm for 20 min (Remi make) and the supernatant was used as the enzyme source. Chitinase activity was measured with colloidal chitin as a substrate. The amount of reducing sugar produced was measured by the Dinitrosalicilic acid (DNSA) method with N-acetylglucosamine (GlcNAc) (Hi media) as a reference compound. A unit of enzyme activity was defined as the amount of enzyme that catalyses the release of 1.0 µmol of GlcNAc per min under the specified assay conditions.

*Estimation of indole acetic acid production by ECM Fungi (Rudawska et al., 1992):* Ectomycorrhizal fungi were inoculated in 50 mL of sterilized Potato Dextrose broth (Hi media) with 0.5% L-Tryptophan in 250 mL conical flask and a control was also maintained. These flasks were inoculated at 27±1°C for a period of 12 days preferably in a dark chamber (IAA is highly light sensitive). After incubation, the cell free culture filtrate was obtained by filtration. The culture filtrate was taken and the pH was adjusted to 2.5 using 1% HCl. The acidic culture filtrate was transferred to a separating funnel and to this equal volume of ice cold diethyl ether was added and shaken vigorously. For complete extraction, it was kept at 4°C in a separating funnel for a period of 4-6 h with intermediate shaking. Of the two layers in the separating funnel, the upper organic layer is saved and collected in a beaker.

Table 2. Antagonistic potential of ECM fungi.

| S.No. | ECM fungi | Plant pathogenic fungi* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
| | | % of inhibition | | | | | | | | |
| 1. | *Alnicola* sp. | 35.71 e | 44.44 c | 37.50 d | 33.33 f | 52.00 b | 31.11 g | 66.67 a | 22.22 h | 44.44 c |
| 2. | *Laccaria fraterna* | 40.32 c | 33.33 e | 32.84 f | 33.33 e | 50.00 a | 27.78 g | 33.33 e | 47.78 b | 38.89 d |
| 3. | *Lycoperdon perlatum* | 47.37 a | 36.67 h | 46.34 c | 41.11 f | 42.86 e | 46.67 b | 38.89 g | 44.44 d | 33.33 i |
| 4. | *Pisolithus albus* | 47.78 a | 33.33 d | 29.27 f | 12.22 h | 38.89 c[#] | 38.89 c | 30.56 e | 27.78 g | 40.79 b |
| 5. | *Russula parazurea* | 38.00 f | 37.78 g | 43.28 d | 50.00 b | 44.00 c | 38.89 e | 37.78 g | 33.33 h | 52.22 a |
| 6. | *Scleroderma citrinum* | 39.55 d | 33.33 g | 32.22 h | 35.72 e | 50.00 b | 52.38 a | 35.33 f | 39.86 c | 27.78 i |
| 7. | *Suillus brevipes* | 41.67 i | 52.22 e | 76.67 c | 42.22 h | 86.67 a | 64.44 d | 51.11 f | 44.44 g | 83.33 b |
| 8. | *Suillus subluteus* | 54.84 d | 33.33 g | 58.21 b | 56.67 c | 52.00 f | 52.22 e | 28.89 i | 31.25 h | 77.78 a |

*1. *Alternaria solani,* 2-*Botrytis* sp. 3-*Fusarium oxysporum,* 4-*Lasiodiplodia theobromae,* 5-*Phytophthora* sp., 6-*Pythium* sp., 7-*Rhizoctonia solani,* 8-*Sclerotium rolfsii,* 9-*Subramaniospora vesiculosa* (= *Trichosporium vesiculosum*), # Means, in a row, followed by common letter (s) is not significantly different at 0.05 level according to DMRT.

The ether phase was evaporated to near dryness by simple evaporation technique in a dark place. Using 2 mL of isopropanol, the residue was collected carefully and preserved in a penicillin vial. The final residue contained the IAA.

*Quantitative estimation of IAA in the final residue (Gorden and Paleg, 1957):* A quantity of 0.5 mL of the final residue was transferred to a clean test tube, 3.5 mL of Salpers Reagent (50 mL of 35% Perchloric acid and 1 mL of 1 M Ferric chloride) was added. The mixture was thoroughly agitated and set aside for 30 min at 4°C in dark. Development of pink color indicated the presence of IAA. The intensity of the pink color was read in UV-Vis Spectrophotometer (Shimadzu Make) at 535 nm and concentration of IAA was estimated using standard curve.

## Results

*Antagonistic effect of ECM fungi against selected plant pathogens:* Data on % inhibition of ECM fungi against plant pathogenic fungi is presented in Table 2. It was observed that the ECM fungus *Alnicola* sp. showed greater inhibitory effect against the plant pathogenic fungus *R. solani* (66.67%). The ECM fungus *L. fraterna* had greater inhibitory effect against the plant pathogen *Phytophthora* sp. (50.00%) and less inhibition against *Pythium* sp. (27.78%). The ECM fungus *L. perlatum* revealed maximum inhibitory action against the plant pathogens like *A. solani* (47.37%), *Pythium* sp. (46.67%) and *F. oxysporum* (46.34%). The same ECM fungus had least inhibition against the pathogen *S. vesiculosum* (33.33%). Another ECM fungus *P. albus* showed greater inhibitory action against the plant pathogen *A. solani* (47.78%) and the least inhibitory action against *L. theobromae* (12.22%). The antagonistic effect of the ECM fungus *R. parazurea* exhibited higher antagonistic potential against *S. vesiculosum* (52.22%) and least inhibition was recorded against *Sclerotium* sp. (33.33%). The ECM fungus *S. citrinum* showed maximum inhibitory action against the plant pathogens *Pythium* sp. (52.38%) and *Phytophthora* sp. (50.00%). The same fungus had least inhibitory action against *S. vesiculosum* (27.78%).

Table 3. Mean percentage of inhibition by ECM fungi.

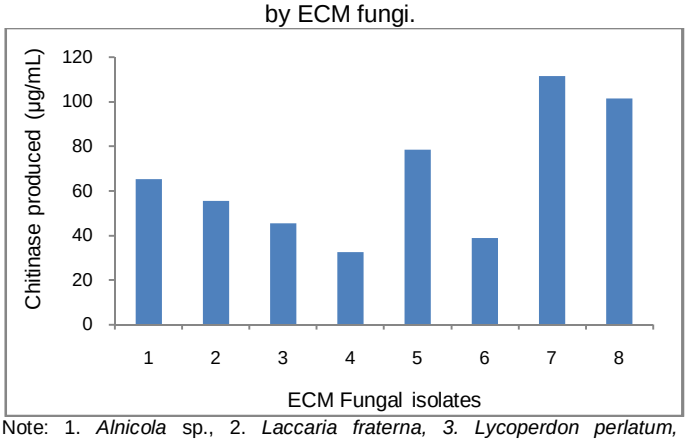
| S. No. | Ectomycorrhizal fungi | Mean of inhibition (%) |
|---|---|---|
| 1. | *Alnicola* sp. | 40.82 |
| 2. | *Laccaria fraterna* | 33.51 |
| 3. | *Lycoperdon perlatum* | 40.85 |
| 4. | *Pisolithus albus* | 33.27 |
| 5. | *Russula parazurea* | 41.75 |
| 6. | *Scleroderma citrinum* | 38.46 |
| 7. | *Suillus brevipes* | 60.31 |
| 8. | *Suillus subluteus* | 49.46 |

Table 4.Mean percentage of inhibition of plant pathogenic fungi.

| S.No. | Plant pathogenic fungi | Mean of inhibition (%) |
|---|---|---|
| 1. | *Alternaria solani* | 38.62 |
| 2. | *Botrytis* sp. | 38.10 |
| 3. | *Lasiodiplodia theobromae* | 38.10 |
| 4. | *Fusarium oxysporum* | 44.50 |
| 5. | *Phytophthora* sp. | 52.10 |
| 6. | *Pythium* sp. | 44.05 |
| 7. | *Rhizoctonia solani* | 40.32 |
| 8. | *Sclerotium rolfsii* | 36.40 |
| 9. | *Subramaniospora vesiculosa* (=*Trichosporium vesiculosum*) | 49.82 |

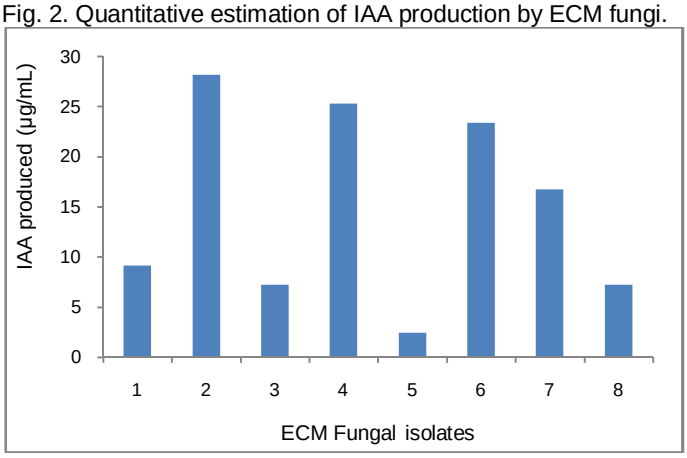
The antagonistic effect of the ECM fungus *S. brevipes* had more inhibition against plant pathogens such as *Phytophthora* sp. (86.67%), *S. vesiculosum* (83.33%), *Fusarium oxysporum* (76.77%) and *Pythium* sp. (64.44%). Minimum inhibitory action of the same ECM fungus was seen against the plant pathogen *A. solani* (41.67%). The antagonistic activity of another ECM fungus *S. subluteus* revealed maximum inhibitory action towards plant pathogenic fungi such as *S. vesiculosum* (77.78%), *F. oxysporum* (58.21%) and *L. theobromae* (56.67%) and the least inhibition was shown against the plant pathogen *R. solani* (28.89%). Among all the eight ECM fungi screened, *S. brevipes* had the maximum mean percentage of inhibition (60.31%) followed by *S. subluteus* (49.46%) and the ECM fungus *P. albus* exhibited least mean percentage of inhibition (33.27%) (Table 3). Among all the plant pathogenic fungi used for the study, the growth of *Phytophthora* sp. was highly inhibited (52.1%) followed by *S. vesiculosum* (49.82%) and the pathogen *S. rolfsii* was least inhibited (36.4%) (Table 4).

Fig. 1. Quantitative estimation of chitinase production by ECM fungi.




Note: 1. *Alnicola* sp., 2. *Laccaria fraterna, 3. Lycoperdon perlatum, 4. Pisolithus albus, 5. Russula parazurea, 6. Scleroderma citrinum, 7.Suillus brevipes, 8.Suillus subluteus.*

Fig. 2. Quantitative estimation of IAA production by ECM fungi.




*Determination of chitinase activity of different ECM fungi:* An experiment was conducted to determine chitinase activity of different ECM fungi and it was observed that the maximum producer of chitinase by the ECM fungus *S. brevipes* (111.6 µg/mL), followed by *S. subluteus* (101.7 µg/mL). Chitinase production by ECM fungi *S. citrinum* (38.99 µg/mL) and *P. albus* (32.38 µg/mL) was the lowest (Fig. 1).

*Estimation of plant growth hormone production ability of ECM fungi:* Another experiment was conducted to estimate IAA production by different ECM fungi under *in vitro* condition. It was observed that the highest producer of plant growth hormone, IAA by the ECM fungi was *L. fraterna* (28.18 µg/mL), followed by *P. albus* (25.32 µg/mL) and *S. citrinum* (23.40 µg/mL), while *S. brevipes* produced only 16.75 µg/mL of IAA. The IAA production was lowest by *R. parazurea* (2.46 µg/mL) (Fig. 2).

## Discussion

The results of the study indicated that all the eight different ECM fungi had antagonistic efficacy against the selected plant pathogenic fungi, but variation was observed in the percentage of inhibition.

It was also recorded that the antagonistic effect was found maximum in the ECM fungus *S. brevipes* (60.31%), followed by *S. subuteus* (49.46%). The least inhibition effect was seen in ECM fungus, *P. albus* (33.27%). Among all the plant pathogenic fungi used in the study, the growth of *Phytophthora* sp. (52.1%) was highly inhibited by all the eight different ECM fungi and pathogens, *S. vesiculosum* (49.82%) and *S. rolfsii* (36.4%) were the least inhibited. This study is in accordance with the findings made by Marx (1969) who carried out antagonistic potential of ECM fungi against the plant pathogen *Phytophthora cinnamoni*. In another study, Marx (1972) had demonstrated that the mantle of ECM fungi of Pine roots not only formed antibiotics and physical barrier but as well increased production by host of volatile and non-volatile compounds which inhibited root pathogens. Such compounds decreased disease incident effectively by increasing the longevity of roots. Roots of Pines colonized by *Pisolithus tinctorius*, *Laccaria laccata*, *Leucopaxillus cerealis* and *Suillus luteus* are known to offer protection against *Phytophthora cinnamoni*. Suh *et al*. (1991) observed that *Pisolithus tinctorius* strongly inhibited growth of *Fusarium solani, Geotrichum candidum, Phanerochaete chrysosporium* and *Verticillium* sp. They also found that the antifungal activity was associated with the production of darkly colored, water soluble phenolic metabolites. Shrestha *et al*. (2005) recorded the antifungal activity and antibacterial action of ECM fungi under *in vitro* condition. They found that the ECM fungi such as species of *Pisolithus* and *Scleroderma* had higher activity against the plant pathogens such as *Pythium* sp., *Rhizoctonia solani*, *Fusarium* sp., *Agrobacterium tumifaciens*, *Pseudomonas solanaserum*, *Klebsiella* sp., *Staphylococcus aureus*, *Shigella dysentriae* and *E. coli.* Maximum production of chitinase enzyme was observed in ECM fungus *S. brevipes* (111.6 µg/mL), followed by *S. subluteus* (101.7 µg/mL). Minimum production of chitinase enzyme was recorded in the ECM fungus *P. albus* (32.38 µg/mL). These results further prove the findings of the antagonistic experiment. *S. brevipes* exhibited maximum antagonism and mechanism of antagonism is by way of maximum chitinase production as observed. This is in accordance with the works carried out by other researchers. Harman (2000) observed that β-1, 3-glucan and chitin are the major components of fungal cell walls of species from subdivisions *Ascomycotina, Basidiomycotina* and *Deuteromycotina.* Extracellular chitinases and β-1,3-glucanases are considered to be the key enzymes in the lysis of this structure. The production of chitinases and β-1,3-glucanases enzymes involved in mycoparasitism by several strains of ectomycorrhizal fungi: *Amanita muscaria* (16-3), *Laccaria laccata* (9-12), *L. laccata* (9-1), *Suillus bovines* (15-4), *S. luteus* (14-7) were assayed spectrophotometrically by adopting Miller's method on different substrates such as colloidal chitin, mycelia of *Trichoderma harzianum, T. virens* and *Mucor hiemalis* was examined by Joanna *et al.* (2005).

Catherine *et al.* (1993) observed that root chitinase activities was induced by the ectomycorrhizal basidiomycete *Pisolithus tinctorius* towards the pathogenic fungus, *Phytophthora cinnamomi,* when 7-day-old seedlings were challenged with the ectomycorrhizal fungus. Root chitinase activity was stimulated already after 6 hours, during the very early stages of ectomycorrhizal colonization. Growth hormone (IAA) production was seen higher in the ECM fungus *L. fraterna* (28.18 µg/mL), followed by *P. albus* (25.32 µg/mL). The IAA production was found least in the ECM fungus *R. parazurea* (2.46 µg/mL). Slankis (1973) observed that the ECM fungi also produce auxins (Plant growth hormones) which may enhance plant growth. The fungal symbiont also supplies the host with growth substances such as auxin, IAA and probably cytokinins and gibberellins. Wallander *et al.* (1992) reported that IAA concentration was higher in the Scots pine roots under high-nitrogen nutrition. Studies by Ek *et al*. (1983) revealed that *Pisolithus tinctorius* 185, a strain previously shown by other workers to give a strong root infection in field experiments, produced the largest amount of IAA. Karabaghli *et al*. (1995) studied that when the Norway spruce (*Picea abies* (L.) Karst.) seedlings were inoculated with the ectomycorrhizal fungus *Laccaria bicolor* ((Marie) Orton), strain S238 N, in auxenic conditions. The presence of the fungus slowed tap-root elongation by 26% during the first 15 days after inoculation and then stimulated it by 13.6%. In addition, it multiplied *in vitro* lateral root formation by 4.3%, the epicotyl growth of the seedlings by 8.4 and the number of needles by 2%. The hypothesis that IAA produced by *L. bicolor* S238 N would be responsible for the stimulation of fungal induced rhizogenesis was tested. It was showed that *L. bicolor* S238 N can synthesize IAA in pure culture. Exogenous IAA supplies (100 and 500 µM) reproduced the stimulating effect of the fungus on root branching.

## Conclusion

The studies on the antagonistic potential of ECM fungi against the selected plant pathogens revealed that the ECM fungi can also be used as a bio-fungicide in tree nurseries and plantations. Further investigations could be done *in vitro* through direct inoculation of ECM fungi. ECM fungi can also be exploited industrially for chitinase production, since they are non-sporulating and some of them are fast growers and higher producers. Further genetic modifications can also be done to improve their yield and growth, thus they may act as one of the best producers of the antifungal enzyme chitinase. The Indole Acetic Acid (IAA) production by ECM fungi further paves the way for further studies of ECM fungi as a biofertilizer and growth promoter for the application in tree nurseries and plantations for quality seedling production.

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