Mass Multiplication of Ectomycorrhizal *Cantharellus* Inoculum for Large Scale Tailoring Nursery Inoculations of Bamboo Seedlings

R. Sharma, R.C. Rajak and A.K. Pandey
Mycological Research Laboratory, Department of Biological Sciences, R.D. University, Jabalpur-482 001, Madhya Pradesh, India

*Corresponding Author: Rohit Sharma, Microbial Culture Collection, Affiliated to National Centre for Cell Science, University of Pune, Ganeshkhind, Pune-411 007 Maharashtra, India Tel: +912026270840*

**ABSTRACT**

The edible ectomycorrhizal Basidiomycetes are difficult to inoculate in field for reforestation of trees and mushroom production due to insufficient mycelial colonization of substrate. Edible mushroom in the genera *Cantharellus* was tested for mycorrhization with *Dendrocalamus* using house waste tealeaves+sand based inoculum under laboratory and green house conditions using polythene bags and plastic boxes. Mycorrhizal seedlings were acclimatized in open pot soil. Dense *Cantharellus* mycelia colonized the substrate in 2-3 weeks. Inoculum survived for atleast six months and retained its viability. Occasionally few bacterial contaminants were observed, which were discarded. The cost effective method developed in present investigation can be used for tailoring large-scale seedling/nursery plantlets and sustainable reforestation of various tree species.

**Key words:** Inoculum, tealeaves, *Dendrocalamus*, regeneration, formulation

**INTRODUCTION**

Ectomycorrhizal (ECM) host trees must be accompanied with their mycorrhizal fungi to survive when planted in forest areas or disturbed sites. Seedlings inoculated in nursery can establish a healthy ectomycorrhiza system before out planting. Inoculation of *Pisolithus tinctorius* significantly increased growth and survival of five Southern pine species planted in various sites (Marx *et al.*, 1982). Commercially produced vegetative inocula of many ectomycorrhizal mushrooms are available in the market. Moreover, these can be stored at low temperature without damage (Lapeyrie and Bruchet, 2006). Several types of natural or laboratory-produced inocula (seedlings with ectomycorrhiza or excised ectomycorrhiza, spores, spherophores) and various methods of application have proved successful through the years (Molina and Trappe, 1982). Most widely used inoculum is soil or humus collected from established plantations. However, transportation of soil inocula is difficult and contains harmful microorganism or noxious weeds. A truffle-producing fungus *Tuber melanosporum* has been established in nursery beds with ECM formed under laboratory conditions. Various authors have demonstrated the use of basidiospores of *Rhizopogon, Scleroderma* and *Pisolithus* as inoculum. Basidiospore inoculum of *Pisolithus tinctorius* and *Rhizopogon* successfully forms ectomycorrhiza on pine seedlings (Molina and Trappe, 1982; Bruns *et al.*, 2008).

Pure culture inoculum of ectomycorrhizal fungi poses many difficulties for wide-scale application restricting to laboratory or green house experiments. However, nursery beds of *Pinus cembra* have been successfully inoculated with pure cultures of *Suillus piorans*. Danell (1994) used...
peat/quartz sand mixture (25/75% v/v) and 20 mL Ingested solution for out planting in vitro formed Cantharellus mycorrhizaes. Generally, medium used to grow ectomycorrhizal fungi is peat moss-vermiculite substrate moistened with modified Melin-Norkrans nutrient solution. Moreover, with four decades of mycorrhizal research, peat moss and vermiculite remains the major substrate for inoculum preparation (Nezzar-Hocine et al., 1998; Yamada et al., 2001; Rincóna et al., 2005; Lapeyrie and Bruchet, 2006). Also, dispersal of ECM fungal inoculum was tested to characterize its distribution and abundance in soil (Thiet and Boerner, 2007). Different isolates of Pisolithus as ectomycorrhizal inoculum have been tried for growth of Acacia (Aggangan et al., 2010). Recently Zhang et al. (2010) have showed that ECM fungal inoculation of Boletus luridus significantly increased the ectomycorrhizal colonization compared with non-inoculated seedlings of Pinus tabuliformis.

Cantharellus tropicalis is an ectomycorrhizal fungus following its observed association with Dendrocalamus strictus and artificial synthesis achieved in laboratory conditions (Sharma, 2008). Synthesis of Cantharellus ectomycorrhiza with Dendrocalamus has been achieved with in vitro germinated seedlings in laboratory (Sharma et al., 2009) and green house. Moreover, mycelia of Cantharellus forms mycorrhiza with laboratory grown seedlings and have positive effect on the growth of Dendrocalamus seedlings (Sharma et al., 2008). Mass production of Cantharellus tropicalis Rahi, Rajak and Pandey inoculum is prerequisite for field application of Dendrocalamus strictus Nees. In present study, attempt has been made to formulate ECM inoculum using cheaper substrate that is easy to use with low production costs and can be made commercially available for re-plantation of tailored bamboo plantlets.

MATERIALS AND METHODS

Fungal isolate: The isolates of Cantharellus tropicalis were obtained in September, 2004 from basidiomata collected near Dendrocalamus strictus. Tissue blocks from the stipe of basidiomata were aseptically excised and cultured on modified Melin-Norkrans Agar Medium (MNM) in petridishes (Straatsma and van Griensven, 1986). The modified MNM medium contained Malt Extract-3.0 g; D-Glucose-2.5 g; KH₂PO₄-0.5 g; MgSO₄.7H₂O-0.15 g; CaCl₂.H₂O 0.05 g; NaCl-0.025 g; (NH₄)HPO₄-0.25 g; FeCl₃-1.2 mL (1% solution); Thiamine HCl-0.1 g; Distilled Water-1000 mL and pH adjusted to 5.8.

Seed preparation: Clean seeds of D. strictus were surface sterilized by rinsing with tap water, shaken in a sealed bottle containing 1% Tween 80 solution for 15 min and subsequently with 4% sodium hypochlorite solution. Thereafter, seeds were imbibed for 30 min in 4-5 changes of sterile distilled water and dried on sterile filter paper. Seeds were then planted aseptically on moist chamber Petri dishes and incubated in the dark at 26±2°C for germination. Seedlings were then transplanted into growth/synthesis system.

Substrate preparation: In this method conducted at Mycological Research Laboratory, Department of Biological Sciences during 2004-2007 as part of ectomycorrhizal studies of central India, substrate was prepared by collecting used waste tea leaves, washed thoroughly with water, and partially air-dried. Good quality sand was collected, washed several times, air-dried and filled in 7×14” and 10×14” autoclavable polythene bags/box. The mixture was sterilized (3 times) at 121±2°C for 15 min. A ratio of 1:1 used tea leaves and sand moistened with a volume of sterile distilled water equal to approximately half the volume of dry substrate proved best. The dimension of container bag allowed sand + used waste tea leaves to stay moist.
**Inoculation:** Two 9 mm fungal plugs of *C. tropicalis* isolates were aseptically placed on substrate surface touching wall. After 3-4 days of incubation at room temperature, the fungal mycelium begun to colonize substrate. At this stage sterilized seedlings were introduced aseptically into it. With the help of forceps seedlings were inserted into hole leaving the seedling near fungal inoculum. The substrate was gently replaced around the root system. After setting the system, they were placed in a growth chamber (Temp. 28±2°C, RH 65-70%, 12:12 h light and dark) and incubated for one month in BOD incubator.

**Colonization:** Fungal colonization of bamboo roots were observed under a binocular stereomicroscope. Established mycorrhiza was sectioned to confirm the presence of intercellular Hartig Net, which constitutes evidence for mycorrhiza.

**RESULTS AND DISCUSSION**

Majority of study on ectomycorrhizal inoculation is done in nurseries for production of container-grown tree seedlings. However, large-scale nursery applications of pure mycelial cultures involving few million seedlings have been severely hampered. It is simple to produce sufficient inoculum in small scale, but difficult for large-scale nursery inoculation in practical forestry program. Because the seedlings are raised for forestation purposes, critical point is effectiveness of inoculum in seedlings survival. *In vitro* culture of inoculum is an immediate and simple method but to keep culture free from bacterial and fungal contaminant is not easy.

In the present investigation sand +used tealeaves was found to be a satisfactory inert medium and carrier for the growth of *Cantharellus tropicalis*. The mycelia grew very fast through the substrate and colonized within 2-3 weeks (1000 g of substrate) producing good fungal inoculum (Fig. 1). Earlier, used tealeaves have been successfully used by Sharma *et al.* (2003) for pure culturing of macro fungi viz., *Pleurotus* species in sterile tubes. The substrate used in the present study allows penetration of mycelia, retains moisture and is low cost. Moreover, it allows smooth ectomycorrhiza formation between *Cantharellus* mycelia and *Dendrocalamus* seedlings (Fig. 2). During the present study, transparent polythene as well as plastic box/polythene was used, which helped in the observation of ectomycorrhiza formation without destruction. In the past, researchers have used several substrates for ectomycorrhization of nursery seedlings. Moser (1958) used pure culture to produce vegetative inoculum of *Suillus placidus* (Bon.) Sing., *S. grevillei* (Klotzsch) Sing., *S. aeruginascens* (Serr.) Snell., *Paxillus involutus*, *Phlegmacium glaucopus*, *Amanita muscaria* and *Lactarius porcinus* Rolland. Takacs (1961) used mycelium to inoculate sterilized

![Fig. 1: Cantharellus inoculum produced on sterilized waste tealeaves+sand](image)
germinated grains of cereals, cereal chaff or peat moss to produce inoculum of Amanita verna (Bull. ex Fr.) Lamarck, Suillus granulatus, S. lutea, Hebeloma crustuliniforme, Russula sp., Scleroderma verrucosum (Bull.) Pers. and S. vulgare. Parke et al. (1984) also grew mycelial cultures of Suillus granulatus and Cenococcum geophilum in cereal grains. Mycelium of fungi fails to grow on substrate like peanut, corn, bark as they release growth inhibitors during autoclaving. Vermiculite and peat moss moistened with modified Melin-Norkrans Medium (MMN) was found to be an excellent substrate by Marx and Bryan (1975). A successful commercial formulation of Fisolithus tinctorius mycelia has been developed by USDA, Forest Service and Abbott Laboratories (Marx et al., 1982). This inoculum trademarked as MycoRhiz®, is also grown in vermiculite-peat moss nutrient medium. Pure cultures of fungi viz., Suillus granulatus, Rhizopogon luteolus, Thelephora terrestris (Ehrh.) Fr. and Fisolithus tinctorius improved survival and growth of seedlings (Marx et al., 1982). However, cost effective method cannot be developed by above methods.

The method used in present study is easy and low cost requiring easily available used waste tealeaves. The differential growth pattern of Cantharellus to lignin and cellulose substrate compared to saprophytic fungi helps in obtaining pure inoculum of ectomycorrhizal mushroom. The mycelia of test fungus C. tropicalis traveled fast through bag and completely covered the substrate rich in cellulose and lignin requiring short incubation period. Sand helps in easy movement of the fungal mycelia through the substrate and breaking the inoculum into pieces during application and easily mixing with nursery soil (Fig. 1). In the absence of sand the substrate becomes solid mass due to the compact colonization. This inoculum can be mixed with soil at a rate of 1-2 kg m⁻². Some bags showed bacterial contamination (due to excess of water) restricted to the bottom which were later discarded. Ectomycorrhiza formation and root development are excellent in above substrate. This can be attributed to better mixing of inoculum to soil with sand allowing good aeration. Mass inoculum can be generated with ease and little technical training at nursery sites. The inoculum of Cantharellus can be stored with little loss of viability of 8-9 weeks at 5-20°C and 6-7 weeks at 25-30°C. Thus this substrate will act in an efficient way as compared to others. However, selection
of substrate is also dependent upon plantation site and plant species (Kumar and Satyanarayana, 2002). Moreover, performance of seedlings tailored with specific mycorrhizal fungi selected on the basis of growth rates, wide host range, and broader tolerance to pH, temperature, salinity and drought have been experimentally demonstrated by different workers. After extensive research, a few ECM fungi viz., Alpova, Amanita, Boletus, Cantharellus, Gomphidius, Laccaria laccata, Suillus luteus (L.:Fr.) Rouss., Hebeloma crustuliniforme, Cenococcum geophilum, Rhizopogon, Scleroderma, Pisolithus tinctorius have been recognized as suitable inoculants for various trees. Axenically grown fungi are produced in sufficient quantities and placed within rhizoplane.

Molina and Trappe (1982) have stressed on the difficulty of transporting, spreading and mixing of vermiculite inoculum as it becomes heavy from water saturation. Sand + used tealeaves inoculum is particularly promising as Cantharellus colonizes entire root system and substrate. Worldwide use of this mass multiplication method using beneficial fungi is quite possible and needs experimentation. The impressive results of this study may stimulate inoculation research programs around the world and a commercial inoculum will be available in market.

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REFERENCES


