Discussion
5. DISCUSSION

Mycorrhizas play a significant role in plant growth. As pointed out by Ruehle (1985); Bougher and Malajczuk (1990) and Natarajan et al. (1995), adequate ectomycorrhizal association is essential for rapid establishment of tree seedlings, and hence ectomycorrhizal fungi like \textit{Pisolithus, Laccaria, Suillus, Paxillus, Hebeloma} and \textit{Thelephora}, to name a few, find extensive application in forestry and revegetation practices. Tree performance in most circumstances depends upon the establishment of mycorrhizal associations that serve many purposes, apart from just facilitating the uptake of nitrogen, phosphorus and water, enhancing the tolerance / resistance to root pathogens, toxic heavy metals, pH fluctuations and even forming a protective barrier against certain edaphic factors. It is believed that the fungal mycelium which extends from the mycorrhizal roots form a three dimensional network which links the roots and the soil environment. It constitutes an efficient system for nutrient uptake and scavenging in nutrient poor conditions (Requena \textit{et al.}, 2001). It also contributes to the formation of water stable aggregates necessary for good soil tilth (Jeffries and Barea, 2000). Thus, mycorrhizal associations are multi-functional (Last, 1999). However, mycorrhizal fungi, like trees, vary in their performance. Similar to trees, this study has therefore been aimed at delving into the relatively less-explored aspect of intraspecific / intra-isolate variation within several isolates of the ectomycorrhizal fungus, \textit{Pisolithus tinctorius} collected from ten different tropical localities of Tamil Nadu, India. Variations have been studied with respect to culture characteristics, \textit{in vitro} and \textit{in vivo} performance with
two different hosts growth, nutrition and biochemical constituents, as also reaction to pH, temperature and carbon sources. The results obtained and the observations recorded have mostly been in concurrence with earlier works reported from various parts of the world.

Variation in culture characteristics among the isolates were distinct. Ten isolates collected from nine different localities of Tamil Nadu viz., Needamangalam, Arunthavapuram, Vaduvoor, Saliyamangalam, Sadayarkoil, Gantharvakottai, Alangudi, Aranthangi and Perungalur were grown under similar conditions, yet showed differences in their growth parameters. Variations were observed in the rate of growth (as shown by the difference in biomass production and colony diameter), total protein content (Table 1), colony morphology (Plate XI) and also in the performance with host plants in vitro (Fig.3). Such differences have often been encountered with ectomycorrhizal fungi like Pisolithus, Hebeloma, Suillus, Scleroderma and Paxillus (Cairney, 1999). From works reported so far, it is clear that considerable intraspecific variation exists within Pisolithus tinctorius, especially, with regard to host specificity, growth form of extramatrical mycelia and organic nitrogen utilization (Cairney and Chambers, 1997). Hence, research today is being made with an attempt towards understanding the infection process and compatibility between P. tinctorius and various hosts using multiple isolates. The present study also focused upon the differences between the ten isolates of P. tinctorius taken under consideration, both in axenic conditions as well as in association with the host plants in vitro and in vivo conditions. Based on the growth rate and other colony parameters considered (Table 1 & 2), it was observed that the alien isolate (Pt(GE)) behaved totally differently from the rest. Although no broad grouping can be made of the other isolates from India, Pt(ALC) and Pt(SC) have shown relatively faster and better growth in both liquid and agar media. Pt(AE), Pt (ALC), Pt(AC) and Pt(PE) have shown similarly in the presence of mycelial cords, but
have varied with respect to other parameters. Thus, the behaviour of the isolates was never similar to each other in all aspects, making groupism a difficult task. This was found to be similar in all the isolates, just as reported by Hutchinson (1990). However, the “source influence” on the isolates could be clearly observed by their association in vitro with the two host plants tested (Fig.3). For instance, Pt(SE) was isolated from *E. tereticornis* plantations and Pt(ALC) from *C. equisetifolia* plantations, and the same isolates have also given the best performance in vitro. In the case of *E. tereticornis*, Pt (GE), Pt(SE) and Pt(AE) (all isolated from *E. tereticornis* plantations) gave high percentage mycorrhization in two of the triplicates, yet, their overall mean value was slightly less. Such a condition has been met with very often by mycorrhizologists. For instance, *P. tinctorius* isolated from carpophores collected in association with *Pinus* spp. have been poor colonizers of *Eucalyptus* spp. (Chilvers, 1973; Malajczuk et al., 1990; Lei et al., 1990a and Burgess et al., 1994). The data of Burgess et al. (1994) also showed significant intraspecific variation of intercompatibility with *P. tinctorius* isolates within the genus *Eucalyptus*, wherein the isolates were derived, not only from different geographical regions, but also from carpophores having different morphological characteristics. According to Cairney and Chambers (1997), these characteristics could even reflect upon the taxonomic position of *P. tinctorius*, which requires detailed systematic studies. Recent methods involving a molecular approach are paving way to solve the unresolved mystery of speciation of *P. tinctorius*, and they indicate considerable polymorphism even within *P. tinctorius* isolates collected from a closer circuit.

France et al. (1981) reported that on *Pinus taeda* in two nurseries inoculated with ectotypically different isolates of *Pisolithus tinctorius* by incorporating vegetative mycelia of each isolate in nursery soil showed that certain isolates were superior to others in enhancing seedling growth
in terms of height, diameter, biomass and ectomycorrhizal infection throughout the growing season. However, the enhanced growth of seedling varied with isolates, with time and type of nursery soil.

Burgess et al. (1995) analysed the electrophoretic patterns of the expressed mycelial proteins from *P. tinctorius* isolates collected from different geographical regions of Australia and observed that they showed much variability in the polypeptide pattern, thereby establishing a correlation between groupings based on polypeptide pattern and geographical origin. In concurrence, there is a possibility that in the present study also, the polypeptide patterns could vary corresponding to the geographical origin. This was also clear from the preliminary screening for protein content done using the method of Bradford (1976). A further probe into this would require a study of the protein profiles of all the isolates using a 2-dimensional SDS-Poly Acrylamide Gel Electrophoresis (PAGE) or by blot analyses. Systematic studies also involve a thorough search of the genetic and molecular intricacies that govern the fungal behaviour. For instance, Lamhamedi and Fortin (1991) observed that reconstituted *P. tinctorius* dikaryons show variability in the growth form of the extramatrical mycelia, in extension rates and in the degree to which they form rhizomorphs. A similar response was also demonstrated with dikaryotic cultures of *Pisolithus* sp., showing great difference in characteristics such as production of mycelial strands, ability to form mycorrhizae (Lamhamedi et al., 1990) and in the production of antibiotic substances (Kope and Fortin, 1989). At the molecular level, polymerase chain reaction enabling direct analysis of target DNA sequences are being increasingly used in fungal systematics (Sims et al., 1999).

Variation also exists among the fungal isolates in their capacity to form ectomycorrhizal associations and promote the growth of the host
plants. Burgess *et al.* (1993) studied the ability of an isolate of *P. tinctorius* Pt H445, in forming ectomycorrhizas with *E. globules* and *E. diversicolor*. Tonkin *et al.* (1989) used two isolates, H98 and H323 and reported variation in their effectiveness. Similarly, Burgess *et al.* (1994) studied the variation in mycorrhizal development and growth stimulation by a larger number of isolates, by inoculating 20 *P. tinctorius* isolates on to *Eucalyptus grandis*. From this experiment, it was found that a large variation exists between isolates and this could be used to categorize the isolates into 6 types. Furthermore, the extent of mycorrhizal development was positively correlated to the growth stimulation in the glasshouse and this could serve as an indicator of aggressiveness and consequently the potential, of an isolate to promote tree growth. Such intraspecific variations in mycorrhizal development have also been observed earlier. Wong *et al.* (1990) found that there were substantial phenotypic differences in the rate and extent of mantle and hartig net formation among four closely related genotypes of *Laccaria bicolor*. Hence it can be concluded that isolates that show variation amongst themselves with respect to morphology and growth, also show variation in association with their host plants. This was substantiated in the present study also, where each isolate performed differently (producing varied number of mycorrhizal tips and hence % mycorrhization) with each of the two host plants tested. Difference was observed within the ten isolates in association with both the host plants. *In vitro* mycorrhization studies are relevant because it is believed generally, that the more aggressive an isolate is *in vitro*, the more rapid is the rate of mycorrhizal development and the greater is the growth stimulation *in vivo*. As put forth by Burgess *et al.* (1994), screening should be done *in vitro*, so as to select only superior isolates for incorporation into nursery inoculation programs for eucalypt plantations. Similarly, Danielson *et al.* (1984) reported that on jack pine seedlings grown for 20 weeks in a peat-vermiculite medium and inoculated with solid carrier mycelial inoculum, the use of low amounts of
fertilizer resulted in seedling size below standards for planting out. This low fertilizer amount, however, permitted infection of more than 90% short roots when inoculated with *Thelephora terrestris, Laccaria proxima, Hebeloma* sp. or E strain fungus (a white spruce mycorrhiza forming fungus), about 50% root colonization when inoculated with *Cenococcum geophilum, Pisolithus tinctorius*, 32% by *Laetorius paradoxus*, and 17% by *Sphaerosporella brunnea*. Inoculation with *Amphenema hyssoides, Hydnum imbricatum* and *Tricholoma flavovirens* showed no infection.

Again, the selection of medium is important. In the present study, both liquid and agar MMN media were used. Palmer (1971) suggested that experience with each isolate is essential to determine if immersion reduces or stops growth, if hyphae will radiate from the plugs, if the hyphae will reflex down and how much time will be required, if the fungus is grown in liquid medium. He also observed that the dry weight of most fungal species is greater in standing than in shake cultures and hence static flask method was only adopted in the present study. This was also in conformity with the hypothesis proposed by Harvey *et al.* (1989). They propounded that static flask cultivation of ectomycorrhizal fungi is a popular method of cultivation for routine laboratory use and for small scale field or nursery trials because contamination of the culture is easy to detect, and determination of the amount of biomass present is very simple.

Thus the characterization experiments and a study of the growth kinetics of the ten *P. tinctorius* isolates involved in this research can be deemed appropriate and important in practical applications, when the results need to be taken from the laboratory to the field to get better returns.
As carried out by Burgess et al. (1994), a follow-up of the behaviour of the \textit{P. tinctorius} isolates in association with its host \textit{in vitro} was carried out \textit{in vivo}, wherein seedlings of \textit{C. equisetifolia} and \textit{E. tereticornis} were grown in a red soil substrate and inoculated with \textit{P. tinctorius} under glasshouse conditions. All the ten isolates were screened for their performance. Growth responses following seedling inoculation with \textit{P. tinctorius} under controlled conditions have been frequently reported (Marx and Bryan, 1970; Beckjord \textit{et al.}, 1985; Heinrich \textit{et al.}, 1988; Bougher and Malajczuk, 1990; Burgess \textit{et al.}, 1994), and a strong influence on the response by the host plants to the fungal genotype has also been observed (Dixon \textit{et al.}, 1987; Lamhamedi \textit{et al.}, 1990; Thomson \textit{et al.}, 1994).

A similar study on ectomycorrhizal inoculation was also carried out by Dell \textit{et al.} (1994) with \textit{Allocasuarina, Casuarina} and \textit{Eucalyptus}, wherein seedlings growth \textit{in vivo} were assessed for ectomycorrhizal association after 90 days of inoculation by various techniques involving Scanning Electron Microscopy, Transmission Electron Microscopy and light microscopy. From their results, it was observed that the infection was not same within the fungus tried, as some of the hosts like \textit{Allocasuarina} did not even get associated with the fungus, although morphologically a fungal sheath was present. In the present study however, ten different isolates were used in association with \textit{C. equisetifolia} and \textit{E. tereticornis}, separately and all the isolates produced mycorrhizae, with the only difference that the percentage varied from one isolate to another (Fig.3). This can again be accounted for by the "aggressiveness factor" of the fungal isolate inoculated. While some isolates show hyper-promoting reaction with the host root, some isolates are docile and slow growing. This in turn led to the difference in the growth enhancement of the seedlings also. It was observed that the local isolate (Pt(GE)) gave the maximum biomass in \textit{C. equisetifolia} (0.9066 g)
and in *E. tereticornis* (0.8548 g) and the maximum percentage mycorrhization in *C. equisetifolia* and *E. tereticornis* respectively at the end of 6 months. Besides, other factors like soil nutrients, soil pH, temperature and relative humidity could have also favoured this isolate, not requiring an acclimatization process. This corroborates with the view of Cairney and Chambers (1997). Accordingly, edaphic factors have a profound influence on the host growth responses under controlled conditions.

However, *P. tinctorius* isolates that show wide intraspecific variation even in requirements like temperature, and pH for axenic growth or *in vitro* mycorrhization, show better root colonization under controlled conditions only in relatively high temperature (above 19°C) (Marx and Davey, 1969; Marx *et al.*, 1970; Cline *et al.*, 1987). It has been observed that strains which prefer low temperature for axenic growth do not perform very well at the temperature *in vivo*, in association with the host. Bougher *et al.* (1990) have reported such a response, wherein, under relatively cool conditions slow growth and consequently poor infection of the newly formed laterals was observed in *E. tereticornis*. Although this is in contradiction to the contention that selection of isolates for *in vivo* studies can be based on small scale *in vitro* experiments, according to Burgess *et al.* (1994), it is likely that performance of the isolates in axenic conditions is much different from that in association with a host plant, either *in vitro* or *in vivo*. Mycorrhizal infection in the field sometimes shows variability for which no cause is apparent (St. John and Coleman, 1983). This could probably explain the reason for the difference in performance by Pt(GE) from Pt(SC) in the nursery, while both isolates behaved almost similarly *in vitro* in axenic conditions. All the isolates produced shiny mycorrhiza and some isolates had mycelial cords extending into the soil, as reported by Burgess *et al.* (1994). The common feature in all the mycorrhizae was the high level of hairiness on the surface.
of the rhizomorphs and the presence of clamp connections in the extending hyphae. This was also reported by Munyanziza and Kuyper (1995) while working with seedlings of *Afzelia quanzensis* in a glasshouse.

In another experiment with the local isolate Pt(GE) to determine the best mode of inoculation and the best soil type, it was observed from the present study that perlite inoculum and unsterilized soil gave the best results over a study period of 10 months (Table 9-26). Two modes of inoculation were tried, viz., the perlite carrier (vegetative mycelial inoculum) method and the bead carrier method. Similarly two types of soil were tried, viz., unsterilized garden soil and steam sterilized soil. In all the seven harvests taken, the perlite inoculated seedlings of *C. equisetifolia* and *E. tereticornis* grown on unsterilized soils only gave the best results. The biomass of the seedlings grown in this treatment was always significantly more than the uninoculated and bead inoculated seedlings grown in both sterilized and unsterilized soil. However, comparing the two soil types alone, unsterilized soil proved to be better. Such results have been encountered in other researches also. For instance, ectomycorrhizal fungal inoculation was reported to have benefited the seedlings of *Acacia nilotica* more in unsterilized soil than in sterilized soil by Natarajan et al. (1995). Although this might at the first instance look incorrect, as sterilized soil is expected to be devoid of all the pathogenic microbes and hence increase the plant growth, but it is not so. It has been proved that unsterilized soil can also harbour some microbes with positive effect like the native Vesicular Arbuscular Mycorrhizal (VAM) fungal propagules and other nitrogen fixing organisms (that will help leguminous plants). In fact, many studies have concentrated upon the effect of dual inoculation, wherein, both an ecto- and an endomycorrhizal fungi are deliberately added, yielding better results than single inoculation (Chen et al., 2000). The advantage here is that the endomycorrhizal fungus
dominates at the early stages and is then taken over by the ectomycorrhizal counterpart, thereby supplying the plant with both the positive effects, in a rather synergistic manner. In the present investigation, however, no separate study was undertaken to assess the VAM colonization in the roots, but the soil was screened for VAM spores, following the method of Gerdemann and Nicolson (1963), before it was taken for the nursery. About 10 spores were found in 100 g of the soil. It could therefore be that these spores enhanced the plant growth in unsterilized soil. VAM associations were not specifically studied, because as put forth by Chen et al. (2000), many experiments have shown that ectomycorrhizal associations are usually more important than VAM associations for Casuarina and Eucalyptus species, although the benefits of both put together can exceed those provided by either one alone. This is the reason for increased incidence of ectomycorrhiza in unsterilized soil. Untreated soil has also exhibited high Ectomycorrhizal Soil Infectivity (ESI) in experiments conducted by Soulas et al. (1997), while finding an alternative method for weeding out pathogens, instead of soil fumigation with chemicals. They introduced the soil solarization method, which is not as harmful as fumigation, yet, found it injurious to some of the ectomycorrhizal fungi included in the pool of beneficial soil microbes. An earlier study by Aggangan et al. (1996a), also revealed similar results. Maximum mycorrhizae formation was relatively higher in unfumigated soils and lowest in the fumigated counterparts in their study. They also observed that ectomycorrhizal fungi should be compatible with resident microflora to effectively colonize the roots. It is thus apparent that sterilization of soil kills most often, if not always, the desirable microflora in the soil, including VAM flora. In this connection, An et al. (1993) have observed that fumigation reduced the species richness and diversity of VAM fungi, which could be the reason for the poor performance of seedlings grown on sterilized soil.
Zhu and Navratil (1987) showed that containerized seedlings of *Larix laricina* representing four provinces and 17 open pollinated families showed that during an 18-week period after inoculation with vegetative inocula of *Laccaria laccata*, *Cenococcum geophilum* and *Pisolithus tinctorius* it formed ectomycorrhizae with 60, 7 and 12 percentage of the total short roots respectively. *Suillus granulotis* failed to form any mycorrhizae. With reference to the best mode of inoculation, several studies have been conducted so far, using different modes apart from the two modes used in the present study. For instance, spore inoculum has been used to produce specific ectomycorrhizae on tree seedlings (Trapppe, 1977). Soil inoculum has been tried and suggested as the commonest method by Harley and Smith (1983). Crushed sporophores and natural soil inoculum have also been used on *Cedrus deodara* seedlings by Singh and Lakhanpal (2000), although they found that these methods yielded only a slight increase than the uninoculated control. Marx et al., (1982,1984) have also used these methods. However, pure vegetative inoculum has been found to have the greatest biological advantage. This mode was also tried by Singh and Lakhanpal (2000) and they concluded that pure culture ectomycorrhizal inoculations have significant effect on improving the growth and development of seedlings of *Cedrus deodara*. According to Marx and Cordell (1989), pure cultures of certain fungi like *Pisolithus tinctorius* can be used to improve survival and growth of tree seedlings on a variety of sites.

The disadvantage of using this method is that vermiculite-peat moss- MMN inoculum used directly from the container and mixed into fumigated nursery soil can be rapidly colonized by other saprophytic microbes that can, on heavy colonization, reduce the positive effect of the ectomycorrhizal inoculum. Leaching of the inoculum before inoculation will aid in removing most of the non-assimilated carbohydrates and can thus reduce this microbial colonization and increase inoculum efficiency.
(Marx, 1980). Leaching of the carrier material was also done in the current study and a fairly dry inoculum was obtained and stored at 4°C until use in the nursery. Perlite particles entrapping the mycelium of *P. tinctorius* were also studied under a microscope for clamp connections and it was confirmed that the inoculum was not contaminated. This method is quite advantageous considering the fact that it is easy to produce mass quantities and the assurance of the presence of only a single desired fungus in the inoculum. In this manner, it is superior to spore inoculum in which many fungal species can be assorted. Another important advantage is the time required for the fungus to colonize – it is faster than any other method, since it does not require any germination of the spores or an inactive period when it remains dormant. Instead, once in contact with the host root, the mycelia from the inoculum establish themselves and begin the infection process.

The other method of inoculation is the bead method, in which the fungal hyphae were immobilized and entrapped within calcium alginate beads. This method allows for long term storage and also serves as an inexpensive method to produce inoculum of constant quantity even for large scale application. This method has been widely used on several ectomycorrhizal fungi (Cheetham *et al.*, 1979; Veliki and Williams, 1981). It has been proved to be a promising method for some species like *Hebeloma* (Le Tacon *et al.* 1985; Mauperin *et al.*1987) and *Laccaria* (Mortier *et al.* 1989). However, this method also has some disadvantages. The quality and viability of the inoculum (Mauperin *et al.* 1987) and also other factors like temperature and humidity that might affect the inoculum storage, have to be constantly monitored (Kuek *et al.* 1992). Hence, adapting this method would be successful only if these factors are looked into, besides finding techniques to maintain high viability of the fragmented mycelia. Experiments to maintain viability have been conducted by Rodrigues *et al.* (1999) and Vijaya and Srivasuki (1999).
They have tried different ratios of the gelling factors (sodium alginate and calcium chloride) and concluded that 0.7M CaCl₂ in combination with a 8:10 solution of alginate – mycelium mixture was found to give the best results. The age of the mycelium was also studied and 20 – 40 day old cultures of *P. tinctorius* favoured better. In the present study also, 0.7 M CaCl₂ and 21 days old cultures were used. The alginate – mycelium combination was 10:10, but this ratio has also performed well in the experiments conducted by Rodrigues *et al.* (1999). Curing of the beads was done for 35 minutes unlike 45 minutes as reported by Mauperin *et al.* (1987), but polymerization was complete despite that. Since the beads were used within a week for inoculation purposes in the present study, not much research was done on the storage of the beads. All the beads were viables as there was 100 per cent germination on MMN medium. In the nursery, the performance of the bead inoculum was not very promising. Not many experiments have been carried out to compare the inoculation modes simultaneously. But, in this study, both perlite and bead inocula were tried on two soil types, and it was observed that the latter performed almost similarly with the control in sterilized soil and even poorer than the control in unsterilized soil in most harvests for most parameters. This could perhaps be due to the relative delay in hyphal germination from beads compared to that of perlite. Another problem faced during this study was a sudden drop in the biomass value of the seedlings during the 6th harvest. This could probably be due to the intense heat and high summer temperatures of a tropical region like Pudukottai resulting in denudation of the plants. Effect was also seen on the plant height and other parameters. But this was not observed in bead inoculated plants. Another reason for this anomaly could also be in the selection of the seedling samples.

The experiments however, proved that variations do exist among the ten isolates in their performance in association with their host plant
in vivo; and within an isolate, it depends on the type of inoculum and soil used. This gives an insight into the selection procedure that is required for future large scale field trials, in which, the appropriate choice of the fungus, and even more, the right strain, will guarantee greater benefits.

Perhaps, few other factors also need to be looked into while choosing a strain. It is likely that the soil conditions may vary even within a single region, and hence a strain which performs well in one area need not necessarily behave so in another. The likelihood of variations in soil factors can be based on the nature of the soil, which includes factors like pH and soil profile that includes the metal content, amongst others. Hence, a probe into the performance of the ten Pisolithus tinctorius isolates under investigation, in various pH and temperature concentrations were studied in vitro.

pH factor plays a key role in judging the performance of an isolate. Experiments to study the variation in the growth between and within species of ectomycorrhizal fungi in response to in vitro pH was also conducted earlier by Hung and Trappe (1983) and it was observed that the fungal growth varied depending on the pH. The fungal mycelia were harvested at the end of 45 days and their dry weights were measured variations were observed in the response shown by the ten isolates used in the study.

Some of the isolates could tolerate high acidic levels (pH 2.0 & 3.0), while some could tolerate very high alkaline levels (pH 8.0). Two isolates, Pt (NE) and Pt(AE) showed preference to acidic pH (3.0); Pt(GC), Pt(GE), Pt(AC) and Pt(SE) showed preference to acidic pH (6.0); by producing the maximum biomass at that level. On the other hand, Pt(SC) and Pt(VC) produced maximum biomass (422.0 mg and 382 mg)
The isolates, Pt(GE), Pt(AC) and Pt(SE) showed maximum growth at the prescribed pH for MMN medium which is pH 6. It is to be noted, however, that each isolate has responded to an increase or decrease from their desired pH level in a unique manner. The growth pattern reveals the high levels of tolerance of Pt(VC), Pt(SC) and Pt(PE) to an increase from the pH 7.0 to 8.0, by only a little decrease in the biomass. Similarly, Pt(NE), Pt(SC), Pt(AC) and (PE) showed a sudden increase in the biomass, from 294.5 mg at pH 5.0 to 412 mg at pH 6.0; 225.0 mg at pH 5.0 to 380.0 mg at pH 6.0: 320.0 mg at pH 5.0 to 472.0 mg at pH 6.0; and 320 mg at pH 5.0 to 423.0 mg at pH 6 respectively.

The pH preference of the isolate could have led to this approximately five fold increase in the biomass production. The response of Pt(PE) has been very uniform with a gradual increase upto pH 8.0. From the screening, it was found that Pt(NE), Pt(AE), Pt(GC), Pt(ALC), Pt(GE), Pt(AC) and Pt(SC) are better adapted to acidic soils, while Pt(VC), Pt(SC) and Pt(PE) prefer alkaline soils.

The colour of the fungal mycelia also changed with a change in the pH. Most isolates showed a pale yellow to white colour in the acid range and were pastel yellow to golden yellow at pH 5.0 and 6.0. The change in hyphal colour has also been reported by Hung and Trappe (1983). The two Pt isolates tested showed a drop from golden yellow (at pH 6.0) to white colour (at pH 3.0) in their study. A reduction in pigment production by ectomycorrhizal fungi near one or both extremes of their pH range in vitro has also been reported for several other ectomycorrhizal fungi by several others (Melin, 1924; Mikola, 1962; Laiho, 1970), but the physiology that governs it, is not known. The only conclusion that can be
drawn is that pH also has an influence on the pigment production apart from biomass production of the fungus.

The growth of the ten isolates of *P. tinctorius* was optimized with respect to different levels of temperature and incubation periods. From this experiment, it is clearly evident that the growth of all the *P. tinctorius* isolates were luxuriant in temperature of 30°C, whereas in temperature of 40°C, the *P. tinctorius* isolates did not grow well.

As far as temperature requirements are concerned, several reports are available stating the intraspecific variation among the isolates of this candidate fungus (Laiho, 1970; Theodorou and Bowen, 1971 and Samson and Fortin, 1986). Variation in the optimal growth temperature of eleven USA isolates of *P. tinctorius*, based on their latitude of origin, has also been clearly reported (Cline et al., 1987). A temperature of 28°C has been found to give maximum biomass production in a *P. tinctorius* isolate for *in vitro* growth in axenic conditions (Marx et al., 1970). Most isolates of the ectomycorrhizal fungus, *P. tinctorius* have been reported to have relatively high temperature option when grown *in vitro*. This is consistent with the fact that mycorrhizal development and survival of forest trees at high temperature was greater when infected with *P. tinctorius* than with *Thelephora terrestris* (Marx et al., 1970). In the present study temperature of 29 to 30°C has been found favourable for Pt(GE) in giving maximum biomass. An increase or decrease from this temperature led to a sudden decrease in the biomass production, though it was not completely curbed.

Of the three incubation periods studied, 45 days period has given the best results. This could be expected because, greater the number of days of incubation, greater the biomass production, except, however, at one critical point and beyond it the growth slows down. Stagnation in
growth beyond this level is because of the increased acidity of the medium. It is believed that fungus like *P. tinctorius* produces a lot of secondary metabolites when in culture, that results in acidification of its culture medium. This in turn decreases the pH of the medium to such an extent that further growth gets retarded.

Mannitol, a six carbon polyol, occurs in vegetative mycelium in spores of ectomycorrhizal fungus, *Pisolithus tinctorius* (Tabler and Tabler, 1982). This fungus and several other ectomycorrhizal fungal species grew on mannitol as a sole carbon source. Nevertheless, all these reports have been limited to a single concentration of mannitol under a single set of environmental conditions (Pons *et al.*, 1986). In the present study, *in vitro* growth on mannitol as a sole carbon source was 50 – 140 per cent of that of equimolar glucose. These observations suggest adaptation of the fungus to mannitol. Similarly, Pons *et al.* (1986) have proposed that conversion of host-derived fructose into mannitol within the fungal mantle of an ectomycorrhizal root serves to sequester carbohydrate from the host, since mannitol can be utilized by the fungus. Another possible function of mannitol in ectomycorrhizal fungi is resistance to environmental stress. Polyol accumulation in several organisms correlates with resistance to stresses caused by temperature and salinity in higher plants and salt stress in fungi. In the present study, *P. tinctorius* grew well on mannitol as a sole carbon source, though not as well as on glucose. Also, media containing mannitol was inoculated with inoculum previously grown on glucose. However, growth on mannitol was slightly, but consistently greater if the inoculum was prepared from a fungus previously grown on mannitol rather than on glucose, when mannitol-grown inoculum was used to inoculate a media containing glucose, growth was the same as that from glucose grown inoculum. Similar results were also reported on the influence of mannitol on the *in vitro* growth,
temperature optimum and subsequent ectomycorrhizal infectivity in *P. tinctorius*.

There are innumerable reports of increase in growth parameters by ectomycorrhizal inoculation of seedlings in forest trees (Beckjord *et al.*, 1985; Bougher and Malajczuk, 1990; Burgess *et al.*, 1994; Dell *et al.*, 1994; Natarajan *et al.*, 1995). In the present study, *P. tinctorius* inoculated seedlings of both test plants showed a significant increase in fresh biomass of the mycorrhizal plants and this could be attributed to the sustained water uptake by mycorrhizae (Lobanow, 1960). Better growth and greater fresh weight of pines and *Casuarina* due to *P. tinctorius* inoculation has been reported recently as well (Marx and Cordell, 1990; Marx, 1990; Rangarajan *et al.*, 1990; Marx *et al.*, 1991; Dell *et al.*, 1994; Natarajan *et al.*, 1995).

In the present study, *Pisolithus tinctorius* inoculated plants of both the test plants were found to possess significantly greater dry weight, greater amount of total chlorophylls, chlorophyll ‘a’ and ‘b’ and greater amount of organic constituents viz., total soluble and reducing sugars, total proteins, total soluble starch, total phenols and total lipids (Fig.11-14). The increase in dry biomass could be due to an increase in organic matter content of the plants. Such an increase in organic constituents viz., increase in total chlorophyll, sugars (Safir, 1968; Krishna, 1981), phenols (Krishna and Bagyaraj, 1984; Selvaraj and Subramanian, 1990) have been recorded in mycorrhizal plants. *P. tinctorius* inoculated plants possessed significantly greater mineral content in tissues examined viz., greater concentrations of N, P and K have been recorded over uninoculated ones (Fig.15). Voluminous literature is available in various reviews about the improved mineral nutrition of mycorrhizal plants (Marks and Kozlowski, 1973; Mikola, 1980; Marx *et al.*, 1991). The inoculated plants of *C. equisetifolia* and
*E. tereticornis* possessed significantly greater quantities of nitrogen than non-mycorrhizal plants. Even in the late 19th century, ectomycorrhizae were believed to be important in nitrogen absorption (Frank, 1894). Hatch (1937) analysed and found the seedlings of pines were not only larger, but also contained greater quantities of three major elements nitrogen, phosphorus and potassium per unit weight than their non-mycorrhizal controls. Similar results were also obtained in *Pinus nigra* (Clements *et al.*, 1977).

The uptake of nutrients are reported to take place through extramatrical mycelium of the mycorrhiza in the soil (Bowen, 1973) which has been earlier proved in a series of experiments using nutrients labeled with isolates (Melin *et al.*, 1958; Harbey and Wilson, 1959). Not only major and mineral elemental uptake but also concentrations of chlorophyll, soluble carbohydrates, proteins and phenols have also been enhanced by mycorrhiza and these changes take place well in advance of physical changes (Marx *et al.*, 1982).

In the present study, the benefits due to mycorrhizal inoculation was observed to be greater in *E. tereticornis* than in *C. equisetifolia* in all the parameters studied. Chlermpongse and Boonyuen (1990) found differential response of two host plant species viz., *E. camaldulensis* and *Pinus caribaea* var. Honduresnis on inoculation with *P. tinctorius*. *Eucalyptus* accumulated more amount of N, P and K in tissue owing to mycorrhizal inoculation than *Casuarina* demonstrating the differential host response to inoculation indicating thereby greater symbiotic association with the former than in the latter.

In India, reclamation of uncultivable lands or waste lands is gaining increased attention and various social forestry programmes have been
taken up by the government in this regard. One such plant species commonly used in waste land reclamation are the *C. equisetifolia* and *E. tereticornis*. In various districts of Tamil Nadu state, both *Casuarina* and *Eucalyptus* seedlings are raised in social forestry nurseries and are planted in adverse soil sites for soil reclamation. Harley (1969) pointed out that the primary colonizers of the barren areas should combine both the ability to fix atmospheric nitrogen arising from symbiosis with bacteria / actinomycetes and mycorrhizal fungi. *C. equisetifolia* is unique in the sense it is a plant with tripartite symbiosis as it possesses root nodules containing dinitrogen fixing actinomycetes (*Frankia* sp.), ectomycorrhiza and arbuscular mycorrhizal fungi (Lamont, 1982).

Thus, the present study deals with the ectomycorrhizal association of *P. tinctorius* with *Casuarina* and *Eucalyptus* plantations providing a clear picture of different aspects like *in vitro*, nursery and field performance. The characterization of the ten *P. tinctorius* isolates, collected from ten different tropical localities of three districts of Tamil Nadu, helps in understanding the behaviour of these isolates when cultured on artificial medium in the laboratory. A knowledge of the growth kinetics and the performance in association with the host plant roots *in vitro* can help in the selection of a suitable isolate for field practices. Selection of a suitable strain and also the mode of inoculation can serve as critical factors in afforestation practices. The present study has paved a way to better understanding of the fungal-host association.