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Ectomycorrhiza like interaction between *Cantharellus tropicalis* and *Dendrocalamus strictus*

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The chanterelle (*Cantharellus tropicalis*) is highly appreciated edible mushrooms of central India occurring primarily within *D. strictus* plantations. Isolates of C. tropicalis were tested for ability to form mycorrhizae in pure culture synthesis with *Dendrocalamus strictus*. The roots of *D. strictus* grown in aseptic condition formed mycorrhizal association with a chanterelle species when this fungus was inoculated to artificial substrate (sand + used tea leaves).

Keyword: *Dendrocalamus strictus, Cantharellus tropicalis,* ectomycorrhiza, artificial synthesis.

Introduction

Dendrocalamus (Graminaceae) comprising 30 species in South and East Asia is an important forest plant/ grass widely distributed in India having great ecological importance within natural forests (Singh *et al.*, 2001). *D. strictus* is commonly found in tropical (central) and temperate (northeastern) regions of India. Because of its light weight and durability it is used for furniture/ house construction, paper pulp, handicrafts etc. In India, more than 100 species of fungi, representing nearly about 10 genera have been reported to form ectomycorrhiza associations with different tree species. In few studies of naturally occurring ectomycorrhizae *Dendrocalamus* spp. has not shown to host naturally occurring ectomycorrhiza.

The chanterelles are much appreciated edible mushrooms found in many countries throughout world. The Indian chanterelle (*Cantharellus tropicalis*) is highly appreciated edible mushrooms of central India occurring primarily within *D. strictus* plantations (Sharma, 2008; Rahi, 2001). The annual harvest of Indian chanterelle is very high around

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Balaghat Madhya Pradesh INDIA, estimated at around 100 tones per year. A method for germination of the spores of *C. cibarius* had been developed but for mycorrhizal synthesis mycelia from tissues are preferred. Pure cultures of *C. tropicalis* have been established on Melins Norkrans nutrient media isolated from stipe tissues of fruiting bodies.

The trophic status of chanterelle (*C. tropicalis*) has been a topic of interest for mycologists of the region and observed mycelia of *C. tropicalis* on *D. strictus* roots. The possibility that it is facultatively mycorrhizal has been debated involving both saprotrophic and mycorrhizal phases. Mycorrhizal phase colonizing fine roots of bamboo and saprotrophic phase colonizing nutrient accumulated rhizome. Development of ectomycorrhiza or formation of mature basidiocarps of *C. tropicalis* has not been previously attempted in pure culture till recently (Sharma *et al.*, 2008).

Cantharellus is consistently associated with *Dendrocalamus* plants in natural forests of Madhya Pradesh. However, not all ectoycorrhizal fungi found associated with adult trees in field form mycorrhizae with young seedlings in nursery. Direct evidence for this association can be obtained by using modified pure culture synthesis technique which requires isolation of fungus into pure culture. Few edible ectomycorrhizal mushrooms have been cultivated under controlled conditions (Danell, 2002; Vaario *et al.*, 1999, 2000; Yamada *et al.*, 1999, 2001; Dahlstrom *et al.*, 2000; Guerin- Laguette *et al.*, 2000). On the other hand *C. cibarius* Fr. sporocarps have been produced under laboratory, green house and exotic plantation conditions respectively (Danell and Camacho, 1997).

Thus the establishment of an artificial cultivation system for *C. tropicalis* is economically important. The purpose of the current study was to isolate *C. tropicalis;* expand upon previous findings of chanterelle mycorrhizae; attempt mycorrhizal synthesis; and study developmental analysis of ectomycorrhiza formation between *D. strictus* and *C. tropicalis*. This investigation would lead to conditions for ectomycorrhizae formation, test the ability of *Cantharellus* to form mycorrhizae with *D. strictus* tree and clarify the mycorrhizal status of *C. tropicalis*.

Materials and methods

Preparation of fungal inoculums

The isolates of *Cantharellus tropicalis* have been obtained in September 2004 from fruit bodies collected near *Dendrocalamus strictus* in Balaghat Madhya Pradesh INDIA. Tissue blocks were aseptically excised from the fruit body and cultured on modified Melin-Norkrans Agar Medium (MNM) in 90mm petridishes and pure culture maintained at 26±2°C. The modified MNM medium contained Malt Extract- 3.0g; D- Glucose- 2.5g; KH_2PO_4- 0.5g; $MgSO_4.7H_2O$- 0.15g; $CaCl_2.H_2O$ 0.05g; NaCI- 0.025g;

$(NH_4)HPO_4$- 0.25g; $FeCl_3$- 1.2ml (l% solution); Thiamine HC1-0.lg; Distilled Water- 1000ml. The pH was adjusted to 5.8 with 1M NaOH before sterilization.

Preparation of plant material

Seeds of *D. strictus* were collected from natural tropical forests of Madhya Pradesh, State Forest Research Institute, Jabalpur and Government Forest Nursery, Seoni. Clean seeds of *D. strictus* were surface sterilized by rinsing briefly with tap water; then shaken gently for 15 minutes in a sealed bottle containing 1% Tween 80 solution using fixed speed shaker (Yorco, INDIA); and subsequently with 4% sodium hypochlorite solution (Qualigens, INDIA). Thereafter, seeds were rinsed and imbibed for 30 minutes (in five changes of sterile distilled water) and dried on sterile filter paper. Seeds were then planted aseptically on moist chamber Petri dishes (approx. 5 seeds/ plate). These were incubated (3-4 days; 26±2°C; in dark) to germinate seeds until radicles is 1cm.

Inoculation for axenic mycorrhizal synthesis

The modified *test tube or flask system* consisted of a wide mouth test tube of 25 x 200mm. (Riviera, GERMANY) and flask of 250ml (Riviera, GERMANY), containing autoclaved mixture of sand + used tea leaves (5% w:w) moistened with sterilized distilled water. The test tube/ flask were autoclaved (121°C for 30min) cooled to room temperature (28±2°C) and inoculated with *C. tropicalis* isolates (two 9mm plugs) aseptically placed on substrate surface touching glass wall. After 3- 4 days, when fungal mycelium had begun to colonize substrate, sterilized seedlings were introduced aseptically into substrate. The seedlings were inserted into a hole with the help of large forceps and slowly withdrawn leaving seedling near fungal inoculum. Substrate was gently replaced around root system and lightly tapped down. The mouth of both kinds of systems was closed with cotton plugs to avoid contamination by other micro- organisms. After setting both systems, they were enclosed in transparent plastic box (Temp. 26 ± 2^0C; R.H. 65- 70%; 12hr: 12hr light and dark) and incubated (1 month) in Plant Growth Chamber (Yorco, INDIA).

Inoculation and growth of pot- grown bamboo seedlings

After harvesting seedlings and roots of *test tube* and *flask system*, a good amount of inoculum is prepared due to mycelial colonization and tried on pot grown seedlings. Seedlings were germinated on sand (2 weeks) and transplanted into inoculum within plastic pots. After setting up *pot system*, they were placed in plant growth chamber (Temp. 26 ± 2^0C; R.H. 65- 70%; 12hr: 12hr light and dark) for one month. After one week, seedlings had

developed a mat of fine roots between sand- used tea leaves. These needed addition of sterilized distilled water to replace loss by evapotranspiration one week after setting the system. Three control replicates (without fungal inoculation) were maintained in each system. Plant fungus interactions were first observed through the *test-tube system* after 3 days. Whole root systems in each combinations of mycorrhizal synthesis system were sampled and removed along with control roots and observed.

Microscopic observation of the bamboo root system

Fungal colonization of roots was observed under a binocular stereomicroscope (Focus, INDIA). Established mycorrhiza was sectioned according to Danell and Fries (1990) to confirm the presence of intercellular Hartig Net, which constitutes evidence for mycorrhiza. When fungal colonization was confirmed, segments of l-2cm in length were dehydrated with EtOH embedded in paraffin wax, cut (3- 15µm) and finally stained with Cotton Blue or 0.5% Toluidine Blue O at room temperatures. Whole or hand sectioned root tips were mounted in Lactophenol on glass slides and stained with Cotton Blue. Further, microscopic observation was done using a compound light microscope (Nikon Eclipse E800, JAPAN).

Re-isolation of fungus from colonized roots

To check the fungal viability on the colonized host roots, mycelium from each combination of mycorrhizal synthesis system were re-inoculated onto MNM agar medium plates.

Results

Mycorrhiza formation

After 4 weeks incubation, both sterilized seedlings and re- rooted seedlings of *Dendrocalamus strictus* were gently removed from the medium and their root system were observed *Cantharellus tropicalis* mycelium grew well in both *test tube* and *flask system method*. Yellow white mycelium/visually extended throughout the substrate moistened with distilled water after inoculation.

After one week incubation in culture plates, *D. strictus* seedling grew one-single main root approximately 5 cm in length in the *test-tube method* and 15cm length in the *flask method*. First order lateral-roots of seedlings were produced after 2 weeks incubation following inoculation with *C. tropicalis* and they were confined to the medium surface. The remaining seedlings, four of which succumbed to contamination, produced neither first nor second order lateral roots.

Microscopic examinations often confirmed mantle or haring net

development or both, in hairless and in bifurcated root tips of *D. strictus* seedlings. Fungal sheath formation was confirmed under the dissecting microscope, 2 weeks after inoculation of isolate on the distilled water moistened substrate (Fig. 1a, b)**.** Bamboo root systems that were heavily covered with mycelium were highly branched & extended and lacked distinct root hairs. Such root systems were observed by the third week on all bamboo seedlings grown in the *test tube/ flask system*. However, 3 week old seedling showed both fungal sheath and cortical hartig net on the lateral roots thereby confirming ectomycorrhiza- like association (Fig. 2)**.** The mantle did ensheath single lateral roots entirely; the proximal ends of the lateral roots and the lower mother root were also covered by ensheathing hyphae. The plectenchymatous mantle possessed a densely woven outer surface and many emanating hyphae. The mycorrhizal lateral roots, approximately 1.25- 2.5 mm in length and 0.1- 0.2mm in diameter, were brown in colour and concolourous from the distal- proximal end. Mycorrhiza was simple, dichotomously branched, thickening at the top of the branch, covered by compact mycelial layer of varied diameter. Mantle was thick, 60- 100 □m in container, covered by a dense aggregate of emanating hyphae which formed a loose external plectenchymatous surface. The outer surface was composed of morphologically undifferentiated hyphae 1.5- 2.5µm in diameter.

Colonization assessment

Seedlings were removed intact from the tubes and plates with any visible contamination noted and washed with de-ionized water to remove substrate. Roots were examined by stereomicroscopy and representative putative colonized roots removed and either sectioned by hand or prepared for microtome- sectioning, free hand sections were stained in cotton blue in aqueous solution and mounted in lactophenol. Roots for microtome sectioning were fixed and post fixed, rinsed and dehydrated in a graded ethanol series and embedded in paraffin (CDH, INDIA). All sections were examined for mantle, hartig net and intercellular and intracellular hyphal development.

Light microscopy

In longitudinal cross sections of an un-inoculated control *D. strictus* root tip grown by the flask tube method (Fig. 3a, b and Fig. 4), the meristematic area was overlaid with an extensive root cap, which extended to cover the juvenile epidermal layers. In contrast, mycorrhizal root tips selected from each culture system possessed a reduced root cap and the tip was en-sheathed.

Mycorrhiza formation system

Sand + Used tea leaves were found to be a satisfactory inert medium carrier. The mycelia grew very fast through the substrate and colonized it within three days and produced good fungal inoculum for pot grown seedling Figure 1a and 1b show a typical synthesis system and examples of mycorrhizal seedlings produced following exposure to inoculum of *C. tropicalis* mycelia which grew well in both *test tube* and *flask method*. Yellow white mycelium visually extended throughout the substrate moistened with distilled water after inoculation.

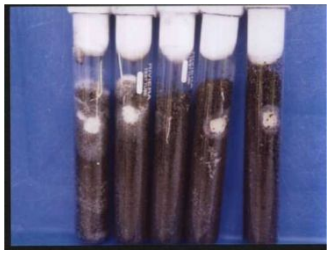

Fig. 1. Tube and Flask growth systems.


a

b

Fig. 2. Ectomycorrhiza formed between *Cantharellus* sp. and *D. strictus* roots after a 4 week incubation showing dichotomous branching and cottony fungal sheath.

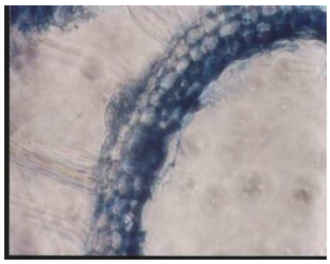

Fig. 3. Light microscopy of *Cantharellus- D. strictus* ectomycorrhiza-. Cross section of infected root showing mantle and hartig net.

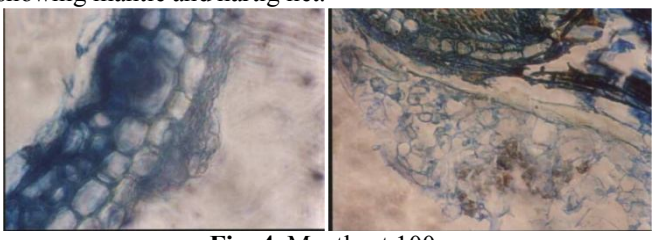

Fig. 4. Mantle at 100x.

Discussion

The root morphology observed in the experiment reveals a mycorrhiza-like relation between the roots of *Dendrocalamus strictus* and *Cantharellus tropicalis* and illustrates ectomycorrhizal association between fungi and monocot plant. Mycorrhizal synthesis with other fungal species and host plants has been reported elsewhere (Guerin-Laguette *et al.*, 2004; Hall *et al.*, 2002; Yamada *et al.*, 2001a,b; Yamada *et al.*, 2006; Parlade *et al.*, 1996). Although *in vitro* mycorrhizal synthesis is difficult in the genus *Cantharellus,* the species formed mycorrhiza- like association in this study. Yamada and Katsuya (1995) also reported mycorrhizal synthesis between *Pinus densiflora* and 21 fungal species, including two species of *Russula*, which are also difficult to manipulate *in vitro*. Mycorrhiza – like association between *Morchella* and 4 tree species of Pinaceae is also known (Dahlstram *et al.*, 2000).

For this association, *in vitro* culture techniques were used. However, nutrient substrate applied to mycorrhizal synthesis experiment was modified to suit the requirement of species and make the technique simple. Prior culture of fungus in MNM solid media did not prevent rapid colonization of new medium. Thus, ordinary MNM medium can be used for culturing and maintaining strains. Mycorrhizas synthesized by this culture system exhibited characteristics typical of ectomycorrhizas. The thin continuous mantle supports hyphae, which penetrates and colonize the host root cortical intercellular spaces. The presence of these defining features in the mycorrhizal roots examined indicates that *C. tropicalis* forms an ectomycorrhiza- like association with *D. strictus* roots under the culture conditions employed. We found that enlarged root tips, along with possessing well developed mantles, exhibited multiple hyphal penetrations. Delay in fine root formation in bamboo can be increased by growth factors such as culture substrate, container form and fertilization rate. A slow development of bamboo root system limits mycorrhization since fewer fine roots are available when fungal inoculum is viable in substrate. However, failure of mycorrhizal infection in some cases may result from either death of mycelium or its inability to infect fine roots. The better effectiveness of *C. tropicalis* mycelium grown on solid inoculum, whatever the form, rather than alginate beads/ liquid culture may be related to the inoculation method rather than to mycelium survival. Receptiveness is greatly influenced by the physical, chemical and biological characteristics of the substrate. Results in this paper demonstrate the higher receptiveness of used tea leaves + sand as substrate than any other substrate or natural soil.

All seedlings that formed mycorrhizas *in vitro* developed new mycorrhizas in open-pot soil; non- mycorrhizal seedlings did not form new mycorrhizas. Moreover, mycorrhization increased tolerance to drought and other environmental stresses (Sharma *et al.*, 2008). This suggests that the environmental setting was suitable for acclimatization of the *in vitro*

mycorrhizas and that the mycorrhizal status is stable under both *in vitro* and open pot soil growth conditions. Some fungi that form mycorrhizae in pure culture fail to form in greenhouse or natural soil conditions (Molina *et al.*, 1997) Mycorrhiza disappearance and succession (contamination) to another symbiont is problematic in the cultivation of mycorrhizal mushrooms. An important aspect of the practice of acclimatization is to protect the inoculated fungus from competitive air borne mycorrhizal fungi in green house conditions. In our study, hairless or bifurcate root tips frequently possessed either or both mantles or hyphal penetration between the cortical cells. Soon after inoculation with pure cultures of the Indian chanterelle, fungal and/ or bacterial contamination, or both appeared within some synthesis systems. Surface sterilization times for the seeds may have been insufficient. Contaminants were examined by light microscopy but were not identified except, *Aspergillus flavus*, *A. niger* and *Penecillium* sp. Most of the contaminants were restricted to the seed cotyledon and careful examinations revealed little association between the contaminant fungus and the roots. Because potential effects of the unidentified contaminants could not be surprised, the possibility exists that they affect the formation of mycorrhizae between the chanterelle isolates and seedlings.

The clearly defined *in vitro* system described here will ensure that sufficient inoculum of *C. tropicalis* can be produced in the laboratory to be applied to practical bamboo forestry situations. However, ecologically the ability of *Cantharellus* to form mycorrhiza- like interaction is significant. Perhaps, chanterelle forms facultative mycorrhizae not only to acquire nutrients from living trees but also to position themselves to decompose fine roots as they senesce or in the event of tree death. In conclusion, it is evident that cultured *C. tropicalis* isolates have the ability to form true ectomycorrhiza- like association on *D. strictus* seedlings. Commercial growth of *C. tropicalis* might be possible through out planting of colonized seedlings like the management of the black truffle *Tuber melanosporum* Vitt. Acclimatization of the colonized seedlings to natural conditions will hold the key to the success of *C. tropicalis* cultivation in future.

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