ISOLATION AND EVALUATION OF ALUMINIUM TOLERANT ISOLATES OF ECTOMYCORRHIZAL FUNGI FOR RECLAMATION OF CONTAMINATED SOILS

A Thesis
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DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY

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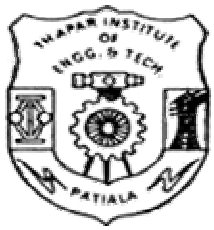

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CERTIFICATE

Certified that the thesis **"Isolation and evaluation of Aluminium tolerant isolates of ectomycorrhizal fungi for the reclamation of contaminated soils."** which is submitted by Ms. Babita Khosla, in fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** in the Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology (Deemed University), Patiala, is a record of the candidate's own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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Dated: *(Babita Khosla)*

Patiala, India.

TABLE OF CONTENTS

Chapter I

1.1 Mycorrhiza and its types

Mycorrhiza, a specialized root organ is the result of many complex interactions leading to a finely tuned symbiosis between a plant and a compatible fungus. As a consequence of this symbiotic relationship, the fungus supplies the basic enzymatic machinery for absorbing, translocating and assimilating major mineral ions. In turn the plant provides the peculiar ecological niche necessary for fungal growth and development including the completion of sexual cycle. The role played by soil borne extramatrical mycelial systems is particularly significant in mobilizing and translocating phosphorus and nitrogen to host plants. The term mycorrhiza**,** which literally means *fungus-root*, was first applied to fungus-tree associations described in 1885 by the German forest pathologist A.B. Frank. Since then we have learned that the vast majority of land plants form symbiotic associations with fungi. An estimated 95% of all plant species belong to genera that characteristically form mycorrhizae. The mycorrhizal condition is the rule among plants, not the exception (Harley and Smith, 1983).

Mycorrhiza can be mainly classified into three categories namely ectomycorrhiza (ECM), endomycorrhiza commonly called arbuscular mycorrhiza (AM) and ectendomycorrhiza. About two-thirds of the plants are symbiotic with AM glomalean fungi, while a relatively small number of plants develop ectomycorrhiza and they dominate forest ecosystems. Ectomycorrhizae are common in both Gymnosperms (Pinaceae, Cupressaceae) and Angiosperms (Juglandaceae, Fagaceae, Betulaceae, Tiliaceae, Salicaceae, Ulmaceae, Rosaceae, Leguminosae, Sapindacea, Aceraceae, Myrtaceae, Ericaceae and Dipterocarpaceae) (Smith and Read, 1997). The fungus partner mainly belongs to higher

fungi, which produce mushrooms and puffballs. Certain Ascomycetes, such as truffles also form ectomycorrhizae (Trappe, 1977). Among the Basidiomycetes there are Hymenomycetes such as *Boletus, Cortinarius, Suillus, Amanita, Laccaria, Lactarius* and *Leccinum*. The Gastromycetes include such examples as *Rhizopogon, Pisolithus* and *Scleroderma* (Smith and Read, 1997). It is estimated that over 2100 species of these fungi can form ectomycorrhizae with forest trees.

Among all the above three, ectomycorrhiza are of great significance to forestry species. The fungus in the mycorrhiza infection zone grows faster than the root and wraps the root up in a pseudo-parenchymatous layer of hyphae called mantle. The fungal mycelium between the root cortical cells is called 'Hartig net' and it may be restricted to the outer most cell layers of the root or can reach in as far as the endodermis (as shown in Figure 1.1).

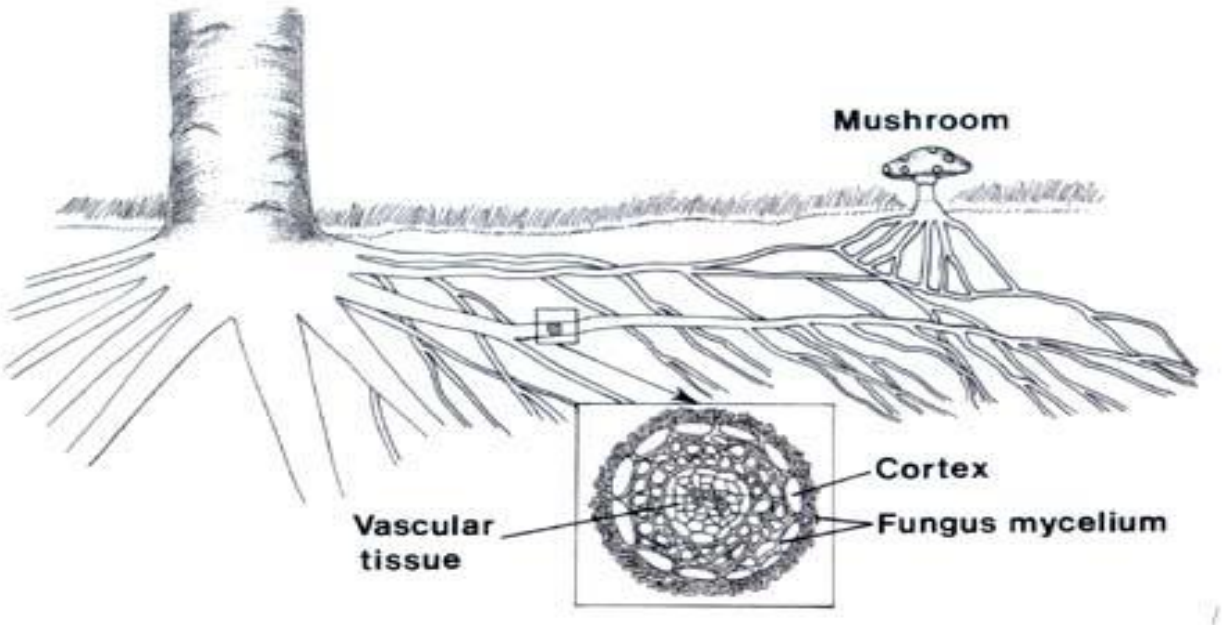

Figure 1.1: Symbiotic association of the fungus and the root of the plant showing the peculiar features of ectomycorrhizal root

Ectomycorrhizal development on forest trees is initiated from propagules (spores or hyphae) of the fungal symbionts in the rhizosphere of the feeder roots. The propagule is stimulated by the root exudates, resulting in hyphal growth over the feeder root surface to ultimately form the mantle. Following the sheath formation, hyphae develop intercellularly in the root cortex to form the Hartig net, which may completely replace the middle lamellae between the cortical cells. The morphology of ectomycorrhizae is determined by the interaction between the host root, the fungal symbionts and the environment. The infected root may be initially simple and non-forked and then proceed through several morphological changes until finally a complex coralloid configuration is achieved. Certain ectomycorrhizae may remain in the non-forked form while other exists as simple bifurcate structures. The host derives many benefits from symbiotic association with ectomycorrhizal fungi. Some of these are (1) longevity of its feeder roots (Smith and Read, 1997) (2) increased rate of nutrients uptake from soil (3) selective absorption of certain ions from the soil (Bowen, 1973) (4) resistance to feeder root pathogens (Marx, 1969) (5) increased tolerance to soil toxins, cold temperatures and high temperatures (Marx *et al.*, 1970; Marx and Bryan, 1971) and (6) increased tolerance to adverse soil conditions. The ectomycorrhizal fungi not only derive the necessary nutrients for their growth from the host, but also find protection and escape from the intense microbial competition in the soil by occupying their specific niche in and on the host root.

1.2 Origin of the problem

Pollution of the biosphere with toxic metals due to man-made activities poses a major environmental and human health problem. In addition to metals of geochemical origin, sometimes reaching high concentrations (Jeng and Bergseth, 1992), the sources of metals

in soil are diverse, including burning of fossil fuels, mining and smelting of metalliferous ores, municipal wastes, fertilizers, pesticides, sewage sludge amendments, the use of pigments and batteries (Darbon *et al.*, 1992). Metals in soil are present as free metal ions, soluble metal complexes, exchangeable metal ions, organically bound metals, precipitated or insoluble compounds such as oxides, carbonates and hydroxides or they may form part of the structure of silicate minerals. The toxicity of metals in soil depends on their bioavailability. According to Hartley *et al.* (1997), metal bioavailability is a function not only of their total concentration but also of physico-chemical (pH, organic matter, clay content) and biological (biosorption, bioaccumulation and solubilization) factors. The problem of toxic metals becomes more severe in acidic soils as the metals are present in the free ionic form in acidic pH range. Acid soil infertility is a major limitation to crop production on highly weathered and leached soils in both tropical and temperate regions of the world (Sanchez, 1976; Von Uexküll and Mutert, 1995). In addition, soil acidification induced by the activities of mankind has become of increasing concern in recent years. Common anthropogenic causes of acidification include leaching with acid rainfall (the result of industrial pollution) and nitrification following applications of nitrogenous fertilizers (Wild, 1988). Two fundamental factors limit the fertility of acid soils; nutrient deficiencies e.g. phosphorus, calcium, magnesium and the presence of phytotoxic substances e.g. soluble aluminium and manganese. In most situations, poor growth in acid soils can be correlated directly with aluminium saturation (Abruna-Rodriguez *et al.*, 1982; Sartain and Kamprath, 1977) and atmospheric pollution leading to soil acidification and elevated concentrations of aluminium is one of the predominant threats to forest ecosystem. Acid soils occupy about 30% of the world's ice-free land area and occur in two main global

regions; the northern cold temperate belt and the southern tropical belt (Von Uexküll and Mutert, 1995). The latter belt includes Southeast Asia, Africa and central South America. In India, acidic soils occupy a considerable area and are found to occur in the Himalayan region, the eastern and north-eastern plains, peninsular region and the coastal plains under varying environmental conditions covering an area of 93.7 million hectares. Assam along with Manipur, Tripura, Meghalaya, Arunachal, Nagaland and Mizoram in the north-eastern part of the country has probably the largest stretches of acidic soils followed by the neighbouring states of West Bengal, Bihar and Orissa. In the coastal state of Kerala, high rainfall and temperature along with rich organic deposits due to the formation of peats under marshy conditions have been responsible for the extension of acid soils (Mandal *et al.*, 1980).

Recently, great attention has been paid to the problem of aluminium that severely contaminate soil leading to wide spread seedling mortality and several decades of delay in revegetation schemes (Ritchie *et al.*, 1985). Concerning the high deposition rates of NO_3^- and SO_4^{2-} are inducing soil acidification and high concentrations of dissolved aluminium has resulted in the screening of many forest tree species for aluminium sensitivity (Schaedle *et al.*, 1989). The tolerance of vascular plants to aluminium is now a major area of research worldwide because aluminium is the third abundant element after Silicon and oxygen in earth crust approximately 8.8% of its weight (The Merck Index, 1989). At toxic concentrations in soil solutions, aluminium can damage roots, interact with the uptake of other mineral elements and impair many physiological processes. The evidence suggests that aluminium interferes with calcium and magnesium uptake, reducing shoot

concentrations of one or both of these elements to levels which are known to cause deficiency symptoms (Jorns and Hecht-Buchholz, 1985).

1.3 Mycorrhizal fungi: a solution to the problem

The response of mycorrhizal fungi to toxic metals is of importance in view of interest in the reclamation of polluted sites and influence on plant growth and productivity. Trees are frequently used for the reclamation of contaminated and strip mined lands, if the community structure of soil microorganisms is significantly affected, this could compromise the success of the site remediation. The rhizosphere hosts a large and diverse community of microorganisms that compete and interact with each other and with plant roots. Soil fungi usually show an optimal pH for growth 1-2 pH units lower than bacteria from the same environment thereby indicating that fungi have a higher tolerance to acidity and to the presence of toxic metals. Fungal survival in the presence of toxic metals mainly depends on intrinsic biochemical and structural properties, physiological and/or genetical adaptations, including morphological changes and environmental modification of metal speciation, availability and toxicity, the relative importance of each often being difficult to determine (Gadd and Griffiths,1978; Gadd, 1990, 1992). The association of ectomycorrhizal fungi with tree roots greatly alters both root morphology and physiology. They have been shown to enhance seedling growth and alter patterns of ion uptake and plant nutrition. The ectomycorrhizal fungi improve the metal tolerance of their host plant by primarily accumulating metals in walls of extramatrical hyphae and extrahyphal slime, thus passage of metals to shoot is restricted (Galli *et al*., 1994). Mycorrhizal fungi also play an important role in providing access to mineral nutrients at all stages of plant development through their ability to mobilize the nutrients from their complex form as found in acidic

soils and by increasing the total absorbing area of the root. Benefits accrued by seedlings in the mycorrhizal state are especially important under adverse rhizospheric conditions where phosphorus is limiting and aluminium and other metals are high (Bjorkman, 1970; Denny and Wilkins, 1987; Jones and Hutchinson, 1988). The survival and growth advantage of mycorrhizal seedlings has been repeatedly reported for seedlings grown on strip mine spoil sites (Marx and Bryan, 1975; Marx and Artman, 1979) and similar advantages would be expected in natural ecosystems where edaphic factors such as high soil acidity may limit plant growth.

1.4 Aim of the study

Although aluminium is the most abundant metal in the Earth's crust, it lacks biological functions and shows a low bioavailability. Acid rain however solubilizes aluminium to toxic levels. Most research on the biological effects of aluminium has been centered on the analysis of aluminium tolerant plants and also several studies have been reported concerning aluminium effects on microorganisms, with more interest to mycorrhizal fungi and soil bacteria. Competition with calcium and magnesium, thus impairing related physiological functions, disturbing phosphorus metabolism and binding to DNA, membranes or cell walls, inhibition of cell division are considered the main toxic effects of aluminium. In the present study, different ectomycorrhizal fungi were screened for their tolerance to aluminium and the mutants were developed which can tolerate high concentration of aluminium, which could be used for reclamation of aluminium contaminated soils.

Objectives:

1. Study the tolerance level of aluminium by different ectomycorrhizal fungi.
2. Isolation of aluminium tolerant mutants of ectomycorrhizal fungi.
3. Study mineral nutrition of aluminium tolerant and wild type isolates in pure culture.
4. Influence of aluminium on plant mineral nutrition in presence of mycorrhizae formed by aluminium tolerant isolates.
5. Influence of ectomycorrhizal fungus on the growth and mineral nutrition of its host plant grown in aluminium contaminated soils.

Chapter II

2.1 Aluminium toxicity

Heavy metal contamination caused by either natural processes or by human activities is one of the most serious environmental problems (Reddy and Prasad, 1990) and because plants function as primarily entry points of heavy metals into food chain, it is important to analyze distribution of different heavy metals in plants. Since most of the plants are mycorrhizal under ecological conditions it seems appropriate to review interactions of mycorrhizal fungi with heavy metals.

Aluminium is the third most abundant chemical element in the Earth's crust with no biological activity. Most aluminium is incorporated into aluminosilicate soil minerals, with only small quantities appearing in soluble forms that can influence living organisms (May and Nordstrom, 1991). However aluminium becomes increasingly soluble as the soil pH decreases below 5.0.

Ulrich (1980) have hypothesized that increased soil acidity leads to enrichment of soil with aluminium, where concentrations of aluminium species can reach levels toxic to biological system and this is a major factor affecting forest soils. Soil acidification results from: imbalances in nitrogen, sulphur and carbon cycles (Bolan and Hedley, 2003; Tang and Rengel, 2003); excess uptake of cations over anions (Tang and Rengel, 2003); continuous use of ammonia- and amide-containing fertilizers (Mahler *et al.*, 1985); and nitrogen-fixation by legumes (Bolan *et al.*, 1991; Coventry and Slattery, 1991; Tang and Rengel, 2003).

Ryan *et al.* (1993), Blancaflor *et al.* (1998), Sivaguru and Horst (1998), Zhang *et al.* (1998), Vazquez *et al.* (1999), Zhang and Rengel (1999), Ahn *et al.* (2001), Ma *et al.*

(2002) reported the inhibition of root growth as one of the earliest and most dramatic symptom exhibited by plants suffering from aluminium stress and this symptom has been observed within hours, or even minutes, of exposure to micromolar concentrations of aluminium in solution cultures. However, with prolonged exposure to aluminium, plants exhibit a myriad of toxicity symptoms on both roots and shoots.

Wallace and Anderson (1984), Horst (1995), Frantzios *et al*. (2001) reported aluminium-induced inhibition of root growth often precedes, or coincides with, a decline in cell division. Therefore, the rapid Al-induced inhibition of root growth is likely to be caused by inhibition of cell elongation rather than cell division.

Lazof and Holland (1999) and Silva *et al.* (2000) reported that the disruption of cell division due to aluminium binding to nuclei of root tip cells leading to decreased cell production, responsible for impedance of root growth during prolonged exposure to aluminium.

Ryan *et al.* (1993) and Kollmeier *et al*. (2000) studied that inhibition of root growth requires the root apex, in particular the distal part of the elongation zone within the apex to be directly exposed to aluminium. These findings indicate that the root apex is a critical site of perception and expression of aluminium toxicity and resistance. Unfortunately, measurements and observations on the root apex exposed to aluminium are relatively rare in the literature on aluminium toxicity and resistance. Most studies report on the whole root systems, providing limited information for understanding of the primary mechanisms of aluminium toxicity and resistance.

Rengel (1996) reported that aluminium enters the cells, probably after damaging the root cell membranes and once within the cell it reacts with phosphorus compounds, and upsets

the plant phosphorus metabolism. The plant tops of aluminium toxic plants appear typically phosphorus deficient. This reflects aluminium dislocation of the plant phosphorus metabolism. The occasional observation of yellow spots or pale flecking of the leaves of grasses or cereals may reflect effects of aluminium on other metabolic processes. The shoot growth is stunted; leaves stubby and die back at tips; leaf colour dull green; leaves and stems develop purple tints similar to phosphorus deficiency. The mechanism of the Al/P interaction is thought to be an adsorption – precipitation reaction between Al and P at the root surface or in the root free space i.e. cell walls (McCormick and Borden, 1974; Naidoo *et al.*, 1978). The phosphorus may be adsorbed by hydroxy-Al already precipitated in the root free space or the phosphorus may be precipitated as insoluble aluminium phosphates; both occurrences are likely. The practical result is that phosphorus deficiency symptoms are common in plants suffering from aluminium toxicity (Foy, 1988; Haynes, 1984). The most characteristic symptom of aluminium toxicity in solution cultures is the development of thickened, stubby and distorted root systems. These symptoms result from the effect of aluminium restricting cell division and cell expansion in the roots. Since root growth is restricted, the ability of the plant to explore the soil volume for nutrients and water is much reduced. As a result, nutrient and/or water stresses are common in plants suffering from aluminium toxicity. Under field conditions, it is often difficult to observe root systems because affected plants are very susceptible to moisture stress and die easily. Among earliest symptoms of aluminium toxicity in different plant systems, reduction of net $Ca^{2+}$and Mg^{2+} uptake (Huang *et al.*, 1992; Rengel, 1992, 1994; Rengel and Elliott, 1992; Jones *et al.*,1995), reductions in P uptake (Huang *et al.*, 1992; Mugwira, 1980), blockage of plasma membrane embedded Ca^{2+} channels (Ding *et al.*, 1993; Huang *et al.*, 1996; Rengel

et al., 1995), interferes in active ion uptake processes functioning across the root-cell plasma membrane (Kochian, 1995; Wright, 1989), reduction in K^+ efflux (Staβ and Horst, 1995), accumulation of callose (Wissemeier and Horst, 1995), and extrusion of malate (Delhaize *et al*., 1993) have been shown to occur within the first 30 minutes of exposure to aluminium.

2.2 Aluminium solubility in soils

Soil chemical factors that limit root growth in acid soils diminish crop production; include the presence of aluminium and manganese cations, and also deficiency or unavailability of calcium, magnesium, phosphorus, molybdenum and silicon. These effects are further complicated by interactions of alumnium with other ions in different plant genotypes and under stress conditions (Foy *et al*., 1978).

Haynes (1984) reported that the practice of liming acid soils, i.e., applying $CaCO_3$, in order to raise soil pH and precipitate exchangeable aluminium as insoluble hydroxy-aluminium derivatives has long been recognized as necessary for ameliorating the toxic effects of aluminium. However, in many acidic soils large quantities of lime e.g. 2–10 tonne/ hectare are commonly required to achieve adequate growth responses. Secondly, the low phosphorus status of highly weathered acidic soils is a particular problem because large amounts of phosphorus need to be applied in order to raise concentrations of available soil phosphorus to an adequate level (Sanchez and Uehara, 1980). This is because such soils contain large quantities of aluminium and iron hydrous oxides which have the ability to adsorb phosphorus onto their surfaces. Thus, much of the added phosphorus is ‘fixed’ and is not readily available for plant use.

Delhaize and Ryan (1995) and Kollmeier *et al.* (2000) documented the cytotoxicity of aluminium in plants. It is generally known that plants grown in acidic soils, due to aluminium solubility at low pH have reduced root systems and exhibit a variety of nutrient-deficiency symptoms, with a consequent decrease in yield.

Wilkins *et al*. (1989) reported that aluminium is an abundant and occasionally toxic component of acidic soils and acid rain may increase concentration of soluble aluminium in soil to toxic levels.

ACID RAIN

$(NH_4^+, SO_4^{-2}, NO_3^-)$

⇓

SOIL ECOSYSTEM

Composition, Texture, Aeration, Microorganisms

$[H^+]$

⇓

$$Al(OH)_3 \Leftrightarrow Al(OH)^{2+} \Leftrightarrow Al(OH)^{2+} \Leftrightarrow Al^{3+}$$

Figure 2.1 Interactions of acid precipitation with soil ecosystem and factors affecting solubilization of aluminium ions from soil. (Piña and Cervantes, 1996)

Baligar *et al*. (1988) reported that at a soil pH 5.5, aluminium form complexes with inorganic phosphorus and inhibit its uptake by the plant. Low pH and poor nutrient availability also enhance the competitive absorption of aluminium over calcium and magnesium by their displacement from cell apoplast exchange sites. In addition to reduced root growth, reduced uptake of calcium and magnesium is a common feature

accompanying aluminium phytotoxicity. The ability of aluminium to substitute for magnesium in biological systems is derived from a high association constant with diverse ligands.

Macdonald and Martin (1988) reported that aluminium binds almost 10^7 times more tightly to ATP (adenosine tri-phosphate) than magnesium does, which means that aluminium concentrations lower than nanomolar are required to compete with millimolar magnesium concentrations. Aluminium may also interfere with phosphorus uptake (Marschner, 1991).

Aluminium exists in soils in many mineral forms including hydrous oxides, aluminosilicates, sulphates and phosphates and is also present in water and air but most of it is incorporated into aluminosilicate soil minerals and only very small quantities (at submicromolar levels) appear in soluble forms capable of influencing biological systems (May and Nordstrom, 1991).

Martin (1986) and Macdonald and Martin (1988) indicated that the proportion of the different oxidation forms of aluminium is a function of the environmental pH and that small variation in acidity cause great changes in the concentration of each of those species mentioned above in figure 2.1.

Bruce *et al*. (1988) found that in soils with a pH of 5.8, the concentration of free aluminium is 6.3µM. Lowering the pH to 4.77 increased the Al^{3+} level to 700 µM, whereas increasing the pH to 6.22 dimnished Al^{3+} concentrations to 5µM. The hydrated aluminium silicates are relatively insoluble and so accumulate in the soil, contributing to the soil acidity (Somani *et al.*, 1996).

Wild (1988) reported that in acidic soils, an appreciable portion of the cation exchange capacity is satisfied by Al ions i.e. the soils have a high aluminium saturation. Whilst these

Al ions are referred to as exchangeable Al^{3+}, they are a mixture of monomeric Al ions [Al^{3+}, $AlOH^{2+}$, Al $(OH)_2^{+}$] with an average charge per Al between 2 and 3, decreasing as pH increases. Different forms of aluminium occur in soil solution: $Al(OH)^{2+}$ and $Al(OH)_2^{+}$ at pH 4–5, Al^{3+} at pH 5.5–7, and $Al(OH)_4^{-1}$ at pH 7–8. The status of $Al(OH)_2^{+}$ and $Al(OH)^{2+}$ is uncertain although experimental results have appeared indicating Al-OH toxicity. Intensification of the process of aluminium compounds solubilization is connected with the degree of soil acidification caused by the washing out of alkaline metals ions (Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}) from the soil and a decrease in the pH of soil solutions.

2.3 Aluminium toxicity and mycorrhizas

The advantages of a diverse and healthy mycorrhizal community include better survival and nutrition of plants in stressed environments. Mycorrhizas in forests are almost universal in vascular plants and occur in most soils. The general consensus suggests that mycorrhizal plants exploit similar sources of soil phosphorus as do nonmycorrhizal plants but have a greater effective absorbing surface area. The hyphae of both ectotrophic and endotrophic mycorrhizas extend from the root surface to much greater distances through the soil than root hairs, and act as a secondary root system that facilitates the uptake of certain nutrients (phosphorus, zinc and possibly sulphur) by the host. Rousseau *et al*. (1994) found that while extramatrical mycelia (aggregates of hyphae) accounted for less than 20% of the total nutrient absorbing surface mass, they contributed nearly 80% of the absorbing surface area of pine seedlings. All mycorrhizas can improve plant growth by enhancing the uptake of phosphorus, and in P-deficient soils mycorrhizal plants grow markedly better than nonmycorrhizal ones (conversely in nutrient-rich soils plants tend to

have very low levels of infection) because the hyphae can explore soil inaccessible to the plant roots and enable plants to overcome limitations to diffusion.

Blaudez *et al*. (2000) studied thirty nine ectomycorrhizal fungal isolates of *Paxillus involutus, Pisolithus tinctorius, Suillus bovinus, S. luteus* and *S. variegatus* on multimetal amended medium to determine their *in vitro* tolerance and found strong interspecific variation in metal tolerance. *S. luteus* and *P. tinctorius* were more tolerant to copper, cadmium and zinc than *Paxillus involutus,* while the reverse was true for nickel.

Smith and Read (1997) reported that ectomycorrhiza are adapted to systems in which nitrogen is limiting, and all nutrients and water taken up by the plant go through the ectomycorrhizal fungus. Further, mycorrhizal associations have been related to ecosystem characteristics, such that ectomycorrhizas are concentrated in the organic layer, and VA mycorrhiza in the mineral layer.

Wit *et al.* (2001) reported aluminium, a key element in critical load calculations for forest and studied the effect of two levels (100 and 200 μmol L^{-1}) of enhanced aluminium concentrations and lowered Ca: Al ratios in the soil solution in a field manipulation experiment in a three year mature spruce stand. The results showed that the only evidence for *in situ* toxicity of aluminium till concentrations of 200 μmol L^{-1} was reduced uptake of magnesium. Fine root production was not affected by aluminium concentration. However, similar concentrations were also reported to reduce root growth and calcium and magnesium uptake for spruce seedlings in nutrient solution studies. Possibly factors like mycorrhiza and rhizosphere processes (exudation of organic acids) play a role in protecting the root from exposure to aluminium in the field.

Rengel (1992) reported that the interactions between Al^{3+} and Ca^{2+} have long been implicated in aluminium phytotoxicity because symptoms of severe aluminium toxicity in the field resemble those of Ca^{2+} deficiency, and supplementation of Ca^{2+} can substantially alleviate Al-stress symptoms. Given the important and critical roles of Ca^{2+} in plant metabolism (Kauss, 1987; Grabski *et al.*, 1998), development and signal transduction (Gilroy *et al.*, 1993; Bush, 1995; Trewavas, 1999; Knight, 2000; Pandey *et al.*, 2000; Plieth, 2001; Sanders *et al.*, 2002), it is not surprising that interactions between Al^{3+} and Ca^{2+} have drawn considerable attention in studying aluminum phytotoxicity.

Cosgrove (1997) reported inhibition of cell elongation, the process that involves cell wall relaxation as one of the most dramatic effects of aluminium on plants. Hence, interactions of Al^{3+} with the cell wall components (Blamey *et al.*, 1990; Horst, 1995; Reid *et al.*, 1995; Rengel, 1996; Blamey, 2001), in particular, displacement by Al^{3+} of Ca^{2+} ions that occupy critical sites in the apoplasm, could be, at least partly, responsible for the observed aluminium toxicity symptoms (Kinraide and Parker, 1987; Reid *et al.*, 1995; Ryan *et al.*, 1997).

Rengel and Zhang (2003) reported that the disruption of cytoplasmic Ca^{2+} homeostasis as a primary trigger of aluminium toxicity. Aluminium causes an increase in cytosolic Ca^{2+} activity, potentially disrupting numerous biochemical and physiological processes, including those involved in the root growth.

Sverdrup and Warfvinge (1993) and Cronan and Grigal (1995) reported that the aluminium contents or the Ca/Al ratio in roots or in ectomycorrhizas as valuable indicators in the assessment of the ecological risk of soil acidification or potential acid soil infertility, in addition to the accepted indicators as the Ca/Al ratio of the soil solution.

Jentschke and Goldbold (2000) reviewed the metal toxicity in ectomycorrhiza. They indicated that the ectomycorrhizas can help in alleviation of metal toxicity in forest tree seedlings and concluded in their review that the role of metal sorption by fungal tissues reduce the metal exposure of the host plant.

Martin *et al.* (1994) reported the accumulation of aluminium by vacuole polyphosphates in the mycelium of the basidiomycete *Laccaria bicolor* grown with 0.5mM $AlCl_3$ and even after a nine day incubation in medium lacking aluminium, the metal remained as Al-polyphosphate complex.

Schier *et al.* (1995) found that aluminium toxicity has been associated with the inhibition of root and shoot growth in ectomycorrhizal and nonmycorrhizal pine seedlings but has less effect on the growth in ectomycorrhizal seedlings. Foliar symptoms of aluminium toxicity (chlorosis and tip necrosis) were more pronounced in nonmycorrhizal than ectomycorrhizal seedlings. Amelioration of aluminium toxicity by mycorrhizal colonization appeared to result from enhanced uptake of nutrients, especially phosphorus rather than to reduce uptake of aluminium. The histological examination of roots showed that aluminium caused greater ultrastructural changes in the roots of nonmycorrhizal seedlings than in the host component of ectomycorrhizal roots, perhaps the fungal sheath protected the host from the detrimental effects of aluminium by acting as a physical barrier to the radial movement of aluminium.

Cumming and Weinstein (1990c) noted that the association of the ectomycorrhizal symbiont *Pisolithus tinctorius* with the roots of the *Pinus rigida* seedlings grown in sand culture led to the maintenance of normal foliar phosphorus concentration under aluminium exposure whereas uninoculated seedlings exhibited altered patterns of foliar phosphorus

accumulation because aluminium complexes inorganic phosphorus thus reducing its availability for plant uptake.

Hartley *et al*. (1997) studied the effects of multiple metal contaminations on ectomycorrhizal Scots pine (*Pinus sylvestris*) seedlings and found that although contaminated and metal amended soil significantly inhibited root and shoot growth of the ectomycorrhizal Scots pine but total root tip density of ectomycorrhiza was not affected.

Wilkins and Hodson (1989) showed in their study the tolerance of Norway spruce to aluminium and found that the presence of *Paxillus involutus* in the rhizosphere (no proper infection) reduced the negative effects of aluminium on plant growth. They also reported large decrease in root Ca and Mg concentration in aluminium treated plants. The mechanism responsible for increased tolerance was not clear.

Taylor (1988) reported that aluminium influences the plant nutrient status, generally manifested by imbalances or deficiencies of calcium, phosphorus and to a lesser extent magnesium. The reductions of these elements in plant tissues may occur at aluminium concentrations well below those causing direct injury.

Zel and Bevc (1993) reported the drastic effects in the mineral content of *Lactarius piperatus* and *Amanita muscaria* mycelia when exposed to elevated concentrations of aluminium. They found increase in aluminium, calcium and sodium contents while decrease in phosphorus, magnesium and potassium contents with increasing Al^{3+} concentration as compared to control mycelium of both the fungi.

Egerton-Warburton and Griffin (1995) studied that *Pisolithus tinctorius* isolates found in rehabilitated and forest-sites showed reduction of mycelial growth with increased concentration of aluminium and lowered levels of primarily calcium and secondarily

magnesium. In ectomycorrhizal trees the impairment of growth caused by aluminium toxicity was found to be associated with the competitive exclusion of calcium, magnesium by aluminium, but inoculation with *Pisolithus tinctorius* benefited plant through an increased plant calcium and magnesium content and reduction in aluminium contents.

Cumming *et al*. (2001) reported the significantly reduced concentrations of calcium, magnesium and iron in mycelia of *Laccaria bicolor* and *Pisolithus tinctorius* by exposure to aluminium and further, the concentration of aluminium increased in the mycelia with increasing aluminium concentrations in the medium.

Reddy *et al.* (2002) studied the influence of aluminium on mineral nutrition of ectomycorrhizal fungi *Pisolithus sp*. and *Canthrellus cibarius* and found that the growth of ectomycorrhizal fungi was inhibited by the presence of aluminium in the culture medium and further exogenous supply of aluminium caused its accumulation in the mycelia of ectomycorrhizal fungi. Aluminium poorly affected calcium, magnesium, potassium and phosphorus level in *Pisolithus sp*. whereas *C. cibarius* accumulated these elements in response to exogenously supplied aluminium. The acid phosphatase activity of hyphae increased in the presence of aluminium.

Turnau and Dexheimer (1995) reported the induction of acid phosphatase activity of ectomycorrhizal fungi on exposure to cadmium and thus suggesting its role in heavy metal detoxification.

Gerlitz (1996) detected higher levels of phosphorus uptake and mobile polyphosphate concentration in *Suillus bovinus* in the presence of aluminium and suggested that Al-'stressed' fungal cells produce polyphosphate to counteract mobile Al ions within hyphae. If one keeps in mind the diversity of substrates of acid phosphatases, these hypotheses are

not exclusive and it can be concluded that the roles of these enzymes in aluminium resistance are probably multiple.

2.4 Mycorrhizas and Aluminium sensitivity of tree seedlings

The studies on ectomycorrhizal fungi have been demonstrated to alleviate growth depressions of tree seedlings due to the toxic effects of aluminium (Cumming and Weinstein 1990a, Hentschel *et al.* 1993, Schier and McQuattie 1995, 1996).

Schier and McQuattie (1995) studied the effect of aluminum on the growth, anatomy and the nutrient content of ectomycorrhizal and nonmycorrhizal seedlings of *Pinus strobus* and found significantly lower potassium, magnesium and boron foliar concentrations and significantly higher phosphorus foliar concentration in *Pisolithus tinctorius* inoculated seedlings than uninoculated seedlings of *P. strobus* over all aluminium levels.

Colpaert *et al.* (1992, 1993) reported that ectomycorrhizal fungi can protect their host trees against heavy metal toxicity. The ultimate goal of a successful revegetation strategy for metal-polluted soils is the establishment of a sustainable ecosystem, whether it is grassland or woodland, in which transfer of metals to aboveground plant parts is restricted, unless phytoextraction of metals is desired. The stability of such a 'managed' ecosystem will depend on the colonization potential, the survival and performance of metal-tolerant representatives of the major functional groups that are characteristic for a terrestrial ecosystem and that are supposed to be essential for its functioning. Ectomycorrhizal fungi are certainly such an essential group in forest ecosystems. They also suggested that the elevated soil metal concentration is responsible for the evolution of adaptive tolerance in ectomycorrhizal symbionts.

Turner (1994), Wilkinson and Dickinson (1995) and Leyval *et al.* (1997) indicated that the colonization of tree roots by ectomycorrhizal fungi can increase tolerance of their hosts to the presence of metals in the soils in toxic concentrations. In addition, ectomycorrhizal fungi and ectomycorrhizal plants might be used to rehabilitate soils containing large quantities of heavy metals.

Kong *et al.* (2000) studied the biochemical responses of mycorrhizas in *Pinus massoniana* with *Pisolithus tinctorius* to combined effects of aluminium, calcium and low pH and found inhibition of enzymes involved in nutritive metabolism particularly acid and alkaline phosphatase in the roots, stems and leaves of plant in response to artificial acid rain and aluminium. Infection with mycorrhizal fungus *P. tinctorius* at the root of *P. massoniana* increased the ability of the plant to resist the toxicity of artificial acid rain and aluminium stress.

Brunner and Frey (2000) reported the localization of aluminium and other heavy metals such as cadmium, copper, nickel or zinc in *Picea abies* seedlings colonized with *Hebeloma crustuliniforme.* They found distinct metal accumulation in the fungal mantle, Hartig net and in the cell walls of the root cortical cells by X-ray microanalysis. Treatment with the highest metal concentrations showed high X-ray counts of metals in cells of the stele but reduced concentrations of the macronutrients potassium, magnesium and phosphorus in roots, indicating a possible disturbance of root and ectomycorrhizal function.

Haynes and Mokolobate (2001) reported that additions of organic residues to acidic soils can reduce aluminium toxicity (thus lowering the lime requirement) and improve phosphorus availability. Complexation of aluminium by the newly-formed organic matter will tend to reduce the concentrations of exchangeable and soluble aluminium present. As

organic residues decompose, phosphorus is released and this can be adsorbed to oxide surfaces. This will, in turn, reduce the extent of adsorption of subsequently added phosphorus thus increasing its availability. The practical implication of the processes discussed is that organic residues could be used as a strategic tool to reduce the rates of lime and phosphoric fertilizers required for optimum crop production on acidic, phosphate-fixing soils.

2.5 Mechanisms of amelioration

Tolerance to aluminium in many higher plants is associated with extra cellular chelation of aluminium by extruded ligands. Organic and inorganic oxyanions released from roots form stable complexes with aluminium in the rhizosphere. Several reports have focused on the production of organic acids by ectomycorrhizal fungi under aluminium exposure and these systems have been linked to aluminium tolerance in higher plants (Delhaize and Ryan, 1995; Kochian, 1995).

Cameron *et al.* (1986) and Hue *et al*. (1986) found that aluminium toxicity is reduced in the presence of inorganic and organic complexing ions in soil solution. Ahonen-Jonnarth *et al.* (2000) found that ectomycorrhizal seedling system exposed to elevated concentrations of aluminium, cadmium, copper or nickel produced organic acids in more concentrations than in the controlled system. The association of ectomycorrhizal fungi with the roots of many trees often alters tree response to metals in the soil environment and reduced metal uptake may result from the preferential binding of metals to fungal mycelia or from the production of extracellular metal chelating compounds (Denny and Wilkins, 1987; Cumming and Weinstein, 1990a; Jones and Hutchinson, 1988).

Ernst *et al.* (1992) reported that metal tolerance of higher plants may be due to a range of potential processes (as shown in Figure 2.2). These may include (1) a reduction of metal exposure by excretion of chelating substances, (2) extracellular sequestration (e.g. by mucilage, pH gradients in the rhizosphere), (3) modified uptake systems at the plasmalemma, or (4) intracellular detoxification. The significance of these processes may vary as a function of the metal involved, its concentration, and the location of the primary lesion caused by the metal. Mycorrhizal fungi may alter metal sensitivity of their hosts, theoretically, by any of the mechanisms outlined above, by either directly affecting metal availability and speciation or indirectly modifying plant physiological processes.

2.5.1 Metal mobility in the fungal apoplast: filtering of toxic metals in the hyphal sheath or Hartig net by adsorption (exclusion mechanism 1)

Gadd (1993) reported that fungi can effectively bind metals to cell walls or extracellular polysaccharides. In addition, intracellular uptake and accumulation of certain metals in vacuoles may be significant. Binding of toxic metals to cell walls has been suggested as a tolerance mechanism both in higher plants (Ernst *et al.*, 1992) and fungi (Ross, 1993). Although the significance of the proposed mechanism is not clear in both fungi (Ross, 1993) and higher plants (Ernst *et al.*, 1992), a similar mechanism has been suggested to protect mycorrhizal roots from metal toxicity. It was suggested that sorption of metals to fungal tissues or intracellular uptake and detoxification in fungal vacuoles subsequently reduced metal uptake into the host plant (Jones and Hutchinson, 1986).

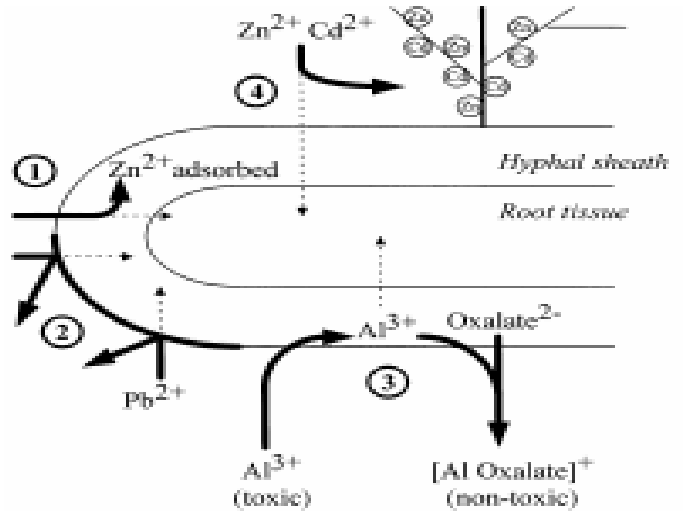

Figure 2.2: Metal exclusion mechanisms possibly operating in ectomycorrhizas (adapted from Jentschke and Godbold, 2000)

2.5.2 Fungal hydrophobicity: restricted metal mobility in the fungal apoplast (exclusion mechanism 2)

Besides metal sorption to fungal cells, metal uptake into host tissues may be affected by the degree of hydrophilicity of the fungal apoplast. The fungal sheath formed by a fungus (Unestam, 1991) could provide a barrier to apoplastic radial transport of water and ions. In ecto- and endomycorrhizal fungi heavy metals were demonstrated to be bound to cell wall components such as chitin, cellulose derivatives and melanin (Galli *et al.*, 1994). Extrahyphal slime and polyphosphate linkage of copper and zinc was observed to be the amelioration mechanism in *Pisolithus tinctorius* (Tam, 1995). All this means that protective effect is directly proportional to the amount of extramatrical mycelium, as has been found in a study of cadmium and mycorrhizal *Pinus sylvestris* (Colpaert and van Assche, 1993).

2.5.3 Chelation by organic acids and other substances released by mycorrhizal fungi (exclusion mechanism 3)

In addition to the large surface area produced by mycorrhizas there are reports that they excrete organic acids which may have a direct influence in the mobilisation of nutrients. Increasing evidence suggests that exudation of organic acids (Jones, 1998) plays a major role in aluminium tolerance of higher plants. In fungi, metal tolerance in some cases has been linked to extracellular chelation by organic compounds (Gadd, 1993). Since mycorrhizal fungi exude a range of organic acids (e.g. Lapeyrie *et al*., 1987) or produce slime capable of binding metals, it is possible that organic compounds released by mycorrhizal fungi are responsible for the amelioration of metal toxicity in mycorrhizal plants.

Ahonen-Jonnarth *et al.* (2000) showed that in contrast to non-mycorrhizal pine seedlings, seedlings colonised with *Suillus variegatus* or *Rhizopogon roseolus* responded to aluminium exposure with a strongly increased exudation of oxalic acid, which is an efficient aluminium chelator. Although these data do not prove that organic acids released by ectomycorrhizal fungi affect the metal sensitivity of the host plant, they highlight the potential role organic acids may have in the amelioration of metal toxicity by mycorrhizas. Oxalate synthesis by fungi could be an efficient way of improving host mineral nutrition. As compared to other organic acids, oxalic acid would be most efficient in mineral weathering because of its complaxant as well as its acid properties. By forming complexes with metal ions, such as calcium, aluminium and iron, oxalate would release phosphate from insoluble phosphates (Cromack *et al*., 1979).

Malajczuk and Cromack (1982) have reported the accumulation of calcium oxalate in the mantle of mycorrhizal roots of *Pinus radiata* and *Eucalyptus marginata* and increases in the phosphatase activity at the surface of mycorrhiza.

2.5.4 Metal sorption on the external mycelium (exclusion mechanism 4)

In recent years, much attention has been focussed on the extent to which the external mycelium reduces net metal exposure. The hypothesis is based on the observation that the efficiency of fungal strains to reduce Zn and Cd translocation to the shoot was correlated with the amount of external mycelium produced by these strains (Colpaert and van Assche 1992, 1993). In addition, Denny and Wilkins (1987) demonstrated that the external mycelium may bind more Zn or Pb than the hyphal sheath or Hartig net. However, direct evidence showed that the binding of metals to the mycelium sufficiently reduces metal uptake into the root tissues has not yet been presented.

2.6 *In vitro* synthesis of ectomycorrhizae

The study of metal toxicity requires relatively low and controlled concentrations of all ions applied to plant. Formation of mycorrhizas in pure culture by a given host and fungus can serve several research purposes, thereby providing insights into the ecology of both associates and their survival index in adverse conditions. It provides defined material for study of mycorrhizal physiology and permits comparison of effects of different mycorrhizal fungi on a given host species. Many methods have been developed and discussed for the aseptic synthesis of ectomycorrhiza on seedling roots (Fortin *et al*., 1983; Peterson and Chakravarty, 1991). Since Melin's (1921) pioneering work on *in vitro* synthesis of ectomycorrhizas on pine seedlings it was easy to establish mycorrhizas *in vitro* on rooting media such as sand, vermiculite/ peat mixture or perlite and the problem was partially

overcome with a technique using sloping plates and flowing nutrient solution (Kahr and Arveby, 1986).

Hacskaylo (1953) introduced the use of vermiculite as a substrate instead of sand to synthesize mycorrhizas between *Pinus virginiana* and *Lepiota rhacodes*. Marx and Zak (1965) improved the usefulness of Hacskaylo's method by introducing the use of peat moss to counteract the high buffering capacity of vermiculite. Marx (1969) developed the technique for production of commercial vegetative inoculum of *Pisolithus tinctorius* and inoculation technique for development of ectomycorrhizas on pine seedlings. They found that a medium of vermiculite and 5- 10% by volume peat moss with nutrient was best for growing mycelial inoculum and the inoculum was most effective after leaching with water to remove nutrients.

Marx and Bryan (1975) developed the vegetative inoculum of *Pisolithus tinctorius* in vermiculite - peat moss nutrient medium and inoculated to *Pinus taeda* seedlings in fumigated nursery soil. They reported excellent success in establishing *P. tinctorius* in the nursery with doubled growth of inoculated seedlings over uninoculated controls. Inoculation with basidiospores of *P. tinctorius* also increased the growth and development of mycorrhizas. Mohan *et al*. (1993) described ectomycorrhizal formation in *Pinus patula* plants inoculated with basidiospores of *Thelephora terrestris*.

2.7 Induction of mutation in ectomycorrhizal fungi

By inducing mutation using chemical and/ or physical mutagens, genetic alterations can be achieved in ectomycorrhizal fungi. Protoplasts are better suited for inducing mutations as these cells lacking cell wall are often more susceptible to mutagenic agents than the intact hyphae. Desired traits such as tolerance to fungistatic agents or stress factors can be

selected from cells/protoplasts exposed to mutagenic agents. Hebraud and Ferve (1988) have identified mutant strains of *Hebeloma cylindrosporum* resistant to several different fungicides after exposure to UV radiation. The frequency of mutation was 10^{-6}. The homokaryotic strains were able to grow at 2-5 times higher than the concentration of the fungicides that completely inhibited growth of the wild type heterokaryotic mycelia. Durand *et al.* (1992) obtained 5- fluoroindole resistant and IAA overproducing mutant strains of *H. cylindrosporum*. Gay *et al*. (1994) showed that the ectomycorrhizal activity of auxin over producer mutants was higher than the wild type h-1 strain.

2.8 Protoplast generation and regeneration

For fungal species which do not or rarely differentiate asexual spores, the possibility of obtaining large number of viable protoplasts may be useful for genetic transformation and to induce mutations on a genetically simple and uniform biological material. The protoplasts can be liberated by the removal of cell wall which acts as a barrier and limits access to the genome of the organism. Hebraud and Fevre (1988) worked on production and regeneration of protoplasts from mycorrhizal fungi and their use for isolation of mutants because the protoplasts are excellent genetic tools. Experimental conditions for the production and isolation of protoplasts from different fungal taxonomic groups are well established (Davis, 1985). Protoplasts have been generated from *Laccaria bicolor, L. laccata, Hebeloma cylindrosporum, H. edurum, H. sinapizans, Suillus bellini* and other ectomycorrhizal fungi (Barett *et al*., 1989; Hebraud and Ferve,1988; Kropp and Fortin, 1986) using enzymes like Novozyme 294. A high proportion of protoplasts (70- 100%) liberated from fungi were enucleate and this reduces the regeneration frequency significantly.

Chen and Hampp (1993) isolated the protoplasts from *Amanita muscaria* and reported the yield of 10^8 protoplasts/g fresh weight of mycelial suspension culture treated with a combination of lytic enzymes as NOVOZYM 234, chitinase and cellulose Onozuka R-10. Later on Dias *et al.* (1996) reported the isolation of protoplasts from the mycorrhizal fungus *Suillus granulatus* by treating the 3-7 days old mycelium with lytic enzyme NOVOZYM 234.

Stulten *et al*. (1995) optimized the method for the isolation and regeneration of protoplasts from the ectomycorrhizal ascomycete *Coenococcum geophilum* and reported the regeneration frequency up to 13% for the isolated protoplasts. However, several reports have mentioned that regeneration of protoplast was achieved only in case of *Hebeloma sp*. and *Laccaria sp.* The best regeneration was reported in *Hebeloma crustuliniforme* approx. 1.6% (Barett *et al*., 1989). The regeneration of protoplasts is needed for molecular genetic experiments. The only genera which showed potential for further experimentation are *Hebeloma sp*. and *Laccaria sp.* Among ectomycorrhizal fungi, only mutants of *H. cylindrosporum* have been reported and all mutations were induced by UV irradiation of protoplasts prepared from homokaryotic strains (Marmeisse *et al*., 1999) and have proved to be a suitable model species for carrying out genetic investigations and alterations of the plant- fungus relationship.

Chapter III

3.1 Biological Material:

3.1.1 Fungal strains

Different ectomycorrhizal fungi were used in this study. Isolates of *Pisolithus albus* (Pt-KN6, Pt-P and Pt-N) were isolated from the basidiomata associated with *Eucalyptus* plantations. The other cultures such as *Pisolithus tinctorius* (Pt-NIC), *Paxillus involutus* (P.I-MAR) and *Hebeloma cylindrosporum* (h-1 monokaryon) were procured from France. The source and location of each isolate was given in the following table.

Table 3.1: Source and the location of ectomycorrhizal fungal isolates used in this study.

Name of Isolate	*Location*	*Host Plant*
***Pisolithus albus* (Pt-KN6)**	**Coastal areas of Chennai, India**	***Eucalyptus tereticornis***
***Pisolithus albus* (Pt-N)**	**Lignite mines of Neyvelli, India**	***E. tereticornis***
***Pisolithus albus* (Pt-P)**	**Near bauxite mines of Orissa, India**	***E. saligna* and *Pinus caribea***
***Pisolithus tinctorius* (Pt-NIC)**	**Puerto Cabezas in Nicaragua**	***Pinus caribea***
***Paxillus involutus* (P.I-MAR)**	**Marmande in France**	***Populus enramericana.***
***Hebeloma cylindrosporum* (h-1)**	**Sand dunes along the coastal area of France**	***Pinus pinaster***

Isolates of *Pisolithus* were used to inoculate *Eucalyptus tereticornis* seedlings. It forms symbiotic association with variety of angiosperm and gymnosperm host plants. *Paxillus involutus,* was used to inoculate *Populus deltoides* seedlings and is having unusual broad host range in nature and laboratory. *H. cylindrosporum*, homobasidiomycete and a predominant fungus occurring in the sand dune ecosystem of France was used to inoculate *Pinus pinaster* seedlings. The dikaryotic mycelium is able to form ectomycorrhizas with a variety of gymnosperm and angiosperm host plants. All the cultures were maintained at 25^0C on modified Melin's media with Heller's micronutrient solution (1953) (Appendix 1).

3.1.2 Plant Material

The host plants used in this study were *Eucalyptus tereticornis, Populus deltoides* and *Pinus pinaster.* The *P. pinaster* plants were obtained as seeds supplied by INRA, Bordeaux, France and the axenically produced *E. tereticornis, P. deltoides* plants were obtained from the tissue culture centre of Thapar Institute of Engineering and Technology, Patiala, India.

3.2 Effect of the addition of aluminium in the medium

Different concentrations of aluminium as $Al_2 (SO_4)_3.16H_2O$ (0, 50, 100, 150, 200, 500, 750, 1000, 2000, 3000 and 5000 mg/L) was amended in the Melin's medium with Heller's micronutrients (pH 5.5), the pH and the concentration of various mineral nutrients (Ca, Mg, K, P and Al) were recorded as the function of aluminium concentration. The addition of aluminium resulted in the decline of pH. So, in another experiment, the pH of the medium was set back to 5.5 after the addition of different concentrations of aluminium and the white precipitates formed was filtered through

Whatman No. 1 filter paper. The concentrations of various mineral nutrients (Ca, Mg, K, P and Al) were recorded in the filtrate by inductively coupled plasma emission spectrophotometer.

3.3 Growth of ectomycorrhizal fungi *in vitro*

To study the growth curve, the different ectomycorrhizal fungi were inoculated (2 x 7 millimeter inoculum) in Melin's medium with Heller's micronutrients (pH 5.5) and incubated at 25^0 C in dark. The growth (as dry weight produced in g/L) and pH of the spent media were recorded at 0, 5, 10, 15, 20 and 25 days of time interval.

3.3.1 Effect of pH on the growth of ectomycorrhizal fungi

The addition of aluminium resulted in the decline of pH in the medium. To see whether the presence of aluminium or the decline of pH was responsible for the inhibition in growth of ectomycorrhizal fungi, an experiment was performed by inoculating the different ectomycorrhizal fungi (2 x 7 millimeter inoculum) in the Melin's medium with Heller's micronutrients without aluminium at two pH ranges *viz.*, 5.5 and 3.15 separately. The growth was recorded as dry weight produced in g/L after the incubation of 20 days at 25^0C in the dark.

3.3.2 Screening of different ECM fungi for their tolerance to Aluminium

Different ectomycorrhizal fungi were screened for their tolerance to aluminium. Fifteen days old mycelial discs (2 x 7 millimeter inoculum) of each fungus cut from the actively growing mycelia were inoculated to 250 ml Erlenmeyer flask containing 50 ml of modified Melin's liquid medium with Heller's micronutrients (pH 5.5). To avoid aluminium stress immediately, and also to allow the fungi to initiate growth in the medium, filter sterilized (0.22 μm) aluminium was added in the form of $Al_2(SO_4)_3.16H_2O$ to give the final

concentrations of aluminium as 0, 200, 500, 750, 1000, 2000, 3000 and 5000 mg/L into the medium after three days of fungal inoculation. The flasks were incubated at 25^0 C in dark and were shaken manually once in a week for 20-30 seconds for uniform distribution of the medium. After 20 days of incubation, the mycelium was harvested and the pH of the spent medium was recorded. The mycelia were analyzed for growth, phosphatase activity and mineral content uptake of calcium, magnesium, potassium, phosphorus and aluminium.

3.3.3 Growth:

The response of different ectomycorrhizal fungi to varied concentrations of aluminium was assessed by growing pure mycelial culture in presence of aluminium. The mycelia was filtered, washed thoroughly with double distilled water and dried at 70^0 C for 48 hours in the oven and weighed. The isolates were ranked for aluminium tolerance based on the substrate concentration at which the mycelial mass was 50% of that in the control (EC_{50}).

3.3.4 Phosphatase activity:

Acid phosphatase activity was determined by using the mycelia grown at different concentrations of aluminium by the method of Tabatabai and Bremner (1969) as mentioned in section 3.9.1.

3.3.5 Mineral nutrition:

The dried mycelium was digested according to the method of Page *et al.* (1982) as mentioned in section 3.9.3 and analyzed for the mineral content such as calcium, magnesium, potassium and aluminium by inductively coupled plasma emission spectrophotometer. The total phosphorus was estimated by the method of Kitson and Mellon (1944) as mentioned in section 3.9.2.

3.4 Isolation of aluminium tolerant mutants of ectomycorrhizal fungi

The different ectomycorrhizal fungi were used for production of aluminium tolerant mutants *in vitro*. The physical mutagen used was U.V light. Two approaches were used for the mutant production.

- Irradiating the mycelium of ectomycorrhizal fungi
- Irradiating the protoplasts isolated from the mycelium of ectomycorrhizal fungi

3.4.1 Irradiating fungal mycelium of ectomycorrhizal fungi

Different ectomycorrhizal fungi were grown on Melin's agar medium overlaid with sterile cellophane sheet for 10-12 days. The mycelium was irradiated with U.V light for different time intervals (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 minutes) at a distance of 20 cm from U.V germicidal lamp so as to optimize the time of irradiation for generation of mutants. The time optimized was 6 minutes with U.V light and then the whole cellophane sheet along with irradiated mycelium was then transferred to Melin's agar medium containing aluminium (1000 mg/L > EC_{50} value for different ectomycorrhizal fungi). The EC_{50} value was determined previously by growing the mycelium on Melin's agar medium containing (50-2000 mg/L Al). The growth initiated from one side of the mycelial colony and the presence of mutants was checked after one week of growth in the dark at 25^0 C. The mutant selected was sub cultured in presence of aluminium, and then grown in Melin's agar medium without aluminium again followed by aluminium amended medium to check the reverse mutations. Each experiment was controlled by transferring non irradiated mycelium to the aluminium containing medium.

3.4.2 Irradiating Protoplasts:

3.4.2.1 Culture conditions and protoplast formation:

The ectomycorrhizal fungal cultures for protoplast formation were grown in a 250 ml Erlenmeyer flasks containing 50 ml of liquid YMG medium (Appendix 1) at 25^0C in dark for 20 days. The medium was decanted after 20 days and the mycelium was macerated in 50 ml of fresh YMG medium using tissue homogenizer (Ultraturrax, T25 basic, Fisher Bioblock Scientific, Germany). Again, macerated the mycelium in fresh medium after 5-6 days of growth. The same procedure was continued 3-4 times till the mycelium covered the whole medium in two days. Mycelia for protoplast formation was collected on Nylon mesh, washed twice with sterile osmotic buffer (0.7 M Mannitol and collected by centrifugation at 5000 x g, 10 min). The packed volume of wet mycelium was measured in eppendorf and transferred to 30 ml of sterile glass tube for incubation with lytic enzyme preparation at 30^0C with shaking (80 shakes/ min). Lytic cocktail was made by mixing Cellulase (2.5 mg/ml) (Sigma-Aldrich) and Driselase (5 mg/ml) (Sigma-Aldrich) in 0.7 M mannitol and filter sterilized through 0.22 μm filters.

Protoplasts were separated from hyphae by filtering through sterile glass wool moistened with osmotic buffer; the protoplasts were pelleted by centrifugation (1500xg, 10 min), washed twice with osmotic buffer and collected each time by centrifugation. The final pellet was resuspended in 1 ml osmotic buffer and number of protoplasts was counted on Neuber's cell counting chamber. The whole procedure was performed under aseptic conditions.

3.4.2.2 Regeneration of protoplasts

Protoplasts were regenerated on osmotically stabilized 0.7%YMG agar medium. An aliquot of protoplasts was plated on 0.7%YMG agar (regeneration medium) and incubated at 25^0C until visible foci of fungal growth appeared. For development of mutants through protoplast approach only *H. cylindrosporum* was used because the protoplasts of *H. cylindrosporum* regenerated back while the protoplasts of *Pisolithus tinctorius, P. albus* and *Paxillus involutus* failed to regenerate.

3.4.2.3 Mutagenesis:

Mutagenic treatments were performed on protoplasts released from the monokaryotic strain of *H. cylindrosporum* (h-1). The aluminium tolerant mutants were obtained from protoplasts irradiated for 60 seconds at a distance of 30 cm with a U.V germicidal lamp (where 90% of living protoplasts died) and plated on regeneration medium containing aluminium (500 mg/L > EC_{50} value of h-1). The mutants selected was sub cultured in presence of aluminium, and then grown in Melin's agar medium without aluminium again followed by aluminium amended medium to avoid reverse mutations. The mutant of h-1 obtained by above procedure was maintained separately on Modified Melin's medium containing 500 mg/L of Al.

3.5 Dikaryotizing the h-1 monokaryon of *Hebeloma cylindrosporum*:

The mutant and the wild type monokaryon h-1 were co cultured with compatible monokaryon h-7 on Melin's medium. The cultures were incubated in the dark at 25^0C for one week and the resultant dikaryotic *H. cylindrosporum* (D2 and mutant $D2^*$) were obtained. The resultant culture showed clamp connections under compound microscope

typical for dikaryotic hyphae. The dikaryons were also studied for their growth, phosphatase activity and mineral nutrition studies in respect to their wild type.

3.6 Mineral nutrition studies of mutant ECM fungi in pure culture

The mutant ectomycorrhizal fungi were grown in Melin's medium with Heller's micronutrients at different concentrations of aluminium viz. 0, 200, 500, 750, 1000, 2000, 3000, 5000 and 7500 mg/L. All cultures were incubated for 20 days at 25^0C in dark. The biomass was harvested by filtration and the parameters such as pH of culture filtrate, dry weight of mycelia and phosphatase activity (by the method of Bremner and Tabatabai, 1969 as mentioned in section 3.9.1) of mycelia were determined. The dried fungal mycelia was digested in nitric and perchloric acid (Page *et al*., 1982 as mentioned in section 3.9.3) and the mineral nutrition (uptake of Ca, Mg and K) and aluminium content of ectomycorrhizal fungi was analyzed by Inductively coupled argon plasma emission spectroscopy. The total Phosphorus was estimated (by the method of Kitson and Mellon, 1944 as mentioned in section 3.9.2). All the values were calculated per gram dry weight of mycelium.

3.7 *In vitro* Experiment

3.7.1 Surface sterilization of seeds:

Axenic culture procedures require surface sterilization of seeds and their subsequent germination in sterile conditions so that no unwanted microbes are present. Seeds of *Pinus pinaster* were surface sterilized by soaking in 30% H_2O_2 for 35 minutes followed by rinsing in sterile distilled water. Seeds were aseptically transferred to water agar (1%) and allowed to germinate for four weeks in plant growth room with photoperiod 14 hour light/ 10 hour dark conditions at 18^0C.

The seedlings of *P. pinaster* and the micro propagated plantlets of *E. tereticornis* and *P. deltoides* were used for the *in vitro* experiments.

3.7.2 Aluminium-mycorrhizal interaction:

A mixture of 100 cm^3 of soilrite and 20 cm^3 of vermiculite (5:1) was placed in each jar. The jars and its contents were autoclaved twice at 121^0C for 60 minutes with 48 hours between sterilization. Afterwards 70 cm^3 of filter sterilized Melin's medium with Heller's micronutrients containing aluminium was added to each jar to supply six levels of Al (0, 50, 100, 200, 400 and 500 mg/L). One half of the seedlings were inoculated with ectomycorrhizal fungi (10x 8millimeter inoculum) grown on modified Melin Norkarn's medium (Appendix 1). Control seedlings received blank agar plugs. Following 60 days of exposure to aluminium solutions, the seedlings were assessed for plant height, shoot and root dry weight, percent mycorrhization, accumulation of aluminium and mineral nutrition content in the plant shoots.

3.7.2.1 Growth Assessments

The plants were harvested after 60 days, the plant height was measured manually with a ruler, the shoot and root were oven dried (at 70^0C, for 48 hours) and dry weight was recorded.

3.7.2.2 Plant mineral Analysis

After drying, the leaves/shoots of each seedling were ground and were digested (according to the method of Page *et al.*, 1982 as mentioned in section 3.9.3) and the contents of Ca, Mg, K and Al were measured using inductively coupled plasma emission spectrophotometer. The total phosphorus was estimated by the method of Kitson and Mellon, (1944) (as mentioned in section 3.9.2).

3.7.2.3 Assessment of seedlings for mycorrhizal formation

The root samples of the harvested seedlings were carefully freed from soilrite by washing in water. The roots were assessed visually under stereomicroscope to count the number of mycorrhizal and nonmycorrhizal tips.

$$\% \text{ mycorrhization} = \frac{\text{Number of mycorrhizal root tips}}{\text{Total number of root tips}} \times 100$$

3.8 Nursery Trials

3.8.1 Preparation of ectomycorrhizal fungal inoculum

The mycelial inoculum of ectomycorrhizal fungus in soilrite - vermiculite carrier was prepared according to the method of Marx and Bryan (1975). The isolate of *Pisolithus tinctorius* (Pt-NIC) was grown at 25^0C for 3 weeks in Petriplates of Modified Melin Norkarn's medium (Appendix 1). Mycelial agar plugs were used to initiate mass cultures for inocula. Erlenmeyer flask (1L capacity) containing 750 cm^3 of soilrite –vermiculite moistened with 375 cm^3 of Modified Melin Norkarn's liquid medium were used as the inoculum containers. The containers were autoclaved for 30 minutes at 121^0C and the flasks were inoculated with 10-12 mycelial discs and incubated in the dark at 25^0C for 30 days. To prepare the mass inoculum for infestation of soil, the mycelium was removed from the flasks and held with two layers of cheese cloth while being leached with cool distilled water to remove the unused nutrients. Excess water was removed by gently squeezing the inoculum wrapped in cheese cloth. The leached inocula were stored at 4^0C till its use. The bauxite mined out soil was mixed with ectomycorrhizal fungal inoculum in the ratio of 50:1.

3.8.2 Soil

Bulk soil of bauxite mined out area of National Aluminium Company, Damanjodi, Orissa, India was collected during June, 2003 and analyzed for the soil characteristics. The pH of the soil was checked by the method of International Society of Soil Science (1930) as mentioned in section 3.9.4. The total organic carbon (by Walkley-Black method as mentioned in section 3.9.5), total nitrogen (by modified Kjeldahl's method as mentioned in section 3.9.7), total phosphorus (by Kitson and Mellon, 1944 method as mentioned in section 3.9.2) and available phosphorus (by Bray and Kurz, 1945 method for acidic soils as mentioned in section 3.9.6) was estimated. The mineral nutrition (Ca, Mg and K) and aluminium content was estimated by Page *et al*. (1982) as mentioned in section 3.9.3.

Table 3.2: Soil characteristics of bauxite mined out soil

pH of soil	**5.5**
Total Organic Carbon (%)	**0.39614 ± 0.065**
Total Nitrogen (%)	**0.0615 ± 0.004**
Total Phosphorus **(mg/kg soil)**	**469.33 ± 8.635**
Available P **(mg/kg soil.)**	**0.493 ± 0.0087**
Magnesium **(mg/g soil)**	**0.8836 ±0.073**
Potassium **(mg/g soil)**	**1.3955 ± 0.153**
Calcium **(mg/g soil)**	**0.6236 ± 0.035**
Aluminium **(mg/g soil)**	**168.18 ± 10.54**

A nursery trial was conducted with *E. tereticornis* plants. The bauxite mined out soil was mixed with vegetative inoculum of ectomycorrhizal fungus *P. tinctorius* (Pt-NIC) in the ratio of 50:1 and filled in the pots. The control was also maintained by mixing bauxite mined out soil with soilrite in the same ratio. *E. tereticornis* plantlets (micropropagated) were transferred to then pots and kept in polyhouse for 15 weeks. The plants were watered regularly. The plants were harvested after 15 weeks by destructive sampling. The plants were analyzed for their growth parameters and also for the different mineral nutrients.

The plant height was measured manually with a ruler, the shoot and root were oven dried (at 70^0C, for 48 hours) and dry weight was recorded. After drying, the leaves/shoots and roots of seedlings were ground and sub sampled. One gram of oven dried ground leaves/shoots and roots were digested (according to the method of Page *et al*., 1982 as mentioned in section 3.9.3) and the concentrations of calcium, magnesium, potassium and aluminium were measured using inductively coupled plasma emission spectrophotometer. The total phosphorus was estimated by the method of Kitson and Mellon (1944) as mentioned in section 3.9.2. The soil after harvesting the plants was collected and analyzed for various physico-chemical characterstics.

3.9 Analytical Procedures

3.9.1 Determination of Phosphatase activity (Tabatabai and Bremner, 1969)

Reagents:

- **Modified Universal buffer 5X** (Skujin's *et al.*, 1962)

Tris (hydroxyl methyl) amino methane	3.025 g
Maleic acid	2.90 g
Citric acid	3.50 g
Boric acid	1.57 g
NaOH (1N)	122 ml
Water	to make volume 250 ml.
pH	5.5

- **0.115M p-nitro phenyl phosphate solution**: Dissolved 4.268 g of p-nitro phenyl phosphate disodium salt hexahydrate in 100 ml of buffer.
- **0.5N NaOH**: Dissolved 20 g NaOH pellets in distilled water and made the volume to one litre.
- **1 mg/ml p- nitrophenol (PNP) solution** in modified universal buffer.

Procedure:

1. The mycelium previously grown in different concentrations of aluminium was filtered and washed aseptically with sterile distilled water followed by a rinse with filter sterilized modified universal buffer.
2. After an aseptic rinse with sterile modified universal buffer, mycelia were placed in sterile 30 ml screw cap tubes with 4 ml of sterile buffer solution.

3. Added 1 ml of filter sterilized 0.115M disodium p- nitro phenyl phosphate solution
4. The contents were incubated at 30^0 C in a water bath for 2 hours in the dark.
5. After 2 hours of incubation, 5 ml of 0.5N NaOH was added to stop the reaction.
6. The mixture was swirled and filtered through Whatman No 1 filter paper.
7. Transferred the filtrate to glass cuvette and measured the yellow color intensity with UV-VIS spectrophotometer (Hitachi U-2001) at 410 nm.
8. The filtered out mycelium from the each vial was dried for 48 hours at 70^0C and the dry weight was recorded separately.
9. Phosphatase activity was indicated as the amount of p- nitrophenol released in the filtrate from the p-nitro phenyl phosphate substrate per gram of the mycelium. The p- nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 10, 20, 30, 40 and 50 µg of p- nitrophenol.
10. To perform controls, followed the procedure described for the assay but made the addition of 1 ml p-nitro phenyl phosphate after the addition of 0.5N NaOH (i.e. immediately before filtration).

Calculation:

$$\text{Phosphatase activity (µM PNP/g mycelium/hour)} = \frac{\text{Concentration of PNP (in µM)}}{2 \times \text{Weight of mycelium}}$$

3.9.2 Total Phosphorus estimation in the mycelium. (Kitson and Mellon, 1944)

Reagents:

- **Vandomolybdate reagent:**

  i) 25 g ammonium molybdate in 400 ml of water

  ii) 1.25 g of ammonium metavandate in 300 ml of boiling water.

  Cooled and added 250 ml concentrated HNO_3. Mix the two solutions and make up to 1L.

- **Saturated solution of 2,4-dinitrophenol** in water
- **5M NH_4OH**
- **50µg Phosphorus/ ml stock solution**: Weighed 0.2917 g of AR grade KH_2PO_4 dried in oven at 60^0C for 1 hour in a one litre beaker; added 500 ml distilled water and five drops of toluene to diminish microbial activity and made the volume to one litre.
- **1µg Phosphorus/ml solution**: Diluted stock solution by 50 times.

Procedure:

1. Placed the mycelial samples in crucible in muffle furnace at 600^0C for 6 hours. After ashing was complete, cooled the crucible in dessicators and moistened the ash with 8-10 drops of water followed by 3 ml of 5M HCl (care was taken to ensure that there was no loss due to effervescence).
2. Placed the crucibles on hot plate at 80^0C and added 0.25 ml HNO_3. Evaporated to dryness in order to solubilize phosphate and to precipitate silica.

3. Moistened the dried salt with 3 ml of 5 M HCl and warm on hot plate. Added 5 ml water and maintained heat for 10 minutes.
4. Transferred the solution while hot, made the volume to 50 ml and filtered through Whatman No. 42 filter paper.
5. Placed 10 ml aliquot and diluted to 30 ml, added 4 drops of saturated solution of 2, 4-dinitrophenol and poured NH_4OH drop wise till yellow color just appeared.
6. Added 10 ml of vandomolybdate reagent and made the volume 50 ml with distilled water. Read the absorbance at 470 nm after 10 minutes.
7. For standard, pipetted out 0, 2, 5, 10, 15and 20 ml of working phosphorus solution (1µg P/ml), in 6 different volumetric flasks and added 4 drops of saturated solution of 2,4-dinitrophenol and poured NH_4OH drop wise till yellow color just appeared.
8. Added 10 ml of vandomolybdate reagent and finally made the volume to 50 ml with distilled water. The phosphorus concentration of these solutions was 0.04, 0.1, 0.2, 0.3 and 0.4 µg /ml respectively.

Calculation:

$$\text{Total Phosphorus in mycelia (mg/g)} = \frac{Q \text{ (in mg)} \times 50}{A \times W}$$

Q = quantity of phosphorus read on X- axis against a sample reading

A = volume of aliquot used (ml)

W = weight of mycelia taken (g)

3.9.3 Determination of Mineral Elements by Inductively coupled plasma emission spectrophotometer (Ca, Mg, K and Al)

Di-acid digestion (Page *et al.*, 1982)

- Concentrated Nitric acid (AR grade)
- 60% Perchloric acid (AR grade)
- 2N HCl (AR grade)
- Standard stock solutions containing 1000 µg/ml of an element from pure metal wire or suitable compounds of the element.
- Multielement secondary standard solutions

Procedure:

1. Took the weighed dried mycelium in a 100 ml conical flask.
2. Added 15 ml of concentrated nitric acid and kept for 1 hour and then added 5 ml of perchloric acid.
3. Kept on a hot plate in acid-proof digestion chamber having fume exhaust system and heated at about 100^0C for first one hour and then raised the temperature to about 200^0C.
4. Continued the digestion until the contents become colorless and only white dense fumes appeared.
5. Reduced the acid contents to about 2-3 ml by continuing heating at the same temperature.
6. Removed from the hot plate, cooled and added diluted HCl.
7. The solution was filtered through Whatman No.42 filter paper and the final volume was made 20 ml with diluted HCl.

8. Measured the concentration of mineral elements in the filtrate using inductively coupled plasma emission spectrophotometer.
9. Prepared the calibration curve for each element by recording absorbance of a series of standard solutions of increasing concentrations.

Calculations:

$$\text{Mineral ion (mg/g mycelium)} = \frac{C \times 20}{W}$$

C = concentration in the sample obtained on X-axis against the reading

W = weight of the mycelium taken

3.9.4 Determination of soil Reaction (pH) International Society of Soil Science, 1930

Reagents:

- Standard buffer solutions of 4, 7 and 9.2 pH

Procedure:

1. Weighed 20 g of air dried soil passed through 2mm sieve into a 100 ml beaker.
2. Added 50 ml of distilled water to it.
3. Thoroughly stirred for 2-3 minutes using a glass rod.
4. Further, stirred suspension four- five times during the next 30 minutes.
5. Allowed suspension to settle down for 30 minutes.
6. In the mean while, switched on the pH meter and after 10 minutes of warming up period, adjusted the pH meter reading to the pH of the buffer solution with the help of standardization knob.
7. Checked the instrument with two buffer solutions of known pH viz. one acidic and other alkaline.

8. Rinsed the electrode with distilled water and carefully wiped with filter paper.
9. Measured the pH of sample by immersing the electrode in supernatant solution.
10. Recorded pH value when the reading had stabilized (usually after 1 minute).

3.9.5 **Total Organic Carbon in soil** (Walkley-Black method, 1934)

Reagents:

- 1 N Potassium dichromate: Dissolve 49.04g of $K_2Cr_2O_7$ in distilled water and made to 1L.
- Concentrated Sulphuric acid
- Anhydrous Sucrose (AR grade)

Procedure:

1. Weighed accurately 1 g of dried soil sample and transferred it into a 150 ml conical flask.
2. Added 10 ml of 1N $K_2Cr_2O_7$ and shake it well to mix.
3. Prepared the blank in which all reagents except soil were added.
4. Kept the conical flask on Teflon /asbestos sheet and added 20 ml of concentrated H_2SO_4 from the sides of the flask. Swirled the flask during addition.
5. Allowed the flask to stand for 30 minutes and there after 70 ml of water was added.
6. Swirled the flask thoroughly and allowed to settle the soil particles overnight.
7. Decant the supernatant and read the color intensity using red filter at 660 nm.
8. For standard weighed 0, 10, 20, 30, 40 and 50 mg of anhydrous sucrose crystals into a 100 ml volumetric flask and repeated the same procedure.

Calculation:

Multiplied the quantities of sucrose (0, 10, 20, 30, 40 and 50 mg) by 0.4207 and found the quantities (%) of carbon present. Because 10 mg sucrose contained 4.207 mg C and if suppose 4.207 mg C was found in 1000 mg of soil, it meant

100 mg soil contained = $\frac{4.207 \times 100}{1000}$ mg carbon

Sucrose (in mg)	**Carbon** (in mg)	**Carbon** (%)
0	0	0
10	4.207	0.42
20	8.414	0.84
30	12.621	1.26
40	16.828	1.68
50	21.035	2.10

Organic carbon (%) = Colorimetric reading x 0.0042

(Note: The quantities of sucrose were multiplied by 0.4207 because sucrose contains 42.07% carbon.

Available Phosphorus in soil (Bray and Kurtz, 1945)

Reagents:

- **Bray's P-1 extractant**: Dissolved 1.110 g of AR grade ammonium fluoride in one litre of 0.025N HCl.
- **Molybdate-tartarate solution**: i) Dissolved 12 g of ammonium molybdate in about 250 ml distilled water.

  ii) Dissolved 0.291 g of antimony potassium tartarate in 100 ml of distilled water.

  Added the two solutions to 1L of 5N H_2SO_4. Mixed thoroughly and made the volume to 2L with distilled water.
- **Ascorbic acid solution**: Dissolved 1.056 g of ascorbic acid in 200 ml of molybdate-tartarate solution. Prepared fresh as and when required.
- **50mg Phosphorus/ L stock solution**: Weighed 0.2917 g of AR grade KH_2PO_4 dried in oven at 60^0C for 1 hour in a one litre beaker, added 500 ml distilled water and five drops of toluene to diminish microbial activity and made the volume to one litre.
- **1mg Phosphorus/ L solution**: Diluted stock solution by 50 times.

Procedure:

1. Weighed 2.5 g of soil sample in a 150 ml conical flask.
2. Added 50 ml of Bray's P-1 extract ant and shake on reciprocating shaker for 5 minutes (soil- to- solution ratio 1:20).
3. Filtered through Whatman No. 1 filter paper quickly so as to collect the filtrate within 10 minutes.
4. Transferred 5 ml of aliquot into 50 ml volumetric flask and diluted to about 20 ml.

5. Added 8 ml of ascorbic acid solution, made the volume 50 ml and shake well.
6. Waited for 10 minutes and then measured the color intensity at 882 nm.
7. Prepared the blank with the extracting solution also (without soil).
8. For standard, pipetted out 0, 2, 5, 10, 15 and 20 ml of working phosphorus solution (1mg P/ L), in 6 different volumetric flasks and added 5 ml of extractant.
9. Added 8 ml of ascorbic acid solution and finally made the volume to 50 ml with distilled water. The phosphorus concentration of these solutions was 0.04, 0.1, 0.2, 0.3 and 0.4 µg/ml respectively.

Calculations:

$$\text{Available phosphorus (mg/kg)} = \frac{Q \times V}{A \times S}$$

Q = quantity of phosphorus in µg read on X- axis against a sample reading

V = volume of extracting reagent (ml)

A = volume of aliquot used (ml)

S = weight of soil sample taken (g)

3.9.7 Total Nitrogen estimation in soil (Modified Kjeldahl's method in Soil chemical analysis by Jackson, 1962)

i) Digestion of soil samples: The method employs a digestion to convert nitrogen present in soil to ammonium sulphate. The ammonium nitrogen was subsequently determined by distillation and titration.

Reagents:

- Conc. H_2SO_4
- Kjeldahl catalyst Tablets: B.D.H. cat No. 33064

  (Each tablet contains 1 g sodium sulphate and 0.1 g copper sulphate)

Procedure:

1. Weighed and transferred 1g of finely ground (air dry < 0.25 mm) soil into a kjeldahl flask.
2. Added 10 ml conc. H_2SO_4 and mixed by swirling.
3. Heated at 200^0C in a digestion block until white vapors were released during digestion.
4. Added 1 Kjeldahl catalyst tablet and heated for 3-4 hours until tablet dissolves (200^0C) to get the precipitation.
5. Removed the digestion tubes from the block and allowed to cool for 5 minutes. "Didn't allow to cool in the heating block- NH_3 from $(NH_4)_2$ SO_4 formed by digestion will be lost"
6. Transferred the whole sample into volumetric flask and made the volume 50 ml.

ii) Determination: (Distillation- Titration method)

Reagents:

- 40% NaOH: Dissolved 400 g NaOH pellets in distilled water and made the volume to one litre.
- 4% Boric acid: Dissolved 40 g H_3BO_3 in a litre of distilled water.
- Mixed indicator: Dissolved 0.066 g methyl red and 0.099 g bromocresol green in 100 ml of 95% alcohol.
- 0.01 N HCl

Procedure:

1. Transferred 5 ml of sample digest to the distillation apparatus.
2. Added 10 ml of 40% NaOH solution, formation of brown precipitates of ferric hydroxide, when the liquids were mixed indicated the neutralization of acid.

3. Took 10 ml of 4% boric acid and 2-3 drops of mixed indicator to a 100 ml Erlenmeyer flask and placed under the delivery tube of the condenser so that the tip was below the surface of the liquid.
4. Closed the sample inlet and drainage outlet and passed steam into the distillation flask. The liquid boiled and the indicator in the boric acid solution changed color from pink to green as soon as ammonia began to distill over.
5. After a minute or two, lowered the flask so that the tip of the delivery tube was clear of the liquid.
6. When about 20-25 ml of distillate had collected, rinsed the tip of the tube with little water and removed the flask.
7. Stopped the entry of steam. The distillation flask will empty automatically and the vacuum generated can be used to rinse the apparatus by immersing the tip of the delivery tube in distilled water.
8. Titrated the distillate against 0.01 N HCl to the pink color of the indicator.
9. Corrected titration for the reagent blank also.

Calculation:

$$\% \text{ Nitrogen in soil} = \frac{\text{Volume of 0.01 N HCl used (in ml)} \times 0.014 \times 50}{\text{Volume of aliquot used (in ml)}}$$

3.10 Statistical analyses:

Three replicates were maintained for each treatment in the screening experiment of different ectomycorrhizl fungi for their tolerance to aluminium. The data was analyzed by one way analysis of variance (ANOVA) for comparison and the means were compared within the fungus by using Duncan's multiple range tests.

For *in vitro* experiment of plant mineral nutrition in presence of mycorrhiza at different concentrations of aluminium, six seedlings for each aluminium level were completely randomized in the experimental system. Data was analyzed by two way ANOVA and the means were compared using student's t-test.

In the nursery experiment of *E. tereticornis* plants in bauxite mined out soil, twenty five replicates were maintained for each mycorrhizal and nonmycorrhizal treatment and were completely randomized in the experimental system. Data was analyzed by ANOVA and the means were compared using student's t-test among the treatment.

Chapter IV

Study the tolerance level of aluminium by different ectomycorrhizal fungi

4.1 Effect of the addition of aluminium on the medium parameters

To see the effect of aluminium on the pH and mineral ion concentration of the medium, different concentrations of aluminium were amended in Melin's medium and the change in pH and availability of mineral ions were recorded. The addition of aluminium to the medium resulted in the decline of pH. The pH of medium was again set back to 5.5 after the addition of aluminium and the white precipitates formed were filtered through Whatman No. 1 filter paper. The medium was analyzed for their mineral ion content.

The addition of 50 mg/L aluminium led to the significant decrease in pH of the medium from 5.5 to 3.55 and with increasing concentrations of aluminium the pH decreased finally to 3.14 at 1000 mg/L. Further addition of aluminium did not decrease the pH of medium (Table 4.1). Compared to the control, the medium containing aluminium (10, 20, 40, 80, 100, 150 and 200 mg/L of Al) was found to have significantly lower levels of phosphorus at pH 5.5 and the phosphorus gets almost exhausted in the medium at 200 mg/L of Al (Table 4.2a). However, there was no significant change found in the phosphorus content of the medium where the pH was not set back to 5.5 (Table 4.2b). Similarly, the aluminium content of the medium was significantly reduced where the pH was set back to 5.5 after the addition of aluminium. In case of other mineral nutrients such as Ca, Mg and K the addition of aluminium and change of pH was not affecting their concentrations in the medium (Table 4.2a and 4.2b). The results indicated that the addition of aluminium resulted in decrease of medium pH but the mineral ion

concentration (Ca, Mg and K) was not affected in the medium due to the addition of aluminium. When the pH of the medium was set back to 5.5, white precipitates were formed and the available phosphorus from the medium gets exhausted while there was no significant difference found in other mineral ion concentrations indicating that aluminium formed complex with phosphorus at pH 5.5 and the white precipitates formed were of aluminium phosphate.

Table: 4.1 Effect of Aluminium concentrations on the pH of the medium.

Conc. of Al ***(mg/L)***	*Al as* $Al_2(SO_4)_3.16H_2O$
0	**5.50**
50	**3.55**
100	**3.39**
150	**3.32**
200	**3.29**
250	**3.26**
500	**3.20**
750	**3.16**
1000	**3.14**
2000	**3.14**
3000	**3.14**
5000	**3.14**

Table 4.2a Addition of different concentration of Aluminium on the mineral nutrient content (mg/L) of the medium when pH was set back to 5.5 and the precipitates were filtered

Al ***(mg/L)***	*P*	*Ca*	*Mg*	*K*	*Al*
0	**143.5**	**6.02**	**10.64**	**12.67**	**0.02**
10	**116.5**	**5.97**	**9.87**	**11.98**	**1.68**
20	**103.1**	**5.49**	**9.58**	**11.67**	**4.51**
40	**95.5**	**5.87**	**9.67**	**10.58**	**12.62**
80	**76.1**	**5.49**	**8.59**	**10.94**	**24.63**
100	**58.2**	**5.42**	**8.94**	**10.95**	**39.61**
150	**30.1**	**5.64**	**8.82**	**10.34**	**45.62**
200	**13.4**	**5.61**	**8.54**	**10.67**	**73.61**
500	**0.0**	**5.47**	**8.64**	**10.65**	**340.2**

Table 4.2b Addition of different concentration of Aluminium on the mineral nutrient content (mg/L) of the medium when pH was not set back to 5.5

Al ***(mg/L)***	*P*	*Ca*	*Mg*	*K*	*Al*
0	**143.5**	**5.77**	**9.9**	**13.65**	**0.02**
10	**142.3**	**6.10**	**8.76**	**14.02**	**8.97**
20	**143.8**	**5.87**	**8.59**	**14.64**	**19.64**
40	**142.6**	**6.27**	**8.67**	**14.25**	**38.72**
80	**141.9**	**5.94**	**9.94**	**13.69**	**75.63**
100	**140.8**	**5.82**	**8.05**	**14.28**	**95.65**
150	**138.5**	**5.69**	**8.21**	**14.67**	**143.12**
200	**139.0**	**5.97**	**9.89**	**14.69**	**195.64**
500	**139.5**	**6.18**	**9.94**	**14.87**	**483.61**

4.2 Growth of ectomycorrhizal fungi *in vitro*

Different ectomycorrhizal fungi were grown in Melin's medium with Heller's micronutrients. The growth of the ectomycorrhizal fungi and the pH of the spent medium were recorded with the time course to follow the growth curve.

The biomass of ectomycorrhizal fungi increased with the due course of time finally reaching the maximum at 15-20 days of incubation and then reached the stationary phase (Table 4.3 and Figure 4.2). So, 20 days was the optimum time selected for the different ectomycorrhizal fungi in following experiments. As the ectomycorrhizal fungi grows the pH of the medium significantly decreased within 5 days of growth depending upon the isolate used (Table 4.4 and Figure 4.3). The pH of the spent medium decreased from 5.5 to 4.3 in case of Pt-NIC, Pt-KN6 and P.I-MAR. The maximum decrease in the pH of spent medium was found in h-1 within 5 days of growth. The pH of the spent medium finally reduced to 3.4-3.5 after 20 days of growth.

To see whether the decrease of pH or the presence of aluminium was affecting the growth of ectomycorrhizal fungi an experiment was set up with different ectomycorrhizal fungi grown in Melin's medium at pH 5.5 and 3.15. The growth was assessed after 20 days and there was no significant change found in the dry weights at the two pH ranges within the fungus (Table 4.5 and Figure 4.4) indicating that the decrease of pH was not influencing the growth of ectomycorrhizal fungi.

4.3 Screening of different ectomycorrhizal fungi for their tolerance to aluminium

To select the aluminium tolerant ectomycorrhizal fungi, different isolates of ectomycorrhizal fungi were grown in different concentrations of aluminium and the biomass was harvested after 20 days of incubation. The various parameters such as growth, phosphatase activity, concentration of aluminium and the mineral nutrients in the mycelium were recorded.

4.3.1 Growth

The growth of ectomycorrhizal fungi was adversely affected with increasing concentration of aluminium (Table 4.6 and Figure 4.5). The growth of *Pisolithus* isolates (Pt-P, Pt-KN6) and *H. cylindrosporum* (h-1) was inhibited by more than 50% (EC_{50}) at 200 µg/ml of aluminium (Table 4.7a and Table 4.7b) and the growth of h-1 was almost completely inhibited at 5000 µg/ml of aluminium concentration. The growth of Pt-N was not affected till 200 mg/L of aluminium and then significantly decreased at 500 mg/L of aluminium with no decrease further till 5000 mg/L. The growth of Pt-NIC was not significantly affected with increasing concentrations of aluminium (Table 4.7b). *Paxillus involutus* (P.I-MAR) also showed tolerance to aluminium with no significant reduction in growth till 750 µg/ml of aluminium and the growth was decreased at further higher concentrations. The growth of P.I-MAR was inhibited by 50% (EC_{50}) at 2000 mg/L of Al concentration. Among the different ectomycorrhizal fungi tested, Pt-NIC showed higher tolerance followed by P.I-MAR. The growth was infact stimulated at 750 µg/ml of aluminium concentration in both of these fungi (Table 4.7a). The Pt-KN6, Pt-P and h-1 isolates were found to be more sensitive to aluminium whereas Pt-N isolate showed moderate tolerance towards aluminium.

Table 4.3 Growth of different ectomycorrhizal fungi (g/L) grown in the Melin's medium (pH 5.5) at different time intervals

Time ***(in days)***	*Pt-KN-6*	*Pt-NIC*	*h-1*	*P.I-MAR*
0	**0.00**	**0.00**	**0.00**	**0.00**
5	**0.11 ± 0.01**	**0.13 ± 0.03**	**0.43 ± 0.07**	**0.18 ± 0.03**
10	**0.31 ± 0.01**	**0.30 ± 0.04**	**1.05 ± 0.07**	**0.69 ± 0.09**
15	**0.57 ± 0.03**	**0.47 ± 0.05**	**1.03 ± 0.02**	**1.01 ± 0.03**
20	**0.59 ± 0.04**	**0.85 ± 0.03**	**1.01 ± 0.02**	**0.89 ± 0.01**
25	**0.60 ± 0.01**	**0.68 ± 0.02**	**1.01 ± 0.01**	**0.81 ± 0.01**

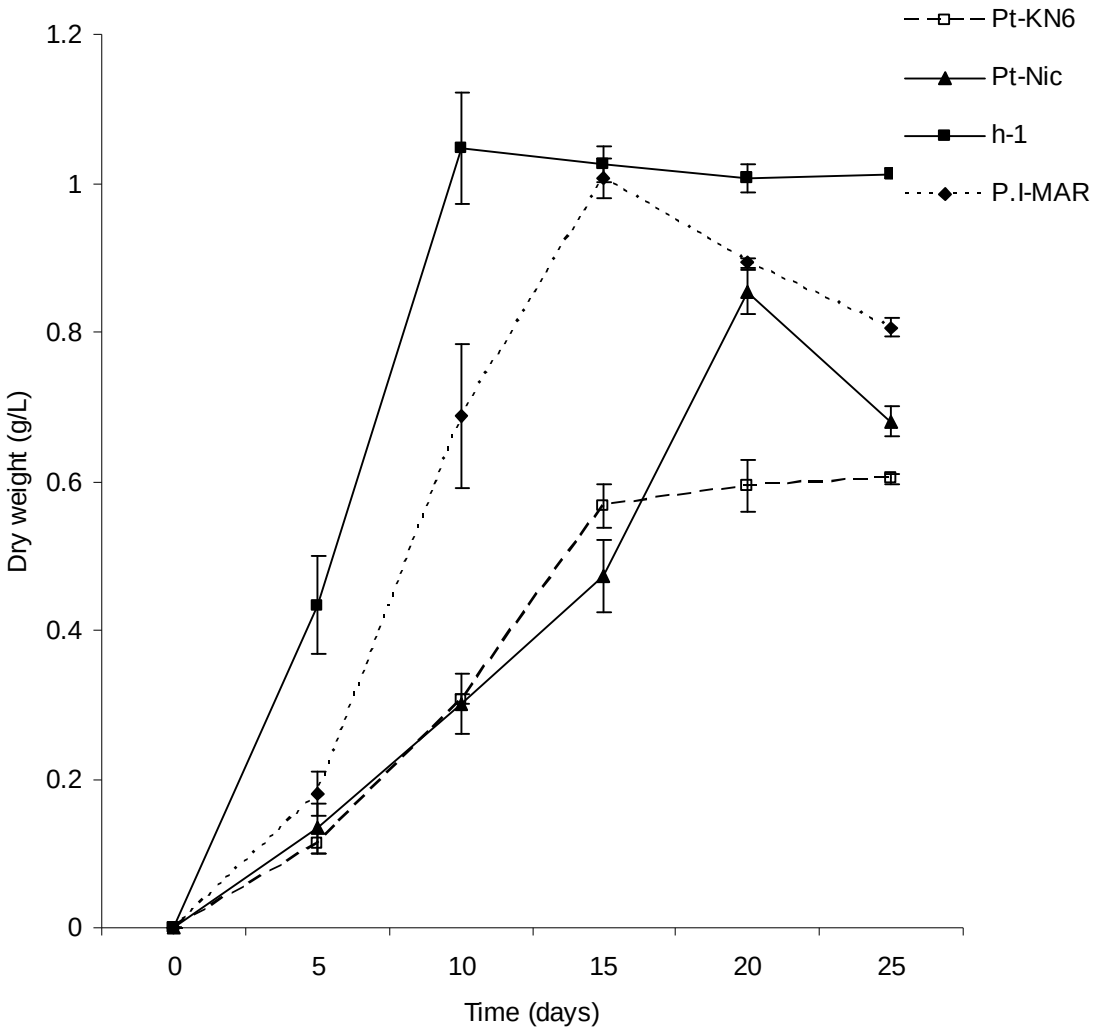

Figure 4.2 Growth curve of different ectomycorrhizal fungi grown in the Melin's medium (pH 5.5). Mean ± SE.

Table 4.4 Decrease in pH of the medium as the ectomycorrhizal fungi grown in the medium

Time ***(in days)***	*Pt-KN-6*	*Pt-NIC*	*h-1*	*P.I-MAR*
0	**5.50**	**5.50**	**5.50**	**5.50**
5	**4.34**	**4.43**	**3.73**	**4.32**
10	**3.57**	**3.63**	**3.45**	**3.47**
15	**3.43**	**3.44**	**3.45**	**3.39**
20	**3.47**	**3.42**	**3.57**	**3.43**
25	**3.60**	**3.52**	**3.54**	**3.60**

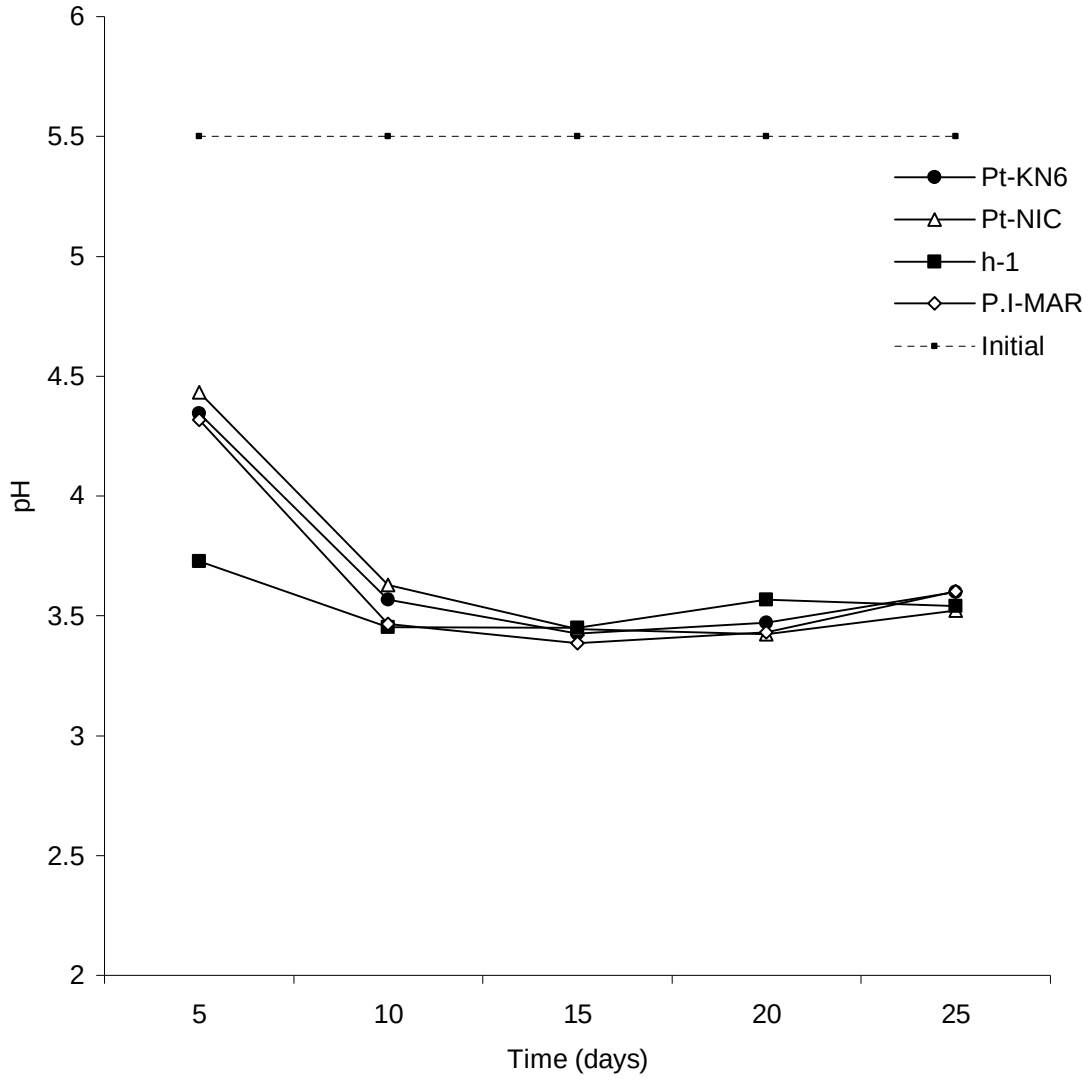

Figure 4.3 Decrease in pH of the medium as the ectomycorrhizal fungi grown in the medium

Table 4.5 Growth of different ectomycorrhizal fungi (g/L) grown in the Melin's medium at pH 5.5 and pH 3.15

	pH 5.5	*pH 3.15*
Pt-KN6	**0.60 ± 0.06**	**0.60 ± 0.05**
Pt-NIC	**0.87 ± 0.02**	**0.78 ± 0.04**
Pt-N	**0.69 ± 0.05**	**0.71 ± 0.05**
Pt-P	**0.56 ± 0.05**	**0.57 ± 0.05**
h-1	**1.02 ± 0.02**	**1.06 ± 0.03**
P.I-MAR	**0.90 ± 0.04**	**0.90 ± 0.05**

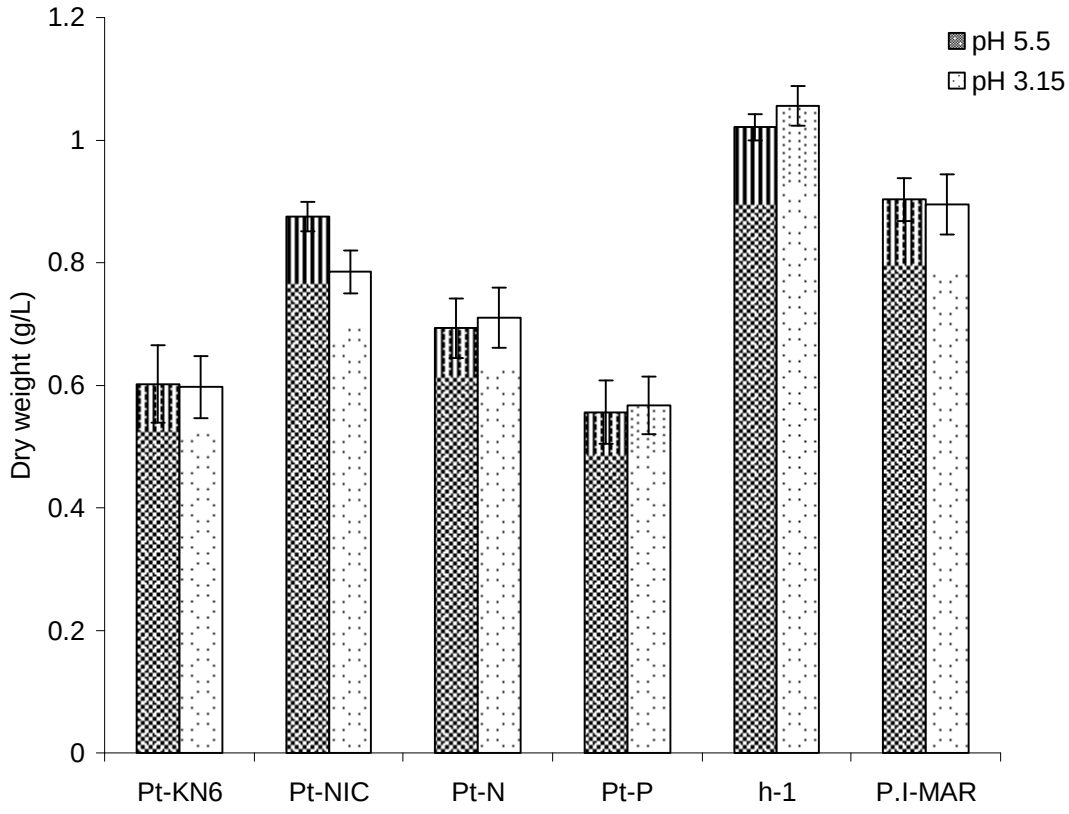

Figure 4.4 Growth of different ectomycorrhizal fungi (g/L) grown in the Melin's medium at pH 5.5 and pH 3.15

Table 4.6 Influence of Aluminium concentrations in the nutrient solution on the ponderal growth (g/L) of different ectomycorrhizal fungi.

Al (mg/L)	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**0.66a**	**0.80a**	**0.70a**	**0.57a**	**1.03a**	**0.87a**
50	**0.49b**	**0.85a**	**0.63a**	**0.57a**	**1.00a**	**0.91a**
100	**0.44b**	**0.86a**	**0.61a**	**0.31b**	**0.80b**	**0.85a**
200	**0.35c**	**0.86a**	**0.68a**	**0.25bc**	**0.49c**	**0.91a**
500	**0.26d**	**0.97a**	**0.49b**	**0.27bc**	**0.30d**	**0.90a**
750	**0.25d**	**1.01a**	**0.51b**	**0.20c**	**0.30d**	**0.92a**
1000	**0.26d**	**0.77a**	**0.51b**	**0.25bc**	**0.22de**	**0.71b**
2000	**0.21d**	**0.75a**	**0.49b**	**0.31bc**	**0.23de**	**0.43c**
3000	**0.22d**	**0.80a**	**0.57ab**	**0.27bc**	**0.13e**	**0.54c**
5000	**0.21d**	**0.79a**	**0.55ab**	**0.25bc**	**0.00f**	**0.44c**

Values sharing a common letter within the fungus are not significantly different at $P<0.05$

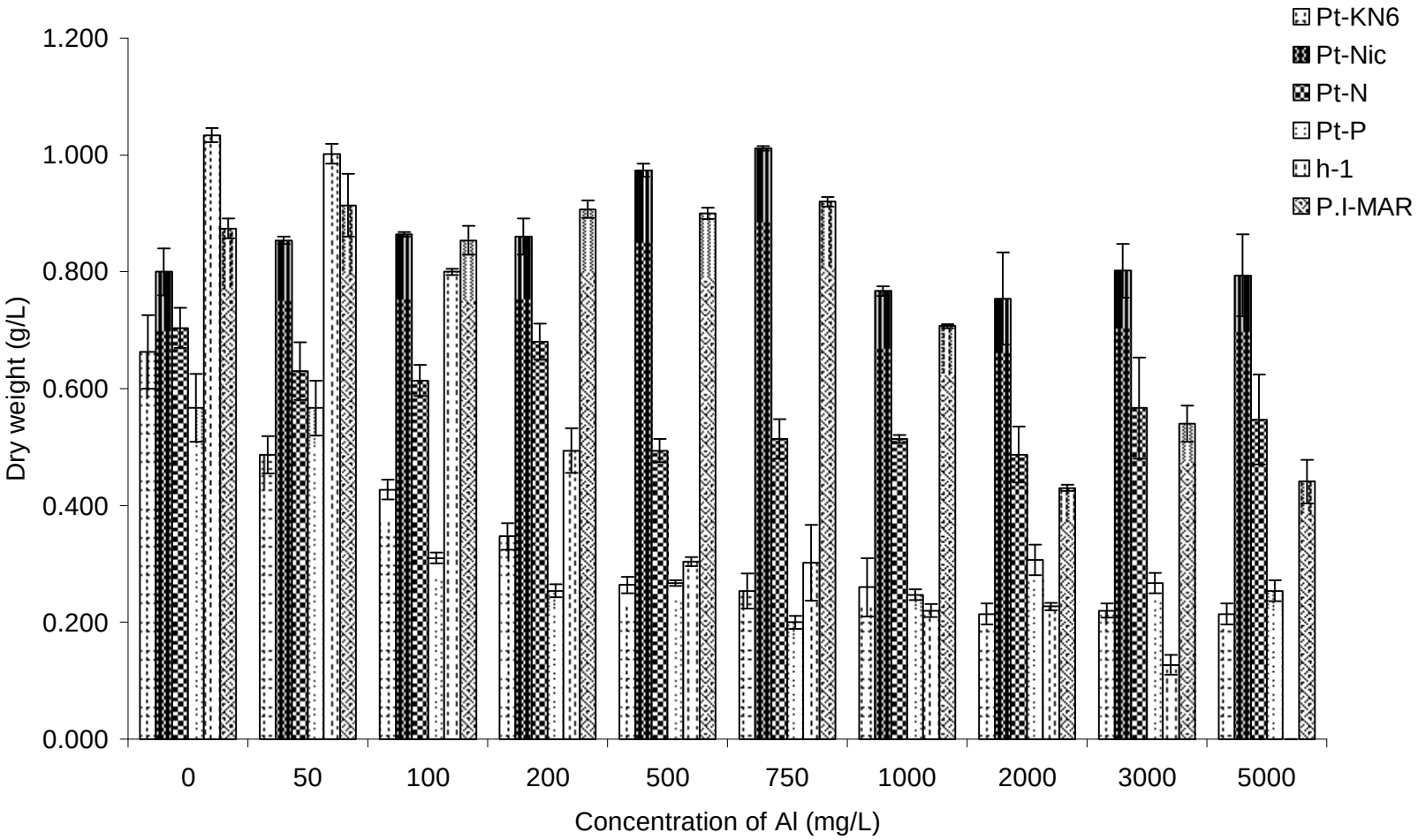

Figure: 4.5 Influence of Aluminium concentrations in the nutrient solution on the ponderal growth of different ectomycorrhizal fungi. Mean value ± SE.

Table: 4.7a Influence of Aluminium concentrations in the nutrient solution on % inhibition of growth of different ectomycorrhizal fungi.

Al (mg/L)	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
50	**26.64**	**-6.67**	**10.44**	**0.00**	**3.29**	**-4.58**
100	**35.68**	**-7.93**	**12.79**	**44.69**	**22.63**	**0.00**
200	**47.74**	**-7.50**	**13.33**	**52.94**	**52.28**	**-3.81**
500	**60.30**	**-21.7**	**29.85**	**55.28**	**70.66**	**-3.05**
750	**61.80**	**-25.0**	**27.01**	**54.71**	**70.99**	**-5.34**
1000	**61.81**	**4.16**	**27.01**	**56.47**	**78.72**	**19.09**
2000	**67.83**	**5.83**	**30.81**	**45.88**	**78.14**	**50.81**
3000	**66.84**	**0.00**	**19.43**	**52.94**	**87.81**	**38.17**
5000	**67.83**	**0.00**	**22.28**	**55.28**	**100**	**48.85**

Table: 4.7b Influence of Aluminium concentrations in the nutrient solution on EC_{50} value of different ectomycorrhizal fungi.

	EC_{50} value of Aluminium
Pt-KN6	**EC_{50}< 200 mg/L**
Pt-NIC	**No significant reduction in biomass till 5000 mg/L**
Pt-N	**22% inhibition in growth found till 5000 mg/L**
Pt-P	**EC_{50} <200 mg/L**
h-1	**EC_{50} <200 mg/L**
P.I-MAR	**EC_{50} <2000 mg/L**

4.3.2 Phosphatase activity

The acid phosphatase activity of Pt-KN6 significantly decreased as the concentration of aluminium increased in the medium upto 200 mg/L and increased at 5000 mg/L of Al concentration when compared to the control mycelium. The acid phosphatase activity of Pt-NIC decreased significantly with increasing concentrations of aluminium when compared to its control mycelium growing in absence of aluminium. Pt-N showed increased acid phosphates activity as compared to the control at all the concentrations of aluminium. In case of Pt-P the activity increased as compared to the control at all the concentrations of aluminium but the maximum enzyme activity was recorded at 100 and 200 mg/L of aluminium. The acid phosphatase activity of h-1 decreased significantly with increasing concentrations of aluminium till 2000 mg/L as compared to control and at 3000 mg/L there was no significant difference in acid phosphatase activity. The acid phosphatase activity of P.I-MAR firstly decreased till 2000 µg/ml of aluminium and then increased at 4000 and 5000 mg/L of aluminium concentration as compared to control. (Table 4.8 and Figure 4.6). These results showed that the maximum acid phosphatase activity was found in Pt-N and h-1 isolate showed minimum activity when grown in presence of aluminium.

Overall the presence of aluminium in the culture medium increased the fungal acid phosphatase activity. Since one of the main effects of aluminium on phosphate in the soil solution is that the freely available phosphate concentration decreased due to the complexation of phosphates with Al^{3+}, the fungal acid phosphatase activity thus enabling the fungi to mobilize phosphorus for its use in phosphorus limiting problematic soils.

Table: 4.8 Influence of Aluminium concentrations in the nutrient solution on the acid phosphatase activity (μM PNP/g dry weight/hour) of different ectomycorrhizal fungi.

Al ***(mg/L)***	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**250.04b**	**125.95a**	**180.17c**	**150.55ef**	**67.06a**	**113.55b**
100	**108.36e**	**92.35b**	**459.97a**	**274.19a**	**33.53c**	**86.180d**
200	**94.74e**	**72.04cd**	**466.88a**	**249.73ab**	**26.66c**	**90.46cd**
500	**182.14c**	**51.10f**	**333.91b**	**203.71cd**	**24.79c**	**89.735cd**
1000	**168.34d**	**56.69f**	**360.53b**	**174.23de**	**30.97c**	**107.34bc**
2000	**196.87c**	**65.01e**	**410.48ab**	**120.96f**	**53.15b**	**101.79bcd**
3000	**253.50b**	**69.52e**	**385.06ab**	**145.47ef**	**57.56ab**	**143.89a**
5000	**393.25a**	**82.49c**	**386.55ab**	**217.40bc**	**0.0d**	**153.96a**

Values sharing a common letter within the fungus are not significantly different at $P<0.05$

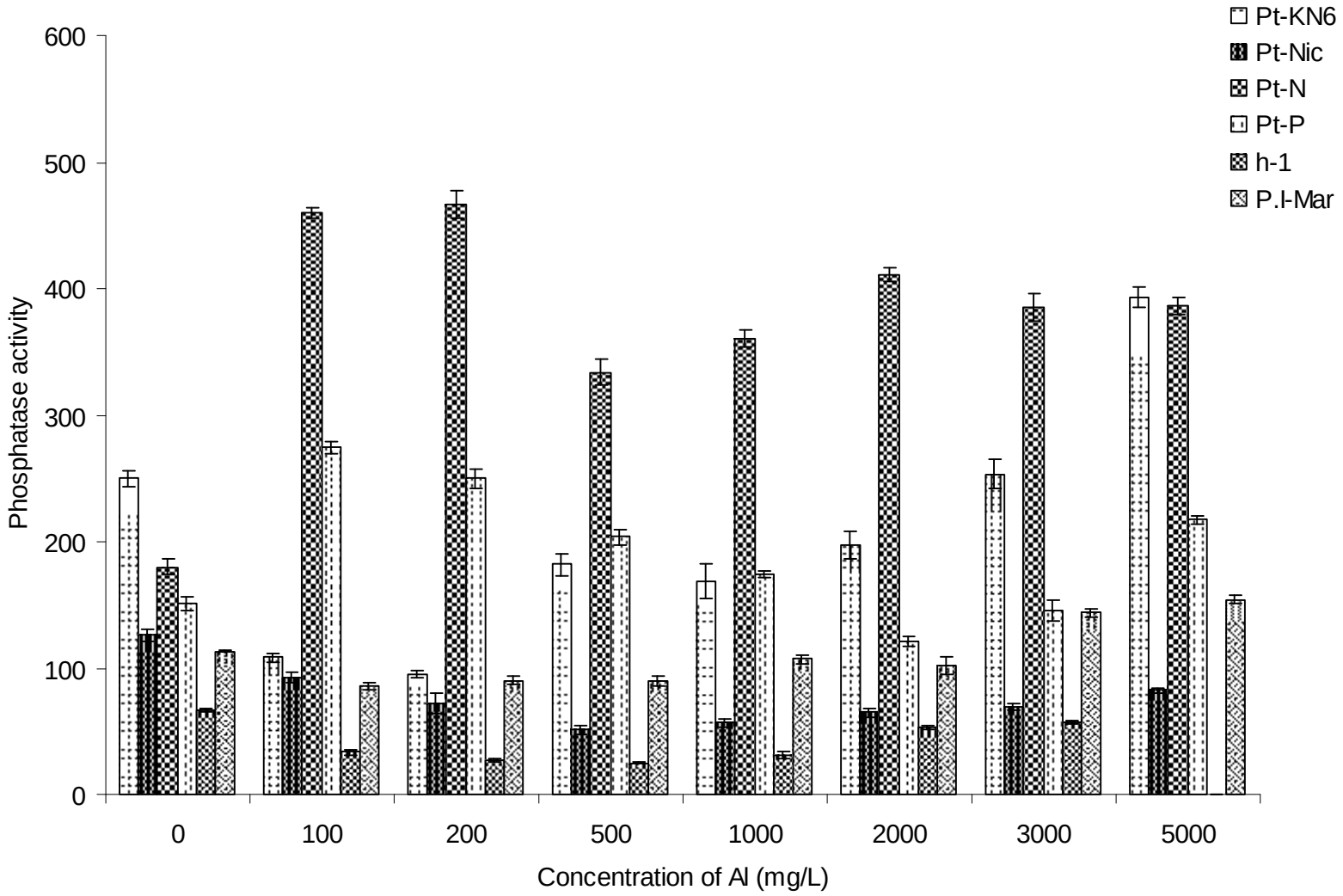

Figure: 4.6 Influence of Al concentrations in the nutrient solution on the acid phosphatase activity of different ectomycorrhizal fungi. Mean value ± SE.

4.3.3 Concentration of aluminium in the mycelium

The aluminium content in the mycelium of ectomycorrhizal fungi increased as a function of aluminium concentration in the growth medium (Table 4.9 and Figure 4.7). The increase in the aluminium content of Pt-KN6 was maximum at 1000 mg/L of aluminium concentration and there after it decreased at higher concentrations of aluminium. In Pt-NIC, the accumulation of aluminium increased significantly with increasing concentrations of aluminium reaching maximum at 500 mg/L of aluminium and then decreased significantly at further higher concentrations. In case of Pt-N and Pt-P the aluminium accumulation in the mycelium increased significantly with increasing aluminium concentrations in the nutrient medium reaching maximum at 3000 mg/L and 5000 mg/L of Al respectively. Similarly, in h-1 maximum accumulation of aluminium was found at 3000 mg/L of aluminium concentration and the fungus did not grow at 5000 mg/L of aluminium. The maximum accumulation of aluminium in case of P.I-MAR was at 750 mg/L of aluminium and it decreased further as concentrations of aluminium increased. Among the different ectomycorrhizal fungi tested, Pt-NIC accumulated maximum aluminium in its mycelium with no significant reduction in biomass.

4.3.4 Mineral nutrient content in the mycelium

The presence of aluminium in the culture medium affected the mineral nutrition of all the ectomycorrhizal fungi. At lower concentrations of aluminium, the phosphorus levels were increased in the mycelium and were significantly reduced at higher concentrations in some fungi. In case of Pt-N, Pt-P, h-1 and P.I-MAR the phosphorus content increased at lower concentrations of aluminium and then decreased significantly at higher concentrations of aluminium when compared to control mycelium. In case of Pt-NIC and

Pt-KN6 the phosphorus content increased significantly in the mycelia with increasing aluminium concentrations as compared to the control mycelium (Table 4.10 and Figure 4.8).

At lower concentrations (0- 500 mg/L of Al), the calcium content in the mycelium was increased in case of *P. albus* isolates (Pt-KN6, Pt-N and Pt-P) and decreased at higher concentrations of aluminium. In case of P.I-MAR, the calcium content increased upto 200 mg/L of aluminium and further decreased as the concentration of aluminium increased in the growth medium. At higher concentrations, the calcium content was decreased significantly in all the fungi except in h-1 where the level of calcium in the mycelium was increased significantly. In case of Pt-NIC the calcium content decreased with increasing concentrations of aluminium when compared to the control mycelium (Table 4.11 and Figure 4.9).

The magnesium content in the mycelium increased upto 1000 mg/L of aluminium concentrations in case of Pt-KN6 and P.I-MAR and then decreased significantly at higher concentrations. In Pt-N magnesium content in the mycelium increased significantly upto 750 mg/L of aluminium concentration and then decreased significantly at higher concentrations. In Pt-P the magnesium level in the mycelium increased significantly with increasing aluminium concentrations upto 2000 mg/L and then decreased. In case of h-1, the magnesium content increased with increasing concentrations of aluminium up to 3000 mg/L as compared to the control and the fungus failed to grow thereafter. However, reverse was found in Pt-NIC where the magnesium content decreased in the mycelium with increasing concentrations of aluminium as compared to the control (Table 4.12 and Figure 4.10).

Table: 4.9 Influence of Aluminium concentrations in the nutrient solution on the accumulation of Aluminium in the mycelium (mg/g dry weight) of different ectomycorrhizal fungi.

Al ***(mg/L)***	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**0.82f**	**0.77f**	**0.89g**	**0.50g**	**0.32e**	**0.64f**
50	**4.43e**	**1.39ef**	**3.64de**	**2.84f**	**1.02e**	**3.11e**
100	**4.60e**	**1.77e**	**3.25ef**	**4.07cd**	**2.01d**	**5.16bc**
200	**4.69e**	**1.71e**	**2.98f**	**3.62de**	**1.77d**	**3.76d**
500	**5.91d**	**16.14a**	**4.14d**	**3.32ef**	**4.03c**	**5.77a**
750	**8.57b**	**13.99b**	**5.46c**	**4.40c**	**3.71c**	**5.43ab**
1000	**10.25a**	**13.54b**	**4.13d**	**3.30ef**	**5.00b**	**4.97bc**
2000	**7.73c**	**10.35d**	**5.79c**	**6.39b**	**8.66a**	**4.82c**
3000	**6.98c**	**10.85d**	**7.25**	**8.52a**	**8.85a**	**4.62c**
5000	**4.79e**	**12.79bc**	**6.50b**	**8.32a**	**0.00f**	**4.96bc**

Values sharing a common letter within the fungus are not significantly different at $P<0.05$

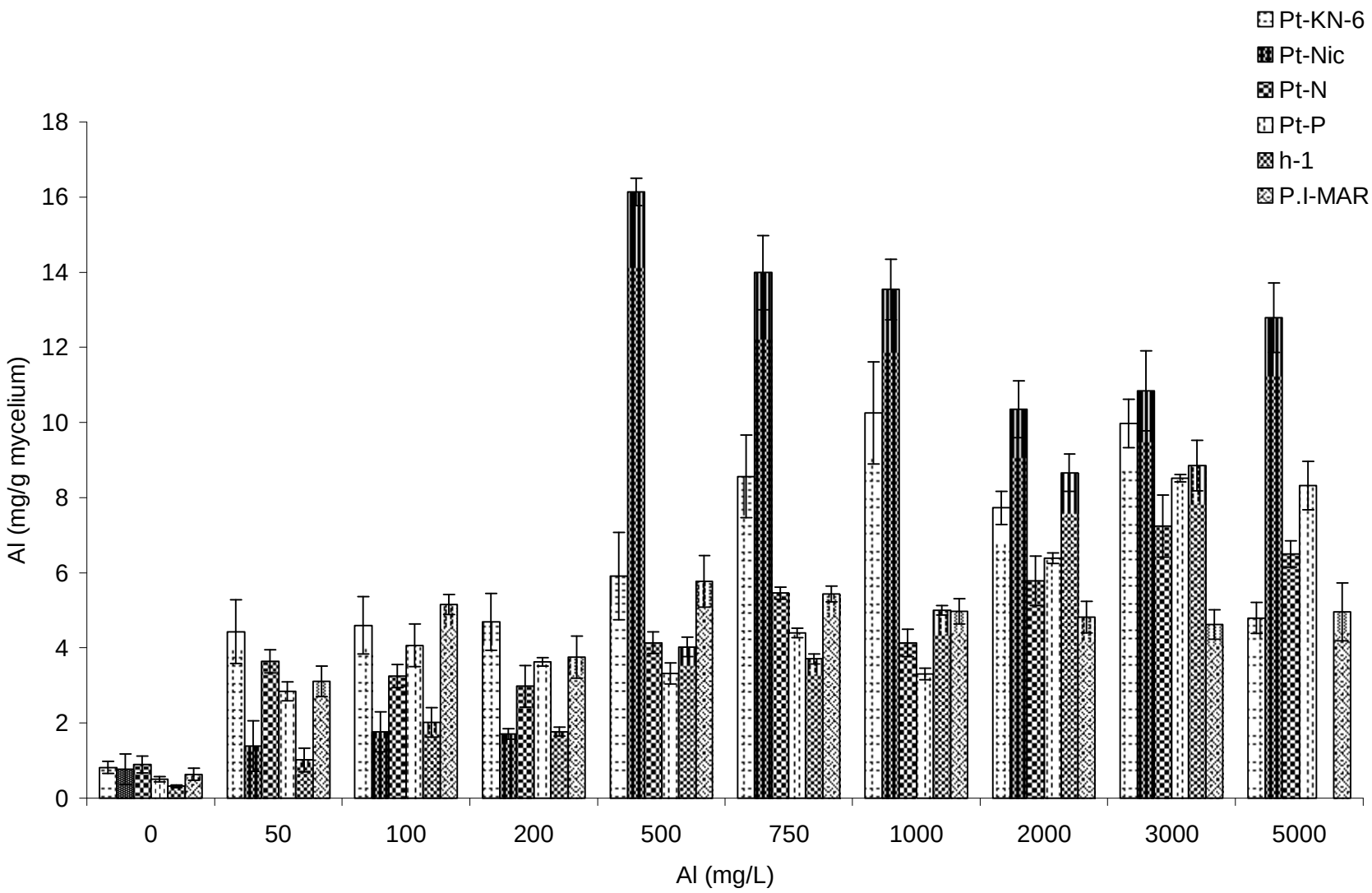

Figure: 4.7 Influence of Aluminium concentrations in the nutrient solution on accumulation of Aluminium in the mycelium of different ectomycorrhizal fungi. Mean value ± SE.

Table: 4.10 Influence of Aluminium concentrations in the nutrient solution on the Phosphorus content in the mycelium (mg/g dry weight) of different ectomycorrhizal fungi.

Al ***(mg/L)***	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**109.84f**	**190.58e**	**181.14c**	**191.75d**	**138.05d**	**73.35ef**
50	**211.50e**	**282.56b**	**212.37a**	**278.41a**	**115.61e**	**175.90c**
100	**240.44d**	**295.12a**	**193.04b**	**271.32ab**	**167.51c**	**224.92b**
200	**301.11bc**	**287.93ab**	**176.25c**	**258.26ab**	**225.56b**	**258.50a**
500	**309.40ab**	**251.02c**	**121.21d**	**251.01b**	**347.27a**	**179.50c**
750	**306.93ab**	**192.08e**	**63.77e**	**219.43c**	**237.13b**	**104.91d**
1000	**321.57a**	**172.84f**	**35.41f**	**173.48de**	**82.41f**	**82 79.50e**
2000	**290.93c**	**189.06e**	**28.24f**	**164.84e**	**80.01f**	**67.45f**
3000	**288.54c**	**215.31d**	**17.52g**	**140.59f**	**58.10g**	**66.05f**
5000	**286.08c**	**188.56e**	**16.49g**	**90.79g**	**0.00h**	**53.95g**

Values sharing a common letter within the fungus are not significantly different at P<0.05

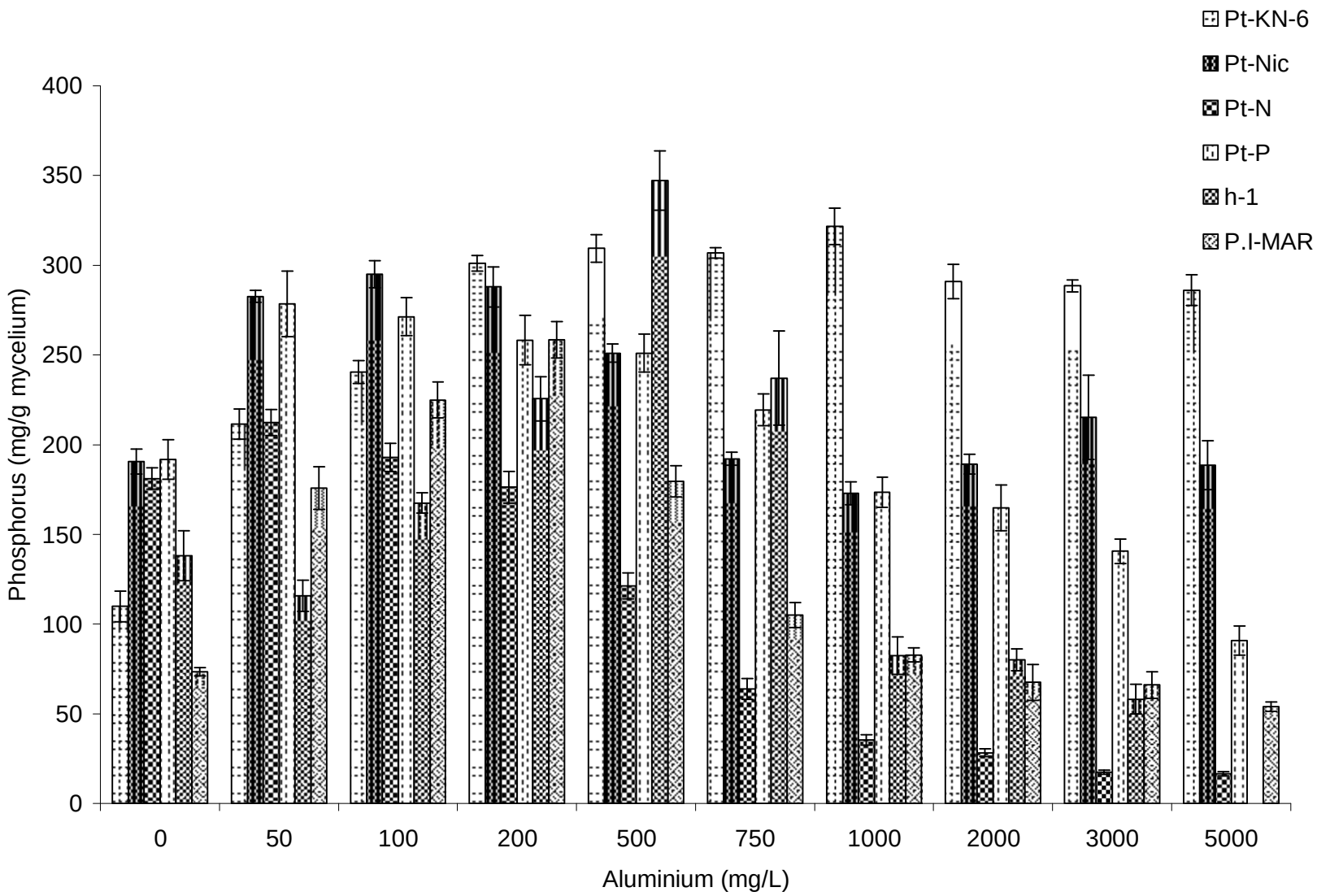

Figure: 4.8 Influence of Al concentrations in the nutrient solution on the Phosphorus content in the mycelium of different ectomycorrhizal fungi. Mean value ± SE.

Table: 4.11 Influence of Aluminium concentrations in the nutrient solution on the Calcium content in the mycelium (mg/g dry weight) of different ectomycorrhizal fungi.

Al ***(mg/L)***	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**1.77g**	**1.90a**	**2.57c**	**0.64c**	**1.04cd**	**4.09b**
50	**1.88g**	**1.77ab**	**1.88d**	**0.72c**	**0.89d**	**4.38b**
100	**2.20f**	**1.66b**	**2.03d**	**0.91b**	**1.04cd**	**4.11b**
200	**3.22e**	**1.16c**	**1.93d**	**1.69a**	**1.77cd**	**4.73a**
500	**5.54a**	**0.90d**	**5.29a**	**0.92b**	**3.40ab**	**2.75c**
750	**5.25b**	**0.82d**	**3.53b**	**0.93b**	**3.74ab**	**2.93c**
1000	**4.53c**	**0.62e**	**2.42c**	**0.29d**	**4.22a**	**1.96e**
2000	**4.12d**	**0.59e**	**1.49e**	**0.28d**	**3.21abc**	**2.28d**
3000	**2.32f**	**0.50e**	**1.12f**	**0.22d**	**3.91ab**	**2.85c**
5000	**0.18h**	**0.09f**	**0.42g**	**0.23d**	**0.00e**	**2.67c**

Values sharing a common letter within the fungus are not significantly different at $P<0.05$

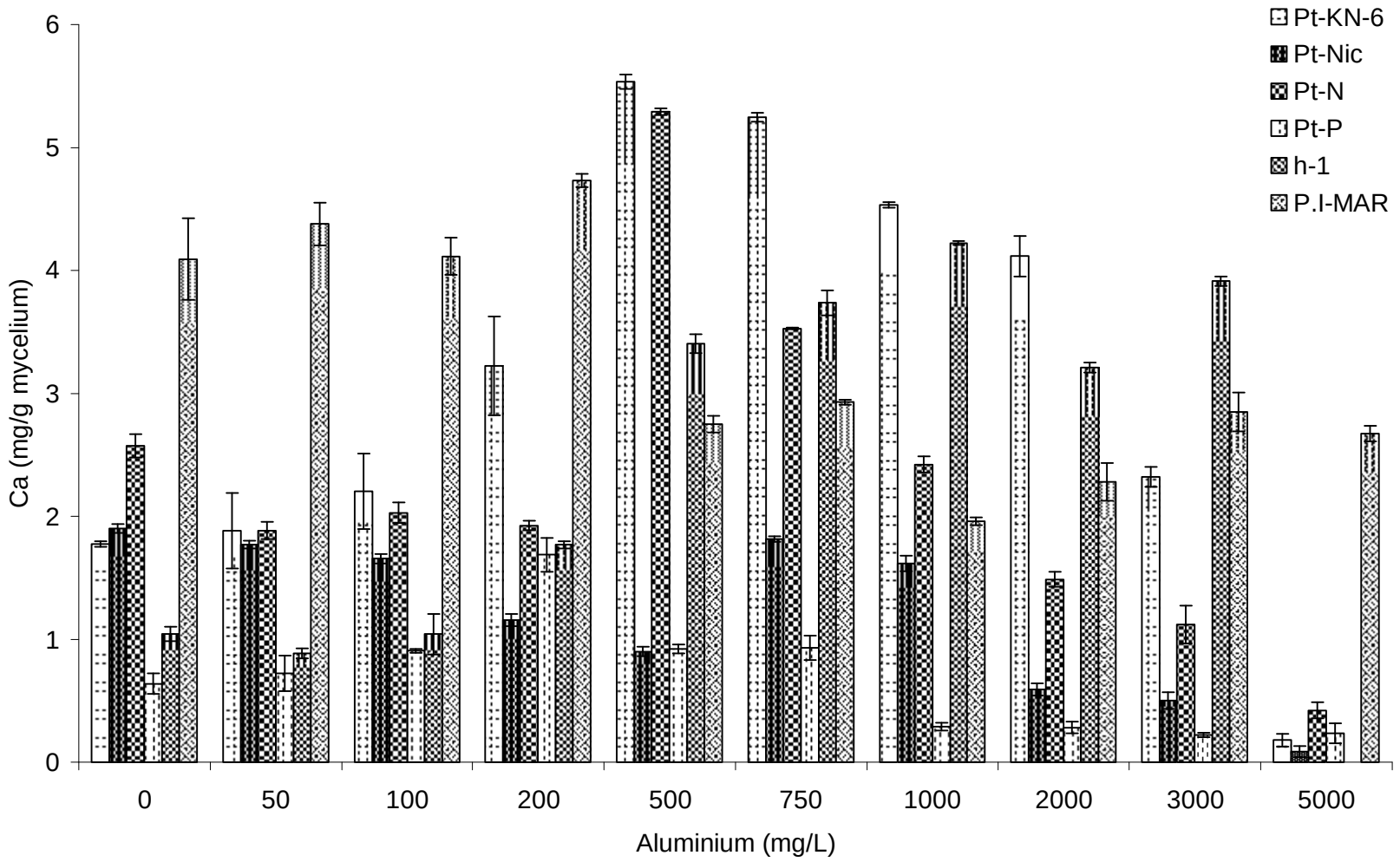

Figure: 4.9 Influence of Al concentrations in the nutrient solution on the Calcium content in the mycelium of different ectomycorrhizal fungi. Mean value ± SE.

Table: 4.12 Influence of Aluminium concentrations in the nutrient solution on the Magnesium content in the mycelium (mg/g dry weight) of different ectomycorrhizal fungi.

Al ***(mg/L)***	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**1.08g**	**2.99a**	**1.70e**	**0.90e**	**0.71f**	**1.42b**
50	**1.22g**	**2.48b**	**1.53f**	**1.71d**	**0.72f**	**1.10c**
100	**1.08g**	**1.73c**	**2.01cd**	**2.27a**	**1.14e**	**1.40b**
200	**1.93f**	**1.57c**	**2.12c**	**1.96bc**	**1.42d**	**1.40b**
500	**2.31e**	**1.03f**	**2.42b**	**2.14abc**	**2.59bc**	**1.12c**
750	**3.47c**	**1.51d**	**3.11a**	**1.99bc**	**2.70b**	**1.07c**
1000	**5.64a**	**1.54c**	**2.10c**	**2.16ab**	**3.09a**	**2.10a**
2000	**4.99b**	**1.11e**	**1.90d**	**2.33a**	**3.12a**	**1.41b**
3000	**3.09d**	**1.23e**	**1.48f**	**1.92bcd**	**3.08a**	**0.93d**
5000	**0.65h**	**0.77f**	**0.78g**	**1.90cd**	**0.00g**	**0.89d**

Values sharing a common letter within the fungus are not significantly different at $P<0.05$

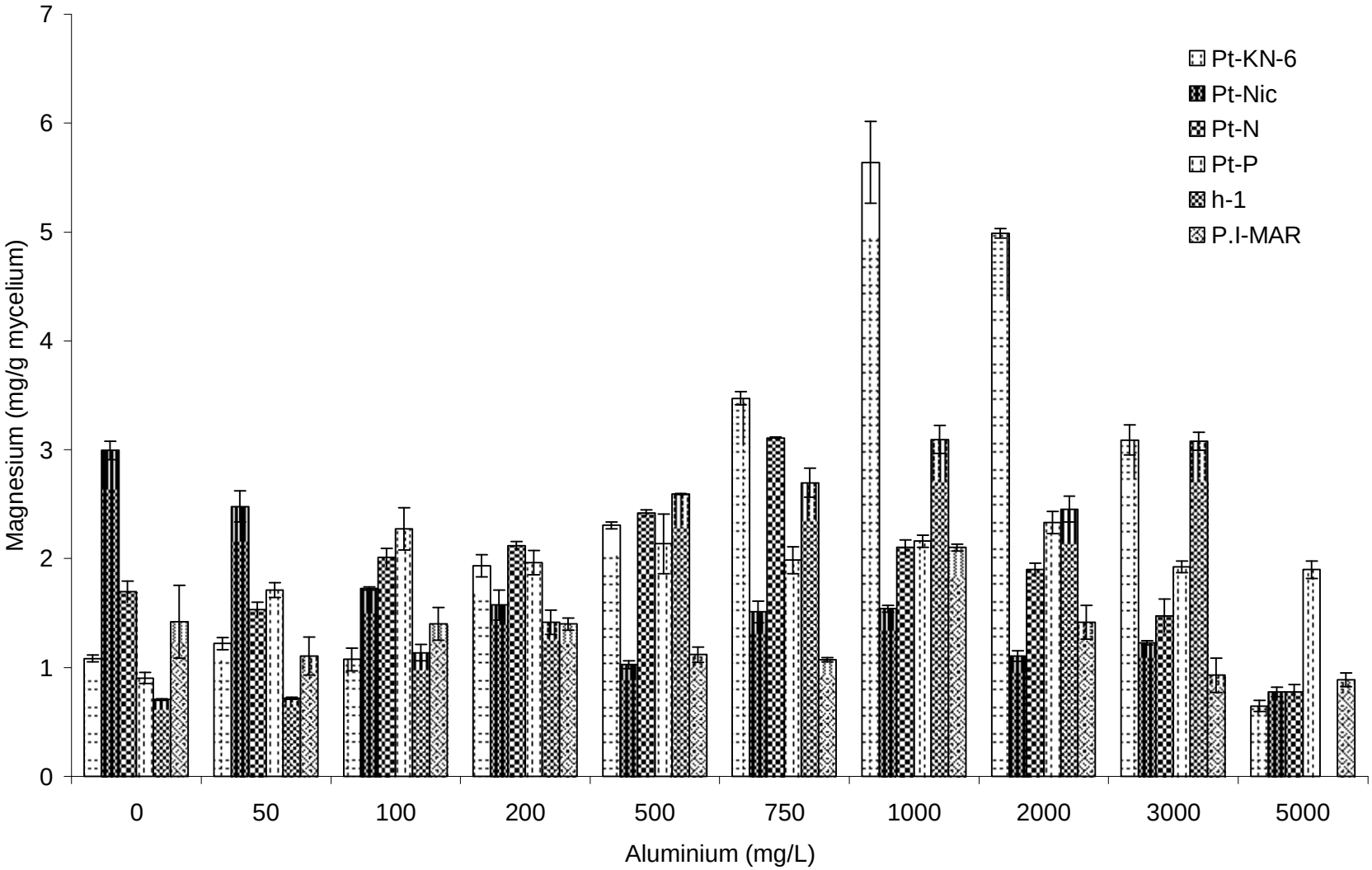

Figure: 4.10 Influence of Al concentrations in the nutrient solution on the Magnesium content in the mycelium of different ectomycorrhizal fungi. Mean value ± SE.

Table: 4.13 Influence of Aluminium concentrations in the nutrient solution on the Potassium content in the mycelium (mg/g dry weight) of different ectomycorrhizal fungi.

Al (mg/L)	*Pt-KN-6*	*Pt-NIC*	*Pt-N*	*Pt-P*	*h-1*	*P.I-MAR*
0	**0.61c**	**10.58a**	**1.01e**	**1.02cd**	**0.17e**	**0.88c**
50	**0.93a**	**10.45a**	**2.58b**	**0.61f**	**0.18e**	**1.48b**
100	**0.90a**	**10.68a**	**2.52b**	**0.70ef**	**0.14e**	**1.99a**
200	**0.61c**	**8.16c**	**4.28a**	**0.61f**	**0.43d**	**0.90c**
500	**0.68b**	**7.63cd**	**1.60c**	**0.83ef**	**0.66c**	**0.62de**
750	**0.51d**	**7.51cd**	**1.58c**	**1.23bc**	**0.63c**	**0.61de**
1000	**0.40e**	**7.98c**	**1.31cd**	**1.19bc**	**0.66c**	**0.68d**
2000	**0.54d**	**7.68cd**	**1.39cd**	**1.31ab**	**0.75b**	**0.53e**
3000	**0.37e**	**7.68cd**	**1.07cde**	**1.52a**	**0.80a**	**0.62de**
5000	**0.18f**	**3.93e**	**0.59e**	**0.70ef**	**0.00f**	**0.62de**

Values sharing a common letter within the fungus are not significantly different at $P<0.05$

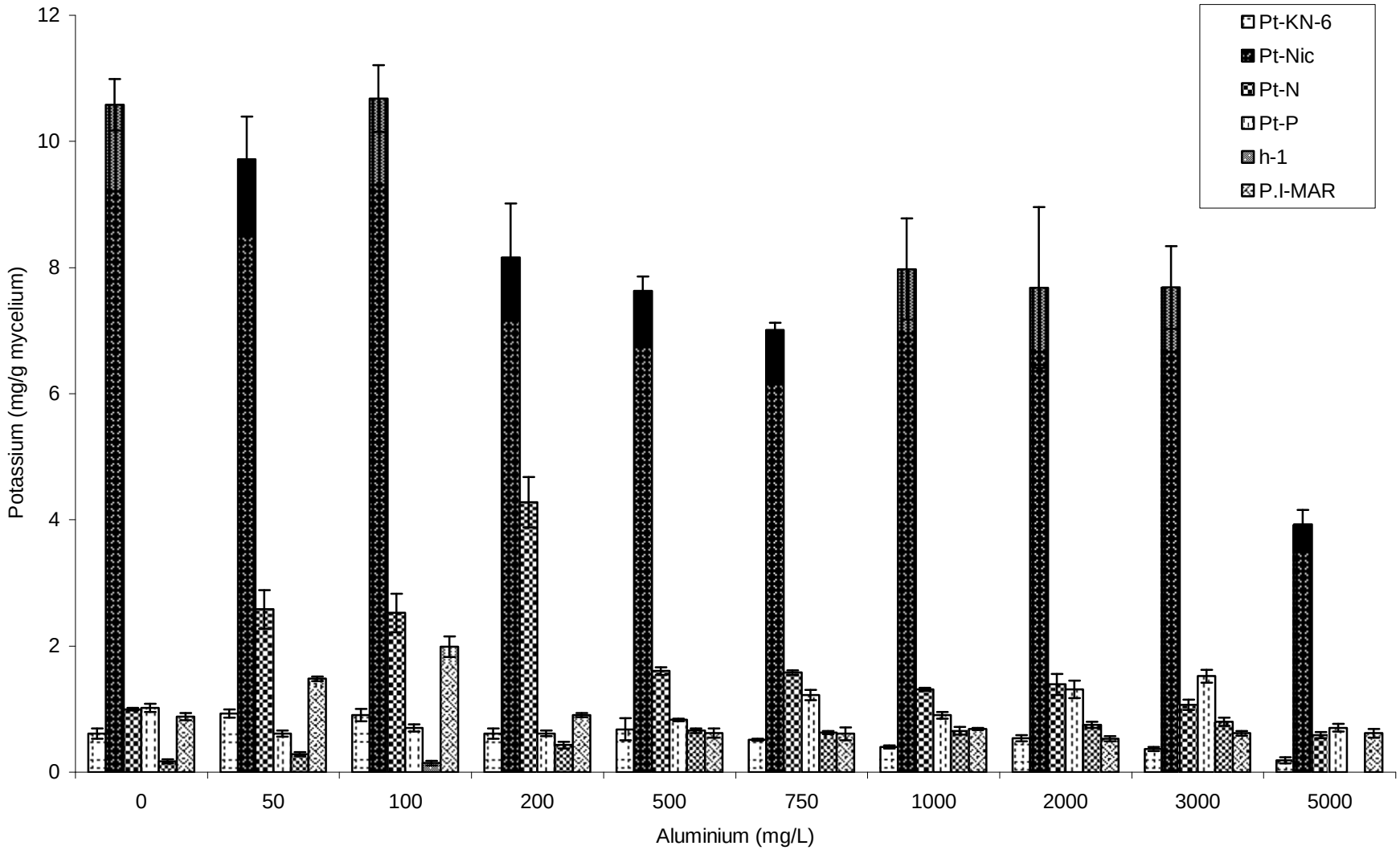

Figure: 4.11 Influence of Al concentrations in the nutrient solution on the Potassium content in the mycelium of different ectomycorrhizal fungi. Mean value ± SE.

The potassium content in the mycelium of Pt-KN6, Pt-N and P.I-MAR was increased at lower concentrations (100-200 mg/L of Al) and the potassium level was decreased significantly beyond 200 mg/L of aluminium. In case of Pt-NIC the potassium content in the mycelium was not significantly affected till 100 mg/L of aluminium concentration and then decreased significantly at 200 mg/L of aluminium and again no significant change in potassium content of the mycelium from 200-3000 mg/L of aluminium concentrations as compared to the control mycelium. The potassium content was significantly lower at 5000 mg/L of aluminium in Pt-NIC. In case of Pt-P, the level of potassium in the mycelium decreased significantly with increasing concentrations of aluminium upto 500 mg/L of aluminium concentration and the increased significantly till 3000 mg/L and again decreased at 5000 mg/L of aluminium concentration. The potassium content in the mycelium of h-1 was not significantly affected upto 100 mg/L of aluminium concentration and then increased significantly till 3000 mg/L (Table 4.13 and Figure 4.11). The above results indicated that Pt-NIC was the most tolerant ectomycorrhizal fungi among the different ectomycorrhizal fungi tested which showed no significant reduction in biomass till 5000 mg/L of aluminium concentration and maximum aluminium accumulatiom in the mycelium as compared to the control and the other fungi used in this study. P.I-MAR was also tolerant to aluminium with no significant reduction in biomass and maximum aluminium accumulation in the mycelium till 750 mg/L of aluminium concentration in the nutrient medium. Pt-KN6, Pt-P and h-1 were the aluminium sensitive ectomycorrhizal fungi with EC_{50} value of 200 mg/L of aluminium.

Pt-NIC accumulated maximum of aluminium and potassium in its mycelium among all the different ectomycorrhizal fungi tested. Pt-KN6 showed maximum uptake of phosphorus and magnesium. The maximum uptake of calcium was found in P.I-MAR.

Chapter V

Isolation of aluminium tolerant mutants of ectomycorrhizal fungi and study their mineral nutrition

5.1 Isolation of mutants of ectomycorrhizal fungi

To isolate aluminium tolerant ectomycorrhizal fungi for reclamation of bauxite mined out soils, ectomycorrhizal fungi were irradiated with U.V light. The fungal mycelium was either directly irradiated or the protoplasts were irradiated with U.V. and regenerated under the selection pressure of aluminium.

5.1.1 Irradiating the fungal mycelium

The ectomycorrhizal fungal mycelium grown in Melin's agar medium overlaid with cellophane sheet was irradiated for 6 minutes with U.V light and then transferred to the medium amended with aluminium at the concentration of 1000 mg/L of Al. The fungal hyphae, which were emerged after the irradiation of the mycelium was cut and transferred on to aluminium amended medium. After exposure to U.V., Pt-KN6 failed to grow even without the presence of aluminium as compared to its non irradiated control mycelium. This could be due to the death of the mycelium in 6 minutes of exposure to U.V light, therefore the Pt-KN6 mycelium was irradiated for different time intervals (0-5 minutes) but Pt-KN6 failed to grow after exposure to U.V.

The growth of irradiated Pt-NIC (Pt-NIC*) was significantly reduced as compared to its non irradiated control which showed no significant reduction in biomass even at 5000 mg/L of aluminium, the highest concentration used in this study. Similarly, the biomass of irradiated P.I-MAR (P.I-MAR*) and Pt-N (Pt-N*) was significantly reduced as compared

to their non irradiated controls which showed no significant reduction in growth till 1000 and 750 mg/L of aluminium respectively. Among the different irradiated ectomycorrhizal fungi, only Pt-P showed an increased tolerance to aluminium when compared to their wild type isolates. The irradiated Pt-P (Pt-P*) had an EC_{50} value < 3000 mg/L when compared to its wild type where its EC_{50} value < 200 mg/L of aluminium showing increased tolerance in terms of growth than its wild type (Table 5.1). Among the different ectomycorrhizal fungi exposed to U.V. only Pt-P showed more tolerance compared to other fungi where the irradiated mycelium showed less growth compared to their respective wild types.

The acid phosphatase activity of irradiated Pt-NIC* fungal mycelium decreased significantly at the low aluminium concentration (0, 100 mg/L) and then there was no significant change found in the phosphatase activity of irradiated and non irradiated mycelia.

Similarly, the phosphatase activity of irradiated P.I-MAR* fungal mycelium decreased significantly up to 2000 mg/L of aluminium concentrations and then there was no significant change found at higher concentrations in the irradiated and non irradiated mycelia. In case of Pt-N the phosphatase activity decreased till 200 mg/L of aluminium and then increased significantly at higher concentrations. The phosphatase activity of irradiated Pt-P* mycelium increased significantly as compared to non irradiated mycelium with increasing concentrations of aluminium (Table 5.2).

Table: 5.1 Influence of Al concentrations in the nutrient solution on the ponderal growth (g/L) of irradiated and nonirradiated ectomycorrhizal fungi.

*Al **(mg/L)***	*0*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**0.80 ± 0.04**	**0.85 ± 0.01**	**0.86 ± 0.01**	**0.86 ± 0.03**	**0.97 ± 0.01**	**1.01 ± 0.01**	**0.77 ± 0.01**	**0.75 ± 0.08**	**0.80 ± 0.05**
Pt-NIC*	**0.38 ± 0.06**	**0.36 ± 0.05**	**0.58 ± 0.01**	**0.48 ± 0.01**	**0.44 ± 0.01**	**0.50 ± 0.03**	**0.36 ± 0.02**	**0.38 ± 0.01**	**0.40 ± 0.01**
Pt-N	**0.70 ± 0.04**	**0.63 ± 0.05**	**0.61 ± 0.03**	**0.68 ± 0.03**	**0.49 ± 0.02**	**0.51 ± 0.03**	**0.51 ± 0.01**	**0.49 ± 0.05**	**0.51 ± 0.09**
Pt-N*	**0.44 ± 0.04**	**0.08 ± 0.05**	**0.04 ± 0.03**	**0.05 ± 0.02**	**0.04 ± 0.03**	**0.04 ± 0.01**	**0.04 ± 0.01**	**0.03 ± 0.01**	**0.03 ± 0.01**
Pt-P	**0.57 ± 0.06**	**0.57 ± 0.05**	**0.31 ± 0.01**	**0.25 ± 0.01**	**0.27 ± 0.01**	**0.20 ± 0.01**	**0.25 ± 0.01**	**0.31 ± 0.03**	**0.27 ± 0.02**
Pt-P*	**0.63 ± 0.04**	**0.51 ± 0.05**	**0.59 ± 0.02**	**0.51 ± 0.01**	**0.47 ± 0.05**	**0.38 ± 0.03**	**0.39 ± 0.01**	**0.36 ± 0.02**	**0.29 ± 0.04**
P.I-MAR	**0.87 ± 0.02**	**0.91 ± 0.05**	**0.85 ± 0.03**	**0.91 ± 0.02**	**0.90 ± 0.01**	**0.92 ± 0.01**	**0.71 ± 0.03**	**0.43 ± 0.01**	**0.54 ± 0.03**
P.I-MAR*	**0.32 ± 0.03**	**0.26 ± 0.05**	**0.22 ± 0.03**	**0.10 ± 0.04**	**0.14 ± 0.02**	**0.14 ± 0.03**	**0.12 ± 0.01**	**0.10 ± 0.01**	**0.08 ± 0.01**
D-2	**0.40 ± 0.03**	**0.42 ± 0.05**	**0.26 ± 0.03**	**0.15 ± 0.02**	**0.15 ± 0.02**	**0.16 ± 0.01**	**0.16 ± 0.01**	**0.13 ± 0.01**	**0.04 ± 0.01**
D-2*	**0.53 ± 0.03**	**0.72 ± 0.03**	**0.62 ± 0.01**	**0.38 ± 0.01**	**0.26 ± 0.02**	**0.18 ± 0.01**	**0.19 ± 0.02**	**0.18 ± 0.02**	**0.17 ± 0.01**

(Mean value ± SE)

Table: 5.2 Influence of Al concentrations in the nutrient solution on the acid phosphatase activity (µM PNP/g/hour) of irradiated and nonirradiated ectomycorrhizal fungi.

*Al **(mg/L)***	*0*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**125.9 ± 4.39**	**92.35 ± 3.76**	**72.04 ± 8.23**	**51.10 ± 3.58**	**57.65 ± 4.58**	**56.69 ± 3.76**	**65.01 ± 3.58**	**69.52 ± 2.55**	**82.49 ± 1.95**
Pt-NIC*	**59.34 ± 0.39**	**59.83 ± 3.56**	**76.20 ± 3.76**	**49.66 ± 8.23**	**52.37 ± 3.56**	**55.69 ± 1.33**	**64.85 ± 3.76**	**70.39 ± 3.58**	**84.35 ± 4.05**
Pt-N	**180.2 ± 6.21**	**459.9 ±4.32**	**466.9 ± 10.6**	**333.9 ± 9.85**	**348.9 ± 10.7**	**360.5 ± 6.85**	**410.5 ± 5.65**	**385.1 ± 10.6**	**386.5 ± 6.85**
Pt-N*	**86.08 ± 2.46**	**151.6 ± 3.22**	**168.9 ± 8.42**	**352.4 ± 7.63**	**372.8 ± 6.09**	**366.7 ± 10.8**	**451.6 ± 9.06**	**405.2 ± 11.6**	**413.6 ± 12.6**
Pt-P	**150.6 ± 5.58**	**274.2 ± 4.57**	**249.7 ± 7.98**	**203.7 ± 6.47**	**185.6 ±7.19**	**174.2 ± 3.26**	**120.9 ± 4.54**	**145.5 ± 7.98**	**217.4 ± 3.26**
Pt-P*	**329.2 ± 62.3**	**325.3 ± 23.7**	**315.1 ± 2.47**	**327.2 ± 27.2**	**333.1 ± 23.6**	**390.5 ±17.9**	**523.2 ± 19.5**	**464.5 ± 44.7**	**530.9 ± 10.4**
P.I-MAR	**113.6 ± 1.00**	**86.18 ± 2.66**	**90.46 ± 3.22**	**89.74 ± 3.63**	**92.62 ± 4.58**	**107.3 ± 3.43**	**101.8 ± 6.40**	**143.9 ± 3.22**	**153.9 ± 3.43**
P.I-MAR*	**67.71 ± 0.88**	**68.36 ± 2.55**	**72.31 ± 3.04**	**72.96 ± 4.35**	**48.70 ± 1.20**	**57.67 ± 0.65**	**83.54 ± 1.04**	**153.1 ± 2.16**	**161.9 ±2.24**
D-2	**88.97 ± 1.53**	**73.11 ± 2.18**	**103.7 ± 6.67**	**97.01 ± 3.06**	**85.64 ±1.26**	**78.69 ± 1.06**	**75.17 ± 0.84**	**75.39 ± 0.98**	**64.13 ± 0.64**
D-2*	**68.99 ± 1.72**	**53.71 ± 3.63**	**56.83 ± 8.29**	**61.66 ± 0.90**	**65.11 ± 2.55**	**79.12 ± 6.13**	**74.07 ± 10.7**	**74.94 ± 6.20**	**68.63 ± 3.04**

(Mean value ± SE)

5.1.2 Irradiating the protoplasts

5.1.2.1 Isolation and Regeneration of protoplasts

The isolation of mutants through protoplasts were carried out only for the ectomycorrhizal fungus *H. cylindrosporum* (h-1) as the protoplasts regenerate back into the fungal hyphae, where as the other fungi used in this study failed to regenerate from the protoplasts. The protoplasts were isolated by incubating the macerated mycelium in the osmotically stabilized lytic enzyme solution (a combination of Cellulase and Driselase enzymes) and then the protoplasts were collected by centrifugation, washed with osmotic buffer (0.7 M mannitol). The kinetics of protoplast formation was greatly affected by the length of time of incubation in lytic enzyme solutions and the concentration of lytic enzymes. The maximum protoplast yield was achieved in 110 minutes of incubation of the fungal macerate in the lytic enzyme solution and after that the yield of protoplasts decreased significantly (Table 5.3 and Figure 5.1). The results of dependence of protoplast yield on the concentration of lytic enzymes Cellulase and Driselase showed that the maximum number of protoplast formation was recorded at 2.5 mg/ml of Cellulase enzyme (Table 5.4 and Figure 5.2) and 5 mg/ml of Driselase enzyme concentration (Table 5.5 and Figure 5.3). As the procedure of isolating mutants involved the regeneration of the protoplasts, regeneration frequency (%) of the protoplasts was studied. The regeneration kinetics of the protoplasts was greatly affected by the length of incubation of the fungal macerate in the lytic enzyme solution and the age of the mycelia. The best protoplast regeneration was 1.2% at 80-110 minutes of incubation in the lytic enzyme solution (Table 5.6 and Figure 5.4) and the optimum age of the mycelia after maceration for getting the maximum number of protoplasts and % regeneration was found to be 3 days. On 4^{th} day the number of

protoplasts isolated was not significantly affected but the regeneration frequency was severely affected (Table 5.7 and Figure 5.5).

Table 5.3 Kinetics of protoplast formation of *H. cylindrosporum* illustrating the dependence of protoplast yield on the length of incubation in lytic enzyme solution.

Incubation time ***(in minutes)***	*No. of Protoplasts* ***($X10^6/cm^3$ wet mycelium)***
0	**0.00 ± 0.00**
20	**0.00 ± 0.00**
35	**29.40 ± 0.88**
50	**69.40 ± 1.34**
65	**106.7 ±1.45**
80	**132.0 ± 2.47**
95	**144.0 ± 1.71**
110	**156.0 ± 2.08**
125	**81.40 ± 1.86**
140	**56.00 ± 1.53**
155	**14.70 ± 0.88**
170	**08.00 ± 0.18**

(Mean value ± SE)

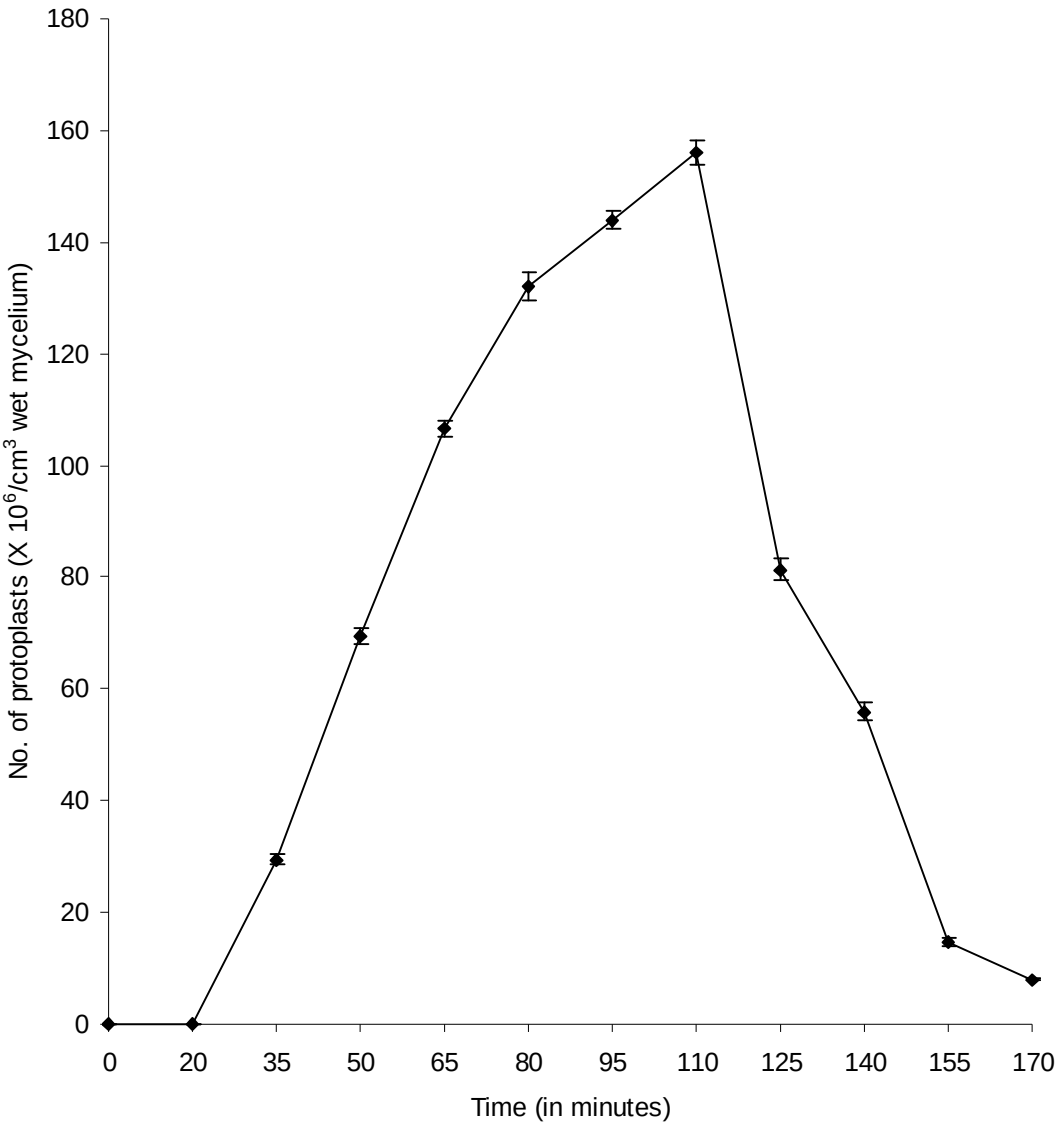

Figure 5.1 Kinetics of protoplast formation of *H. cylindrosporum* illustrating the dependence of protoplast yield on the length of incubation in lytic enzyme solution. Mean ± SE.

Table 5.4 Kinetics of protoplast formation of *H. cylindrosporum* illustrating the dependence of protoplast yield on the concentration of lytic enzyme (cellulase) in presence of 5 mg/ml of Driselase.

Concentration of Cellulase ***(mg/ml.)***	*No. of Protoplasts* ***($X10^6/cm^3$ wet mycelium)***
1.5	**82.0 ± 5.32**
2.0	**98.0 ± 4.46**
2.5	**116.0 ± 7.32**
3.0	**43.0 ± 5.72**

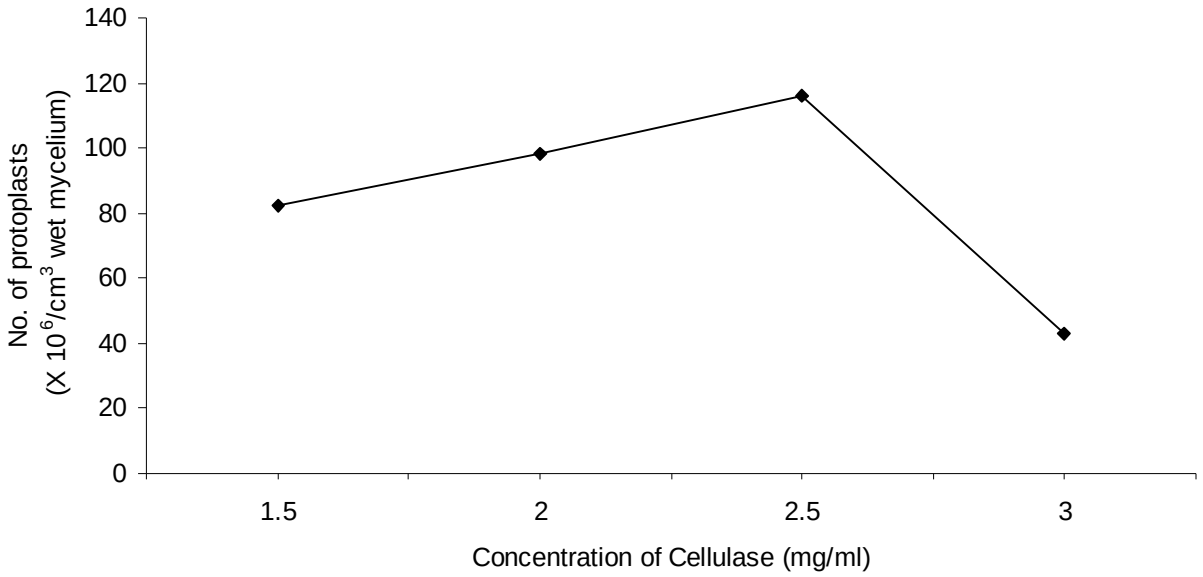

Figure 5.2 Kinetics of protoplast formation of *H. cylindrosporum* illustrating the dependence of protoplast yield on the concentration of lytic enzyme (cellulase) in presence of 5 mg/ml of Driselase.

Table 5.5 Kinetics of protoplast formation of *H. cylindrosporum* illustrating the dependence of protoplast yield on the concentration of lytic enzyme (Driselase) in presence of 2.5 mg/ml of Cellulase.

Concentration of Driselase ***(mg/ml)***	*No. of Protoplasts* ***($X10^6/cm^3$ wet mycelium)***
3.0	**22.0 ± 1.46**
4.0	**86.0 ± 4.22**
5.0	**125.0 ± 8.24**
6.0	**64.0 ± 3.28**

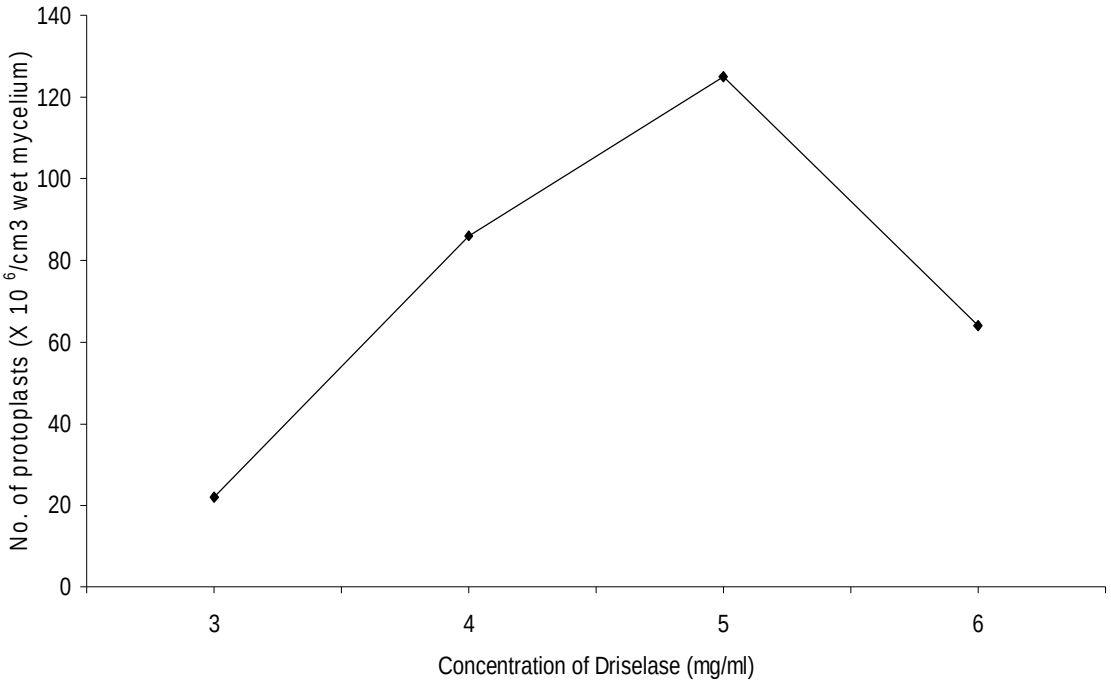

Figure 5.3 Kinetics of protoplast formation of *H. cylindrosporum* illustrating the dependence of protoplast yield on the concentration of lytic enzyme (Driselase) in presence of 2.5 mg/ml of Cellulase.

Table 5.6 The effect of length of incubation in lytic enzyme solution on % regeneration of protoplasts of *H. cylindrosporum*.

*Incubation time **(in minutes)***	*% Regeneration*
0	**0.0**
20	**0.2**
35	**0.6**
50	**0.7**
65	**0.8**
80	**1.2**
95	**1.2**
110	**1.2**
125	**0.6**
140	**0.1**
155	**0.0**
170	**0.0**

$$\% \text{ Regeneration} = \frac{\text{No. of fungal foci X 100}}{\text{Total no. of protoplasts plated}}$$

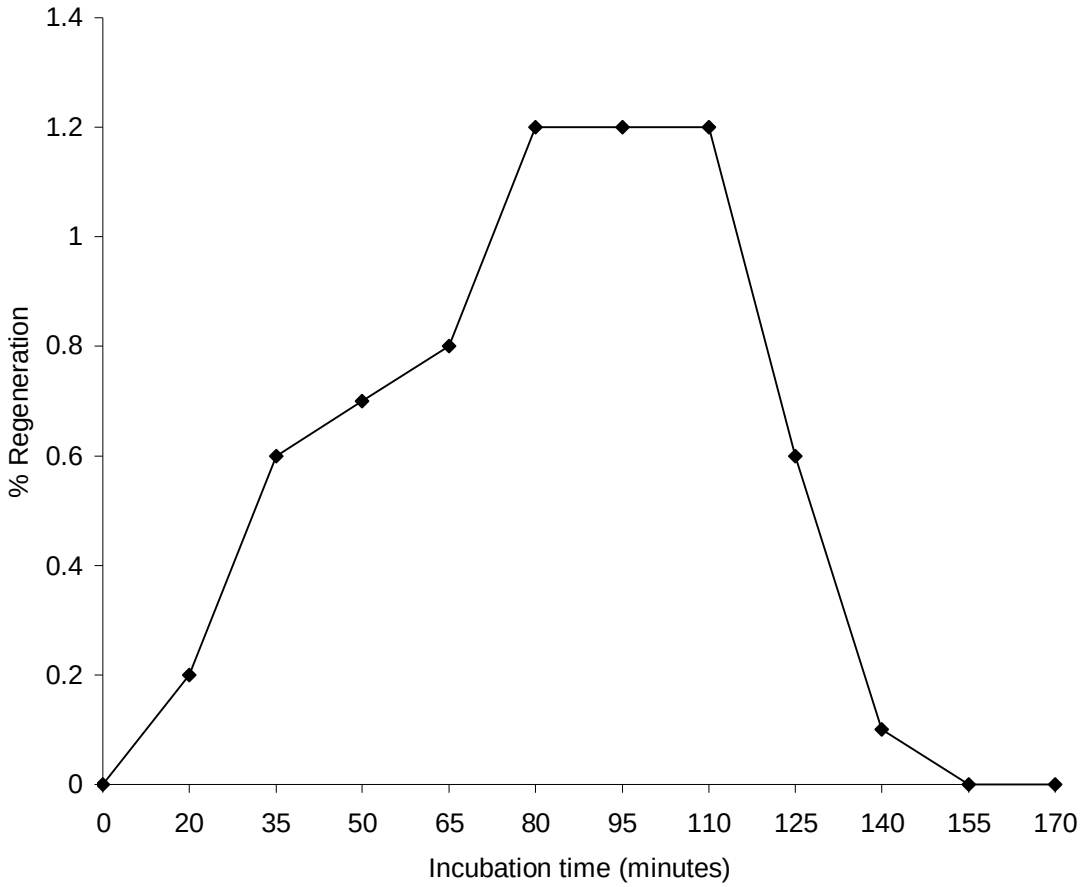

Figure 5.4 The effect of length of incubation in lytic enzyme solution on % regeneration of protoplasts of *H. cylindrosporum*.

Table 5.7 Influence of the age of the culture after maceration and the length of incubation in lytic enzyme solution on the number of protoplasts of *H. cylindrosporum* isolated and their regeneration frequency.

Mycelial Age ***(in Days)***	*No. of Protoplast* ***($X10^6/cm^3$ mycelium)***	*Regeneration* ***(%)***
0	**10.80 ± 0.02**	**0.0**
1	**9.80 ± 0.07**	**0.0**
2	**78.0 ± 0.38**	**0.6**
3	**116.0 ± 0.71**	**1.2**
4	**120.0 ± 0.74**	**0.8**
5	**80.0 ± 0.36**	**0.2**
6	**20.0 ± 0.14**	**0.0**
8	**0.0 ± 0.00**	**0.0**
10	**0.0 ± 0.00**	**0.0**

$$\% \text{ Regeneration} = \frac{\text{No. of fungal foci X 100}}{\text{Total no. of protoplasts plated}}$$

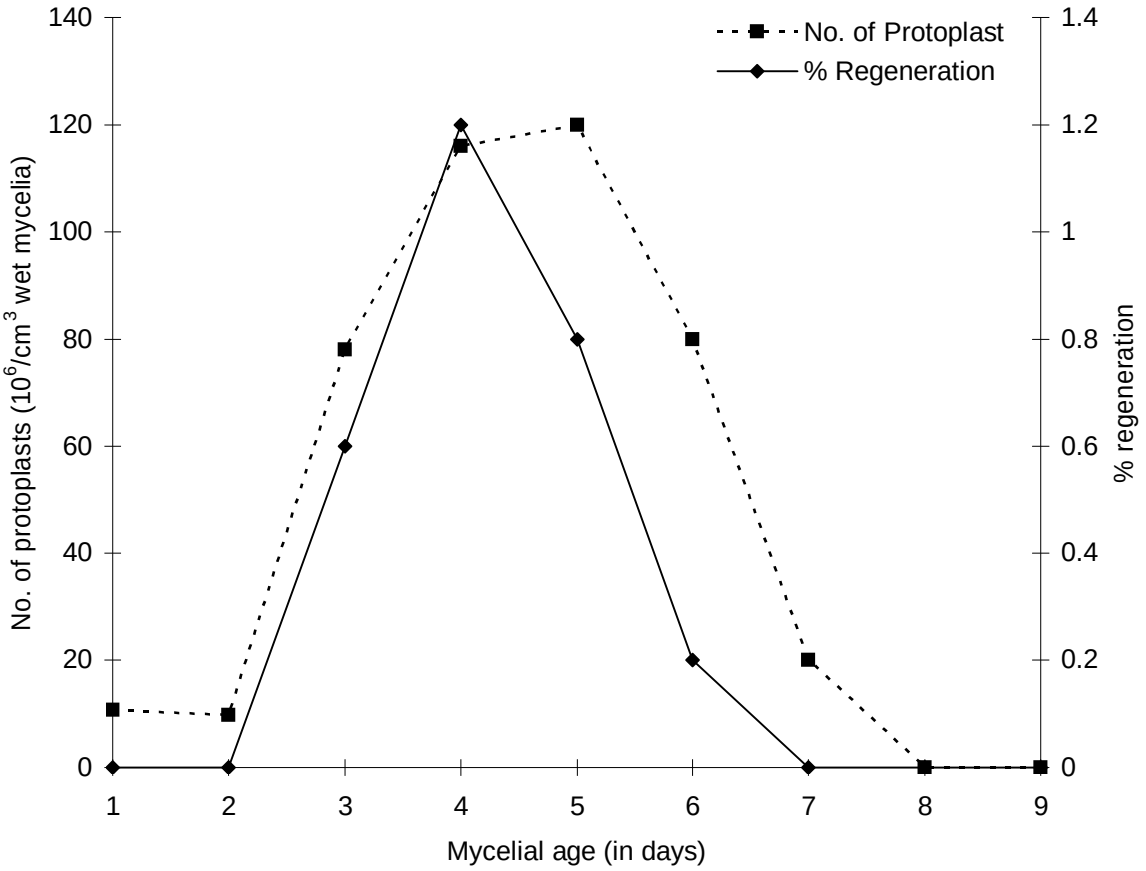

Figure 5.5 Influence of the age of the culture after maceration and the length of incubation in lytic enzyme solution on the number of protoplasts of *H. cylindrosporum* isolated and their regeneration frequency.

5.1.2.2 Mutagenesis

The protoplasts isolated were irradiated with U.V light to induce mutations for different time intervals (0-80 seconds) and plated on the aluminium amended regeneration medium. The population of 10^3 protoplasts/ microlitre/ plate was used. Assuming 1% regeneration (as evident from the results of Table 5.6) only 10 protoplasts per plate were expected to regenerate. At time zero 11 protoplasts regenerated on the medium and were assumed 100% survival of the protoplasts. As the time of irradiation increased the % survival of the protoplasts decreased significantly (Table 5.8 and Figure 5.6). At 60 seconds of irradiation 10% survival of the protoplasts was recorded as only one focus of fungal growth was observed per plate. So, 60 seconds was the time optimized for induction of mutation in the protoplasts as 90% of living protoplasts were killed with U.V light and 10% protoplasts were regenerated under selection pressure of 1000 µg/ml aluminium.

5.1.2.3 Dikaryotization of the mutant monokaryon h-1

The emerging hyphae from these regenerated protoplasts were dikaryotized with compatible monokaryon h-7 of *H. cylindrosporum*. The resultant dikaryon (D2*) had an EC_{50} value <750 µg/ml of aluminium as compared to its wild type dikaryon having EC_{50} value < 200 µg/ml of aluminium (Table 5.1) showing increased tolerance in terms of growth than its wild type (D2). The phosphatase activity of irradiated D2* fungal mycelium decreased significantly up to 750 mg/L of aluminium concentrations and then there was no significant change found at further higher concentrations in the irradiated and non irradiated types (Table 5.2).

5.2 Mineral Nutrition of the ectomycorrhizal fungi

The irradiated and their respective wild type ectomycorrhizal fungi were grown in Melin's medium with different concentrations of aluminium. The uptake of mineral nutritient ions and aluminium accumulation in the mycelium was compared for irradiated and nonirradiated mycelia.

5.2.1 Aluminium concentration in the mycelium

The accumulation of aluminium in the mycelium of the irradiated and its respective wild type ectomycorrhizal fungi was recorded. There was no significant difference found in the aluminium content of irradiated and wild type mycelia of *Pisolithus* isolates (Pt-N and Pt-NIC), *P. involutus* (P.I-MAR) and *H. cylindrosporum* (D2). Only, the uptake of aluminium by irradiated Pt-P* was significantly lower when compared to its wild type isolate at higher concentrations of aluminium (beyond 2000 mg/L of Al) (Table 5.9). The less accumulation of aluminium by Pt-P* at higher concentrations of aluminium might be the reason behind its more tolerance to aluminium as compared to its control.

5.2.2 Mineral content of the mycelium

The mineral nutrition of ectomycorrhizal fungi irradiated with U.V. light was studied and compared with the respective wild type isolates. Comparative analysis of mineral uptake of phosphorus (Table 5.10), calcium (Table 5.11) and magnesium (Table 5.12) did not show any significant difference between the irradiated and wild type ectomycorrhizal fungi. So, among the irradiated ectomycorrhizal fungi, the Pt-P* isolate of *P. albus* and D2* of *H. cylindrosporum* was selected for further *in vitro* plant- fungus interaction studies as they showed increased tolerance to aluminium apart from the tolerant isolates of Pt-NIC and P.I-MAR.

Table 5.8 Influence of the exposure to U.V light on the %age survival frequency of protoplasts of *H. cylindrosporum*.

Irradiating time ***(in seconds)***	*No. of Protoplast regenerated*	*%Survival*
0	**11.0 ± 1.53**	**100**
5	**9.30 ± 0.88**	**83.3**
10	**9.01 ± 1.73**	**80.0**
20	**7.30 ± 0.33**	**43.3**
30	**4.60 ± 0.67**	**40.7**
40	**3.30 ± 0.33**	**33.4**
50	**1.60 ± 0.33**	**16.7**
60	**1.00 ±0.00**	**10.0**
70	**1.00 ±0.00**	**10.0**
80	**0.00 ±0.00**	**0.0**

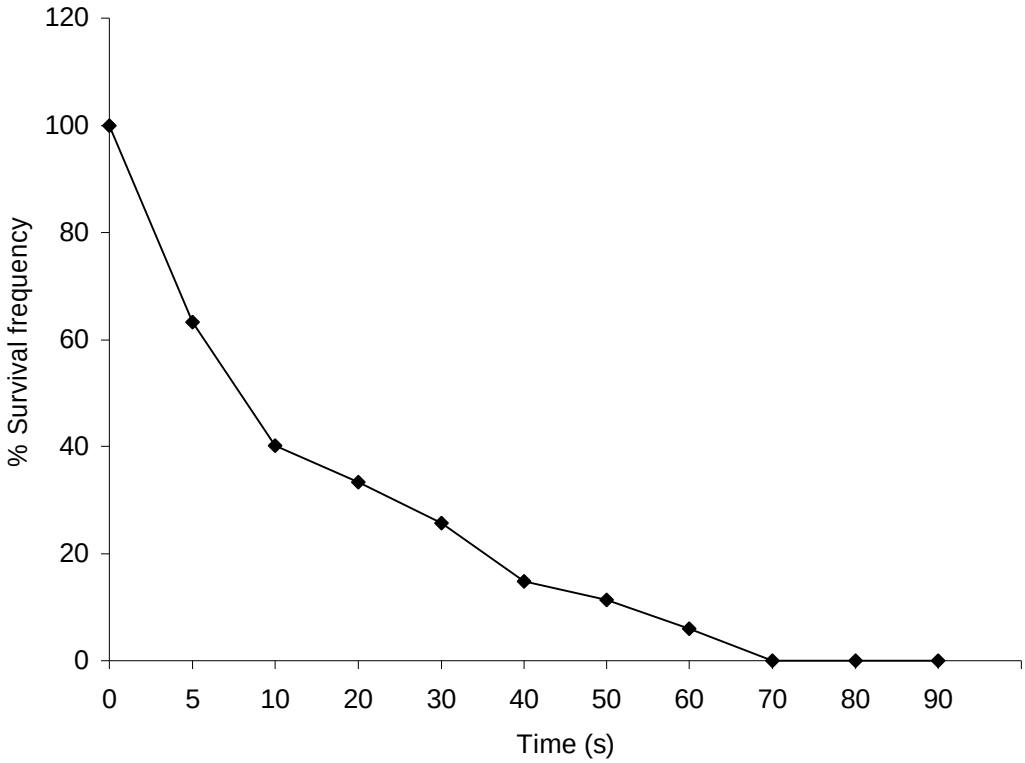

Figure 5.6 Influence of the exposure to U.V light on the %age survival frequency of protoplasts of *H. cylindrosporum*.

Table: 5.9 Influence of Al concentrations in the nutrient solution on its accumulation in the mycelium (mg/ g dry weight) of irradiated and nonirradiated ectomycorrhizal fungi.

Al (mg/L)	*0*	*50*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**0.77 ± 0.11**	**1.39 ± 0.67**	**1.77 ± 0.53**	**1.71 ± 0.14**	**16.1 ± 0.36**	**14.0 ±0.98**	**13.5 ± 0.80**	**10.3 ± 0.75**	**10.8 ± 1.06**	**12.8 ± 0.92**
Pt-NIC*	**0.67 ± 0.11**	**1.46 ± 0.48**	**1.88 ± 0.56**	**1.66 ± 0.26**	**17.3 ± 0.28**	**13.0 ±1.06**	**12.5 ± 0.91**	**10.7 ± 0.81**	**11.7 ± 1.14**	**15.9 ± 1.05**
Pt-N	**0.89 ± 0.23**	**3.64 ± 0.31**	**3.25 ± 0.31**	**2.98 ± 0.55**	**4.14 ± 0.29**	**5.46 ± 0.16**	**4.13 ± 0.37**	**5.79 ± 0.65**	**7.25 ± 0.82**	**6.50 ± 0.35**
Pt-N*	**0.89 ± 0.11**	**5.09 ± 0.37**	**5.12 ± 0.49**	**5.85 ± 0.61**	**5.49 ± 0.45**	**5.67 ± 0.25**	**5.95 ± 0.54**	**5.87 ± 0.53**	**9.57 ± 1.26**	**9.64 ± 0.66**
Pt-P	**0.50 ± 0.07**	**2.84 ± 0.25**	**4.07 ± 0.57**	**3.62 ± 0.11**	**3.32 ± 0.27**	**4.40 ± 0.25**	**3.30 ± 0.14**	**6.39 ± 0.13**	**8.52 ± 0.49**	**8.32 ± 0.67**
Pt-P*	**0.43 ± 0.08**	**1.57 ± 0.07**	**1.82 ± 0.09**	**1.63 ± 0.03**	**2.01 ± 0.41**	**1.82 ± 0.11**	**3.96 ± 0.63**	**2.47 ± 0.25**	**2.46 ± 0.56**	**3.30 ± 0.81**
P.I-MAR	**0.64 ± 0.16**	**3.11 ± 0.40**	**5.16 ± 0.26**	**3.76 ± 0.57**	**5.77 ± 0.68**	**5.43 ± 0.21**	**4.97± 0.33**	**4.82 ± 0.42**	**4.62 ± 0.39**	**4.96 ± 0.77**
P.I-MAR*	**0.65 ± 0.09**	**4.07 ± 0.52**	**5.28± 0.32**	**5.22 ± 0.57**	**5.49 ± 0.62**	**5.64 ± 0.65**	**5.87 ± 0.67**	**5.03 ± 0.57**	**5.17 ± 0.27**	**5.29 ± 0.35**
D-2	**0.51 ± 0.06**	**1.36 ± 0.25**	**1.98 ± 0.26**	**2.55 ± 0.45**	**3.06 ± 0.37**	**3.59 ± 0.57**	**3.48 ± 0.29**	**3.00 ± 0.55**	**3.15 ± 0.95**	**4.19 ± 0.65**
D-2*	**0.58 ± 0.16**	**1.33 ± 0.32**	**1.55 ± 0.25**	**2.28 ± 0.55**	**2.96 ± 0.25**	**2.55 ± 0.62**	**2.56 ± 0.22**	**2.25 ± 0.35**	**2.05 ± 0.55**	**3.06 ± 0.64**

(Mean value ± SE)

Table: 5.10 Influence of Al concentrations in the nutrient solution on the Phosphorus content in the mycelium (mg/ g dry weight) of irradiated and nonirradiated ectomycorrhizal fungi.

Al (mg/L)	*50*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**1.77 ± 0.03**	**1.65 ± 0.04**	**1.66 ± 0.05**	**0.90 ± 0.04**	**1.82 ± 0.03**	**1.62 ± 0.06**	**0.59 ± 0.05**	**0.50 ± 0.07**	**0.09 ± 0.01**
Pt-NIC*	**1.84 ± 0.03**	**1.73 ± 0.04**	**1.11± 0.05**	**0.95 ± 0.04**	**1.87 ± 0.03**	**1.64 ± 0.07**	**0.56 ± 0.05**	**0.50 ± 0.07**	**0.07 ± 0.01**
Pt-N	**1.88 ± 0.07**	**2.03 ± 0.08**	**1.93 ± 0.04**	**5.29 ± 0.03**	**3.53 ± 0.01**	**2.42 ± 0.07**	**1.49 ± 0.06**	**1.12 ± 0.15**	**0.42 ± 0.01**
Pt-N*	**1.99 ± 0.08**	**2.01 ± 0.08**	**2.00 ± 0.04**	**5.55 ± 0.03**	**3.57 ± 0.02**	**2.35 ± 0.02**	**1.38 ± 0.06**	**1.18 ± 0.13**	**0.49 ± 0.02**
Pt-P	**0.72 ± 0.14**	**0.91 ± 0.01**	**1.69 ± 0.14**	**0.92 ± 0.04**	**0.93 ± 0.10**	**0.29 ± 0.03**	**0.28 ± 0.05**	**0.22 ± 0.15**	**0.23 ±0.05**
Pt-P*	**0.76 ± 0.13**	**0.96 ± 0.05**	**1.67 ± 0.09**	**0.99 ± 0.05**	**0.98 ± 0.14**	**0.32 ± 0.05**	**0.30 ± 0.06**	**0.24 ± 0.10**	**0.27 ± 0.06**
P.I-MAR	**4.38 ± 0.17**	**4.11 ± 0.15**	**4.73 ± 0.06**	**2.75 ± 0.07**	**2.93 ± 0.02**	**1.96 ± 0.03**	**2.28 ± 0.15**	**2.85 ± 0.15**	**2.67 ± 0.06**
P.I-MAR*	**4.49 ± 0.18**	**4.11 ± 0.12**	**4.78 ± 0.09**	**2.92 ± 0.09**	**2.95 ± 0.05**	**2.07 ± 0.06**	**2.20 ± 0.09**	**2.94 ± 0.14**	**2.76 ± 0.07**
D-2	**0.98 ± 0.16**	**1.04 ± 0.06**	**1.54 ± 0.13**	**0.85 ± 0.05**	**0.76 ±0.07**	**0.36 ± 0.07**	**0.32 ± 0.06**	**0.33 ± 0.13**	**0.33 ± 0.05**
D-2*	**0.98 ± 0.15**	**1.12 ± 0.07**	**1.61 ± 0.13**	**0.86 ± 0.06**	**0.77 ± 0.08**	**0.36 ± 0.04**	**0.35 ± 0.06**	**0.33 ± 0.12**	**0.35 ± 0.05**

(Mean value ± SE)

Table: 5.11 Influence of Al concentrations in the nutrient solution on the Calcium content in the mycelium (mg/ g dry weight) of irradiated and nonirradiated ectomycorrhizal fungi.

Al (mg/L)	*0*	*50*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**1.90 ± 0.04**	**1.77 ± 0.03**	**1.65 ± 0.04**	**1.66 ± 0.05**	**0.90 ± 0.04**	**1.81 ± 0.02**	**1.62 ± 0.06**	**0.59 ± 0.05**	**0.50 ± 0.07**	**0.09 ± 0.00**
Pt-NIC*	**1.94 ± 0.02**	**1.84 ± 0.03**	**1.73 ± 0.04**	**1.11 ± 0.05**	**0.95 ± 0.04**	**1.87 ± 0.03**	**1.64 ± 0.07**	**0.56 ± 0.05**	**0.50 ± 0.07**	**0.07 ± 0.01**
Pt-N	**2.57 ± 0.09**	**1.88 ± 0.07**	**2.03 ± 0.08**	**1.93 ± 0.04**	**5.29 ± 0.03**	**3.53 ± 0.01**	**2.42 ± 0.07**	**1.49 ± 0.06**	**1.12 ± 0.15**	**0.42 ± 0.01**
Pt-N*	**2.53 ± 0.08**	**2.01 ± 0.08**	**2.01 ± 0.08**	**2.00 ± 0.04**	**5.55 ± 0.03**	**3.57 ± 0.02**	**2.35 ± 0.02**	**1.38 ± 0.06**	**1.18 ± 0.13**	**0.49 ± 0.02**
Pt-P	**0.64 ± 0.08**	**0.72 ± 0.14**	**0.91 ± 0.02**	**1.69 ± 0.14**	**0.92 ± 0.04**	**0.93 ± 0.10**	**0.29 ± 0.03**	**0.28 ± 0.05**	**0.22 ± 0.15**	**0.23 ± 0.04**
Pt-P*	**0.68 ± 0.07**	**0.76 ± 0.13**	**0.96 ± 0.05**	**1.67 ± 0.09**	**0.99 ± 0.05**	**0.98 ± 0.14**	**0.32 ± 0.05**	**0.30 ± 0.06**	**0.24 ± 0.10**	**0.27 ± 0.06**
P.I-MAR	**4.09 ± 0.33**	**4.38 ± 0.17**	**4.12 ± 0.15**	**4.73 ± 0.06**	**2.75 ± 0.07**	**2.93 ± 0.02**	**1.96 ± 0.03**	**2.28 ± 0.16**	**2.85 ± 0.15**	**2.67 ± 0.06**
P.I-MAR*	**4.23 ± 0.22**	**4.49 ± 0.18**	**4.11 ± 0.12**	**4.78 ± 0.09**	**2.92 ± 0.09**	**2.95 ± 0.05**	**2.07 ± 0.06**	**2.20 ± 0.09**	**2.94 ± 0.14**	**2.76 ± 0.07**
D-2	**0.94 ± 0.09**	**0.98 ± 0.16**	**1.04 ± 0.06**	**1.54 ± 0.13**	**0.85 ± 0.05**	**0.76 ± 0.07**	**0.36 ± 0.07**	**0.32 ± 0.06**	**0.33 ± 0.13**	**0.33 ± 0.05**
D-2*	**0.96 ± 0.08**	**0.98 ± 0.15**	**1.12 ± 0.07**	**1.61 ± 0.13**	**0.86 ± 0.06**	**0.77 ± 0.08**	**0.36 ± 0.04**	**0.35 ± 0.06**	**0.33 ± 0.13**	**0.35 ± 0.05**

(Mean value ± SE)

Table: 5.12 Influence of Al concentrations in the nutrient solution on the Magnesium content in the mycelium (mg/ g dry weight) of irradiated and nonirradiated ectomycorrhizal fungi.

Al (mg/L)	*0*	*50*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**2.99 ± 0.08**	**2.48 ± 0.14**	**1.73 ± 0.01**	**1.57 ± 0.14**	**1.03 ± 0.04**	**1.51 ± 0.10**	**1.54 ± 0.03**	**1.11 ± 0.05**	**1.23 ± 0.02**	**0.77 ± 0.05**
Pt-NIC*	**2.92 ± 0.05**	**2.70 ± 0.16**	**1.79 ± 0.06**	**1.60 ± 0.15**	**1.02 ± 0.04**	**1.53 ± 0.15**	**1.50 ± 0.15**	**1.20 ± 0.06**	**1.33 ± 0.06**	**0.71 ± 0.06**
Pt-N	**1.70 ± 0.10**	**1.53 ± 0.07**	**2.01 ± 0.08**	**2.12 ± 0.04**	**2.42 ± 0.03**	**3.11 ± 0.01**	**2.10 ± 0.07**	**1. 90 ± 0.06**	**1.48 ± 0.15**	**0.78 ± 0.07**
Pt-N*	**1.66 ± 0.08**	**1.48 ± 0.08**	**2.00 ± 0.12**	**2.18 ± 0.06**	**2.52 ± 0.04**	**3.16 ± 0.06**	**2.09 ± 0.09**	**1.95 ± 0.08**	**1.43 ± 0.17**	**0.80 ± 0.08**
Pt-P	**0.90 ± 0.05**	**1.71 ± 0.07**	**2.27 ± 0.20**	**1.96 ± 0.11**	**2.14 ± 0.27**	**1.99 ± 0.12**	**2.16 ± 0.05**	**2.33 ± 0.10**	**1.92 ± 0.05**	**1.90 ± 0.08**
Pt-P*	**0.89 ± 0.06**	**1.73 ± 0.09**	**2.35 ± 0.21**	**1.91 ± 0.08**	**2.18 ± 0.18**	**1.96 ± 0.15**	**2.31 ± 0.07**	**2.45 ± 0.24**	**1.89 ± 0.10**	**1.95 ± 0.11**
P.I-MAR	**1.42 ± 0.33**	**1.10 ± 0.17**	**1.40 ± 0.15**	**1.40 ± 0.06**	**1.12 ± 0.07**	**1.07 ± 0.02**	**2.10 ± 0.03**	**1.42 ± 0.15**	**0.93 ± 0.16**	**0.89 ± 0.06**
P.I-MAR*	**1.45 ± 0.22**	**1.17 ± 0.07**	**1.41 ± 0.16**	**1.41 ± 0.03**	**1.15 ± 0.08**	**1.05 ± 0.08**	**2.09 ± 0.07**	**1.47 ± 0.18**	**0.98 ± 0.14**	**0.92 ± 0.08**
D-2	**1.09 ± 0.09**	**1.06 ± 0.11**	**1.65 ± 0.13**	**2.06 ± 0.19**	**1.95 ± 0.15**	**1.86 ± 0.17**	**1.84 ± 0.08**	**1.70 ± 0.16**	**1.58 ± 0.05**	**1.25 ± 0.07**
D-2*	**1.13 ± 0.08**	**1.10 ± 0.10**	**1.70 ± 0.05**	**2.17 ± 0.14**	**2.01 ± 0.14**	**1.91 ± 0.15**	**1.88 ± 0.07**	**1.77 ± 0.13**	**1.62 ± 0.08**	**1.58 ± 0.10**

(Mean value ± SE)

Table: 5.13 Influence of Al concentrations in the nutrient solution on the Potassium content in the mycelium (mg/ g dry weight) of irradiated and nonirradiated ectomycorrhizal fungi.

Al (mg/L)	*0*	*50*	*100*	*200*	*500*	*750*	*1000*	*2000*	*3000*	*5000*
Pt-NIC	**10.6 ± 0.40**	**9.72 ± 0.67**	**10.7 ± 0.53**	**8.16 ± 0.85**	**7.63 ± 0.23**	**7.01 ± 0.12**	**7.98 ± 0.81**	**7.68 ± 1.28**	**7.68 ± 0.66**	**3.93 ± 0.23**
Pt-NIC*	**10.9 ± 0.36**	**9.78 ± 0.50**	**11.1 ± 0.46**	**8.07 ± 0.68**	**7.23 ± 0.26**	**6.99 ± 0.11**	**7.93 ± 0.55**	**7.32 ± 0.88**	**7.35 ± 0.51**	**4.01 ± 0.32**
Pt-N	**1.01 ± 0.02**	**2.58 ± 0.31**	**2.52 ± 0.31**	**4.28 ± 0.40**	**1.60 ± 0.06**	**1.58 ± 0.03**	**1.31 ± 0.02**	**1.32 ± 0.16**	**1.07 ± 0.08**	**0.59 ± 0.05**
Pt-N*	**0.99 ± 0.06**	**2.64 ± 0.29**	**2.88 ± 0.31**	**4.60 ± 0.36**	**1.66 ± 0.07**	**1.69 ± 0.06**	**1.26 ± 0.05**	**1.45 ± 0.16**	**1.07 ± 0.10**	**0.63 ± 0.07**
Pt-P	**1.02 ± 0.07**	**0.61 ± 0.05**	**0.70 ± 0.06**	**0.61 ± 0.04**	**0.83 ± 0.02**	**1.23 ± 0.08**	**0.90 ± 0.05**	**1.34 ± 0.14**	**1.52 ± 0.10**	**0.71 ± 0.06**
Pt-P*	**0.91 ± 0.06**	**0.60 ± 0.17**	**0.70 ± 0.07**	**0.64 ± 0.18**	**0.81 ± 0.05**	**1.21 ± 0.07**	**0.88 ± 0.03**	**1.33 ± 0.15**	**1.35 ± 0.11**	**0.78 ± 0.06**
P.I-MAR	**0.88 ± 0.06**	**1.48 ± 0.04**	**1.99 ± 0.16**	**0.90 ± 0.03**	**0.62 ± 0.07**	**0.61 ± 0.10**	**0.68 ± 0.01**	**0.53 ± 0.04**	**0.62 ± 0.04**	**0.62 ± 0.07**
P.I-MAR*	**0.92 ± 0.08**	**1.51 ± 0.08**	**2.03 ± 0.05**	**0.91 ± 0.06**	**0.65 ± 0.08**	**0.62 ± 0.06**	**0.66 ± 0.04**	**0.59 ± 0.03**	**0.59 ± 0.06**	**0.68 ± 0.06**
D-2	**1.03 ±0.07**	**0.95 ± 0.08**	**0.86 ± 0.08**	**0.88 ± 0.12**	**0.69± 0.07**	**0.85 ± 0.07**	**0.73 ± 0.03**	**0.65 ± 0.05**	**0.78 ± 0.04**	**0.65 ± 0.05**
D-2*	**1.06 ± 0.08**	**0.97 ± 0.14**	**0.87 ± 0.11**	**0.86 ± 0.11**	**0.71 ± 0.06**	**0.89 ± 0.06**	**0.76 ± 0.04**	**0.67 ± 0.05**	**0.79 ± 0.05**	**0.67 ± 0.05**

(Mean value ± SE)

Chapter VI

Influence of Aluminium on plant mineral nutrition in presence of mycorrhizae formed by metal tolerant mutants

Aluminium influences the plant mineral nutrition. Mycorrhizal fungi play an important role in protecting the plants from the toxicity of metals. In the present study influence of aluminium on plant mineral nutrition was studied in presence of ectomycorrhizal fungi which are tolerant to high levels of aluminium.

6.1 Effect of aluminium on the growth of *P. deltoides in vitro*

The micro propagated plantlets of *P. deltoides* were grown at different concentrations of aluminium (0, 50, 100, 200, 400 and 500 mg/L) in soilrite-vermiculite moistened with Melin's medium. The plantlets were inoculated with ectomycorrhizal fungus *P. involutus* (P.I-MAR) and harvested after 60 days of exposure to aluminium. The plantlets were analyzed for growth parameters, uptake of mineral nutrients and aluminium concentration in the plant shoots. The results showed that the growth of the plantlets was significantly affected by aluminium. The response of nonmycorrhizal and ectomycorrhizal plantlets to increasing concentrations of aluminium indicated that nonmycorrhizal plantlets were more sensitive to the presence of aluminium. *P. involutus* formed ectomycorrhizas with the roots of *P. deltoides* and colonized 61.3 % of lateral roots in aluminium unamended medium. The mycorrhizae formed were simple with numerous irregularly spaced branches, typically occurring along one main axis (Figure 6.1). The mycorrhizae were

pale white to yellowish when young and turned brown on ageing. The mycorrhizal colonization decreased significantly as the concentration of aluminium increased in the growth medium (Table 6.1).

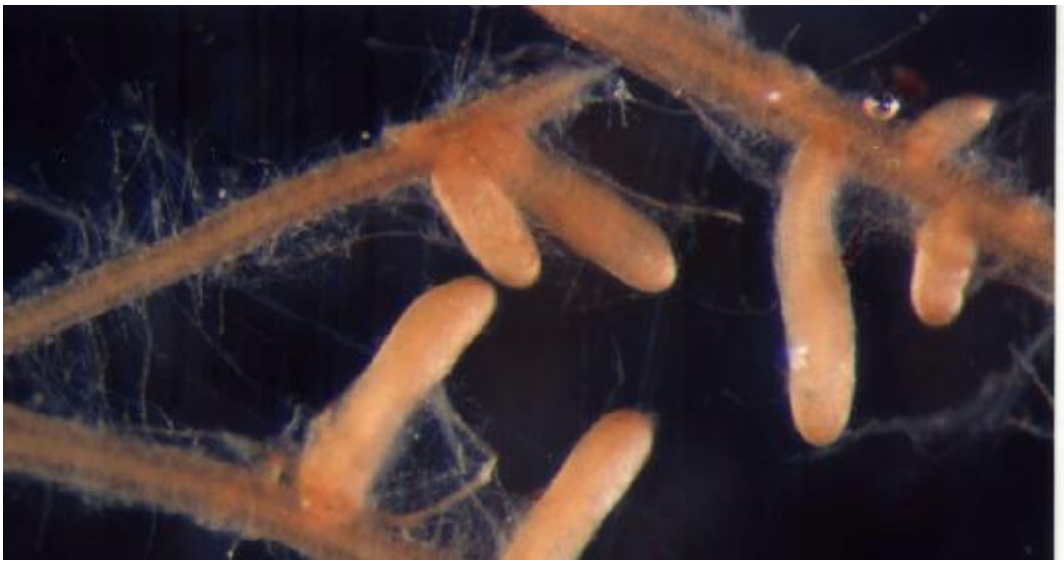

Figure: 6.1 Ectomycorrhizal roots of *Populus deltoides* colonized by *Paxillus involutus.*

Table: 6.1 Influence of Aluminium concentrations on the % mycorrhization of *P. deltoides*

plants inoculated with *P. involutus.*

Al ***(mg/L)***	*% mycorrhization*	*% inhibition*
0	**61.32 ± 0.63 a**	**0**
50	**45.67 ± 1.78 b**	**25.5**
100	**44.47 ± 3.01 b**	**27.5**
200	**35.43 ± 2.05 c**	**42.2**
400	**24.86 ± 2.41 d**	**59.5**
500	**24.82 ± 3.24 d**	**59.5**

Values sharing a common letter are not significantly differerent at P<0.05

The percent inhibition in colonization was calculated by using following formula.

$$\% \text{ inhibition in colonization} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

Control meant the plants growing without aluminium

Test meant the plants growing in presence of different concentrations of aluminium.

The inoculation of ectomycorrhizal fungus *P. involutus* to *P. deltoides* plantlets improved their growth in presence of aluminium as compared to non-inoculated plantlets. Compared with the control (0 mg/L Al), relative reduction in plant height was not significant till 100 mg/L of Al in ectomycorrhizal plants while in nonmycorrhizal plantlets significant reduction in plant height was observed at 50 mg/L Al concentration (Table 6.2 and Figure 6.2). Significant differences were observed in the mycorrhizal and nonmycorrhizal treatments as well as aluminium concentrations (Table 6.2). The plant height was significantly higher in mycorrhizal treatment when compared to nonmycorrhizal plants in all the concentrations of aluminium.

The shoot dry weight (Table 6.3 and Figure 6.3) and root dry weight (Table 6.4 and Figure 6.4) of the plantlets decreased with increasing concentrations of aluminium as compared to control plantlets. The shoot and root dry weights of the ectomycorrhizal plantlets were significantly more than nonmycorrhizal plantlets in all aluminium concentrations. The highest concentration of aluminium applied (500mg/L of Al), completely inhibited the shoot and root growth of nonmycorrhizal plantlets and the plantlets died showing the acute toxicity symptoms such as

production of short stubby roots and necrotic lesions on shoot tips while the ectomycorrhizal plantlets survived.

Table: 6.2 Influence of Aluminium concentrations on the shoot height (cms) of *P. deltoides* (P.d)

plants inoculated with P .I-MAR.

Al (mg/L)	*P. d*	*P. d + P .I-MAR*
0	**9.67 ± 0.94**	**9.47 ± 1.35**
50	**7.97 ± 0.20**	**9.10 ± 0.32**
100	**7.97 ± 1.39**	**9.10 ± 0.61**
200	**5.17 ± 0.23**	**6.63 ± 0.44**
400	**4.93 ± 0.88**	**5.00 ± 0.44**
500	**2.13 ± 0.12**	**3.87 ± 0.18**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F value*
Interaction	**5**	**4.542**	**0.9084**	**0.5684 ns**
Treatment	**1**	**7.111**	**7.111**	**4.449 ***
Concentration	**5**	**193.6**	**38.71**	**24.22 ***
Residual	**24**	**38.36**	**1.598**	

* P<0.05

*** P<0.001

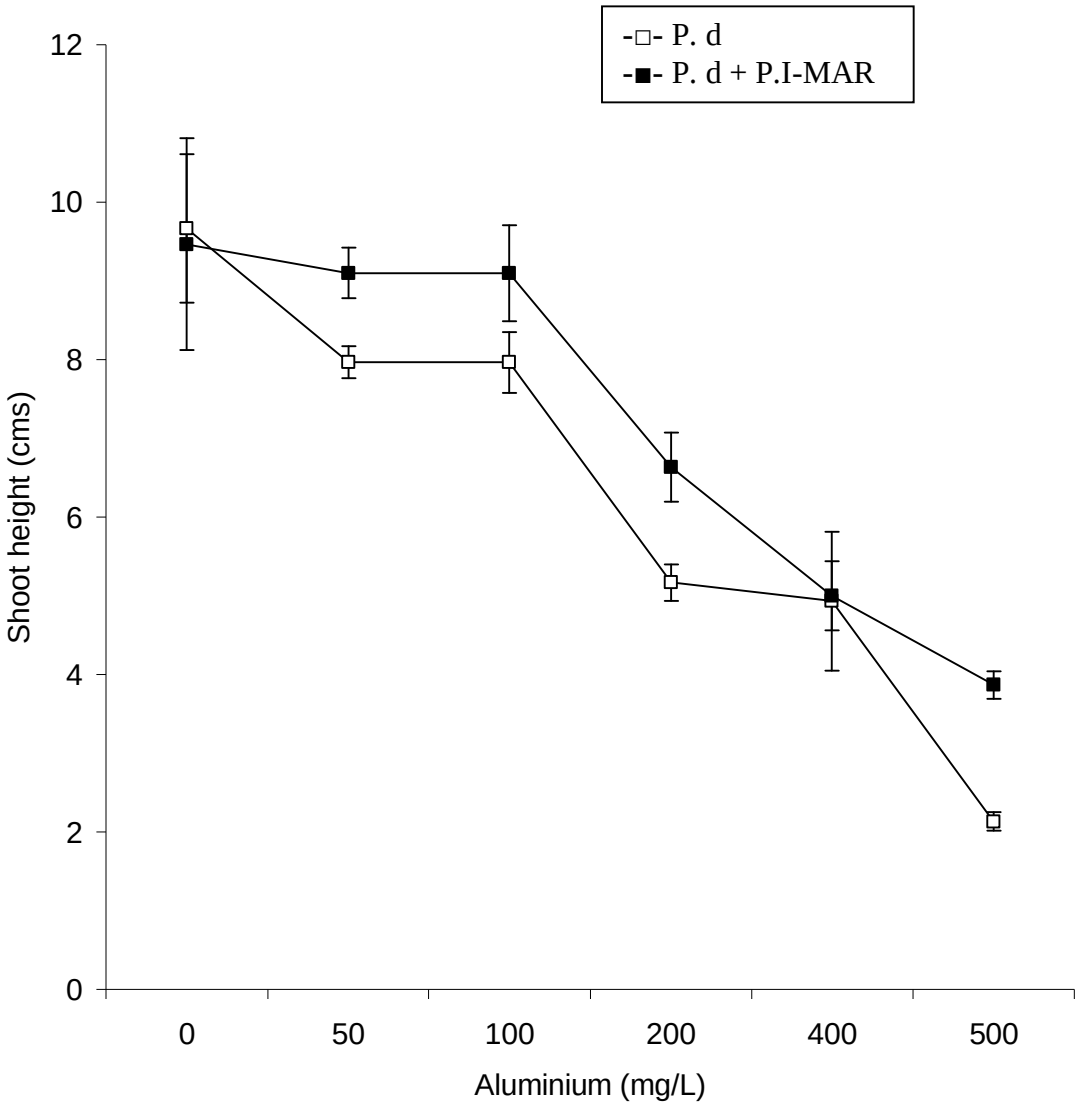

Figure: 6.2 Influence of Aluminium concentrations on the shoot height (cms) of *P. deltoides*

plants inoculated with P.I-MAR. Mean value ± SE.

Table: 6.3 Influence of Aluminium concentrations on the shoot dry weight (mg) of *P. deltoides*

(P.d) plants inoculated with P .I-MAR.

Al ***(mg/L)***	*P. d*	*P. d + P.I-MAR*
0	**42.33 ± 0.88**	**54.33 ± 1.20**
50	**34.00 ± 1.15**	**44.00 ± 0.58**
100	**23.33 ± 0.67**	**30.33 ± 0.67**
200	**13.00 ± 0.58**	**23.67 ± 0.88**
400	**10.00 ± 0.58**	**22.33 ± 0.88**
500	**8.33 ± 0.33**	**12.00 ± 0.58**

Analysis of Variance

Source of Variation	***Df***	***Sum-of-squares***	***Mean square***	***F value***
Interaction	**5**	**83.81**	**16.76**	**9.006 *** **
Treatment	**1**	**774.7**	**774.7**	**416.3 *** **
Concentration	**5**	**6440**	**1288**	**692.0 *** **
Residual	**24**	**44.67**	**1.861**	

*** P<0.001

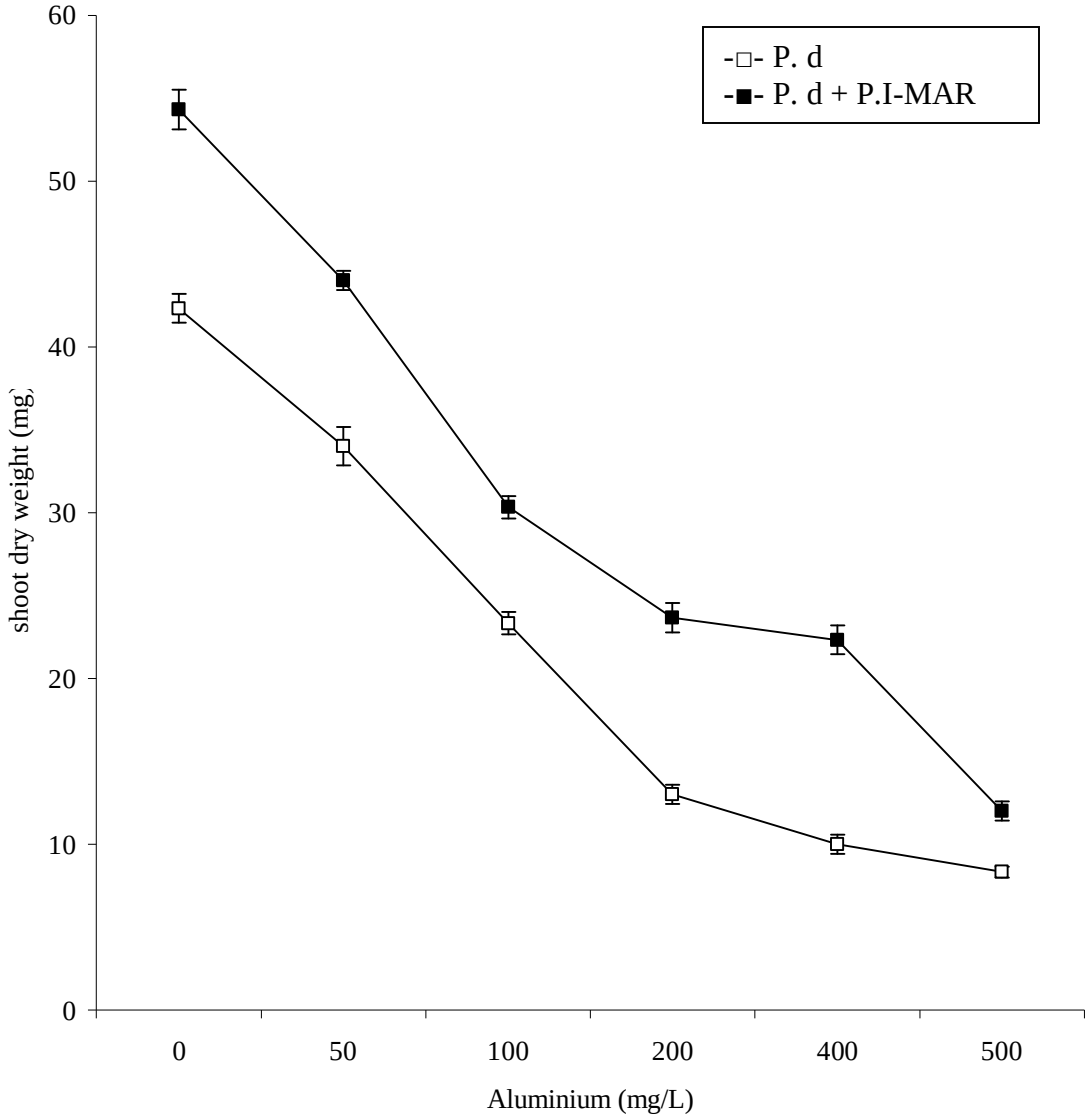

Figure: 6.3 Influence of Aluminium concentrations on the shoot dry weight (mg) of *P. deltoides*

plants inoculated with P .I-MAR. Mean value ± SE.

Table: 6.4 Influence of Aluminium concentrations on the root dry weight (mg) of *P. deltoides*

(P.d) plants inoculated with P .I-MAR.

Al (mg/L)	*P. d*	*P. d + P.I-MAR*
0	**10.00 ± 0.58**	**17.33 ± 1.20**
50	**8.67 ± 0.33**	**13.67 ± 0.88**
100	**7.00 ± 0.58**	**10.00 ± 0.58**
200	**5.67 ± 0.33**	**10.00 ± 0.58**
400	**2.67 ± 0.33**	**8.33 ± 0.33**
500	**2.33 ± 0.33**	**8.67 ± 0.33**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**17.47**	**3.494**	**3.311***
Treatment	**1**	**250.7**	**250.7**	**237.5*****
Concentration	**5**	**312.1**	**62.43**	**59.14*****
Residual	**24**	**25.33**	**1.056**	

* P<0.05

*** P<0.001

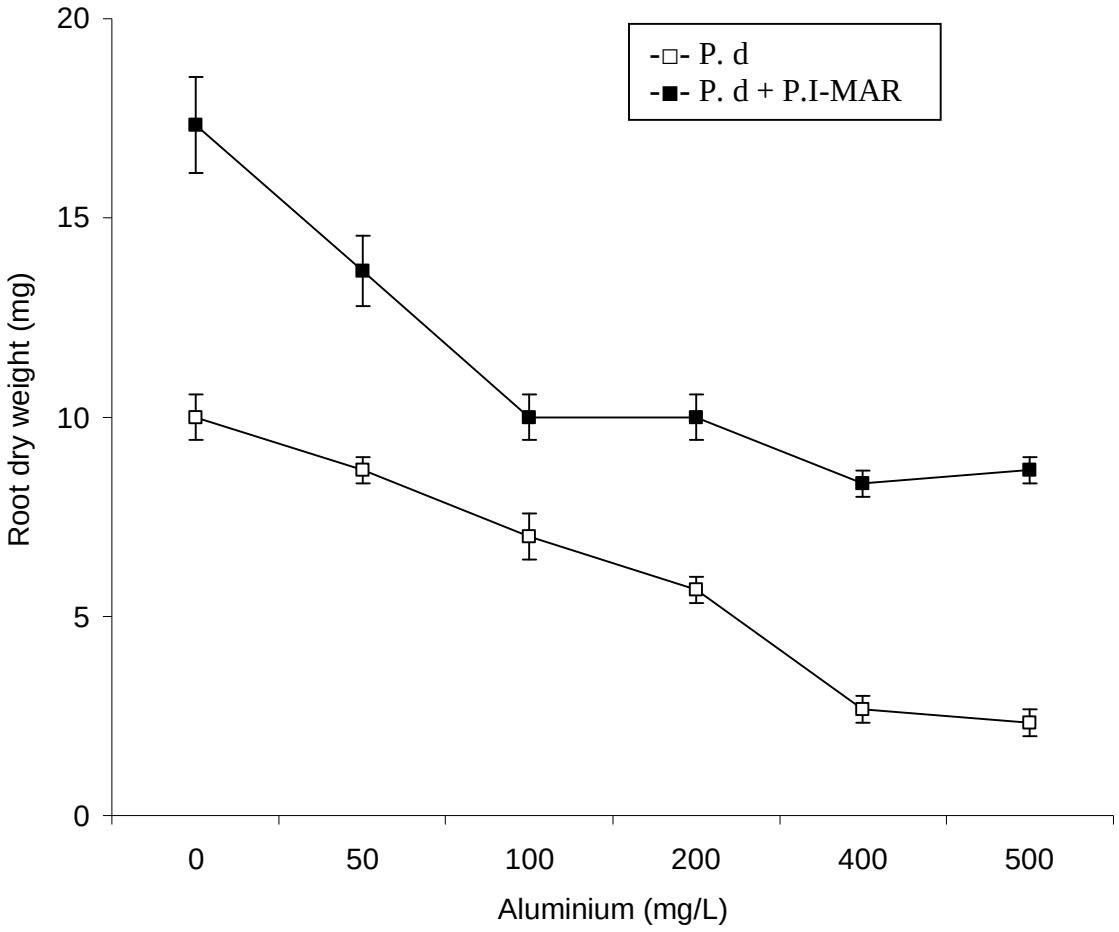

Figure: 6.4 Influence of Aluminium concentrations on the root dry weight of *P. deltoides* plants inoculated with P .I-MAR. Mean value ± SE

6.2 Aluminium concentration in plant shoots

The ectomycorrhizal and nonmycorrhizal *P. deltoides* plantlets were harvested after 60 days and were analyzed for the accumulation of aluminium in their shoot tissues. The concentrations of aluminium in the plant shoot of ectomycorrhizal and nonmycorrhizal plantlets increased with increasing concentrations of aluminium in the medium (Table 6.5 and Figure 6.5). When compared to nonmycorrhizal plantlets, the mycorrhizal plantlets showed very less aluminium content even at higher aluminium concentrations.

Table: 6.5 Influence of Aluminium concentrations on its accumulation (mg/g dry weight) in plant shoot of *P. deltoides* (P.d) plants inoculated with P .I-MAR.

Al (mg/L)	*P. d*	*P .d +P. I-MAR*
0	**0.33 ± 0.01**	**0.15 ± 0.01**
50	**2.32 ± 0.12**	**0.40 ±0.04**
100	**2.80 ± 0.51**	**0.37 ±0.02**
200	**3.09 ± 0.49**	**1.27 ±0.18**
400	**17.04 ± 0.64**	**2.62 ±0.06**
500	**21.71 ± 0.97**	**4.89 ±0.86**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**242.2**	**48.44**	**73.58 *****
Treatment	**1**	**234.2**	**234.2**	**355.7 *****
Concentration	**5**	**1304**	**260.9**	**396.3 *****

Residual	**24**	**15.80**	**0.6584**

*** P<0.001

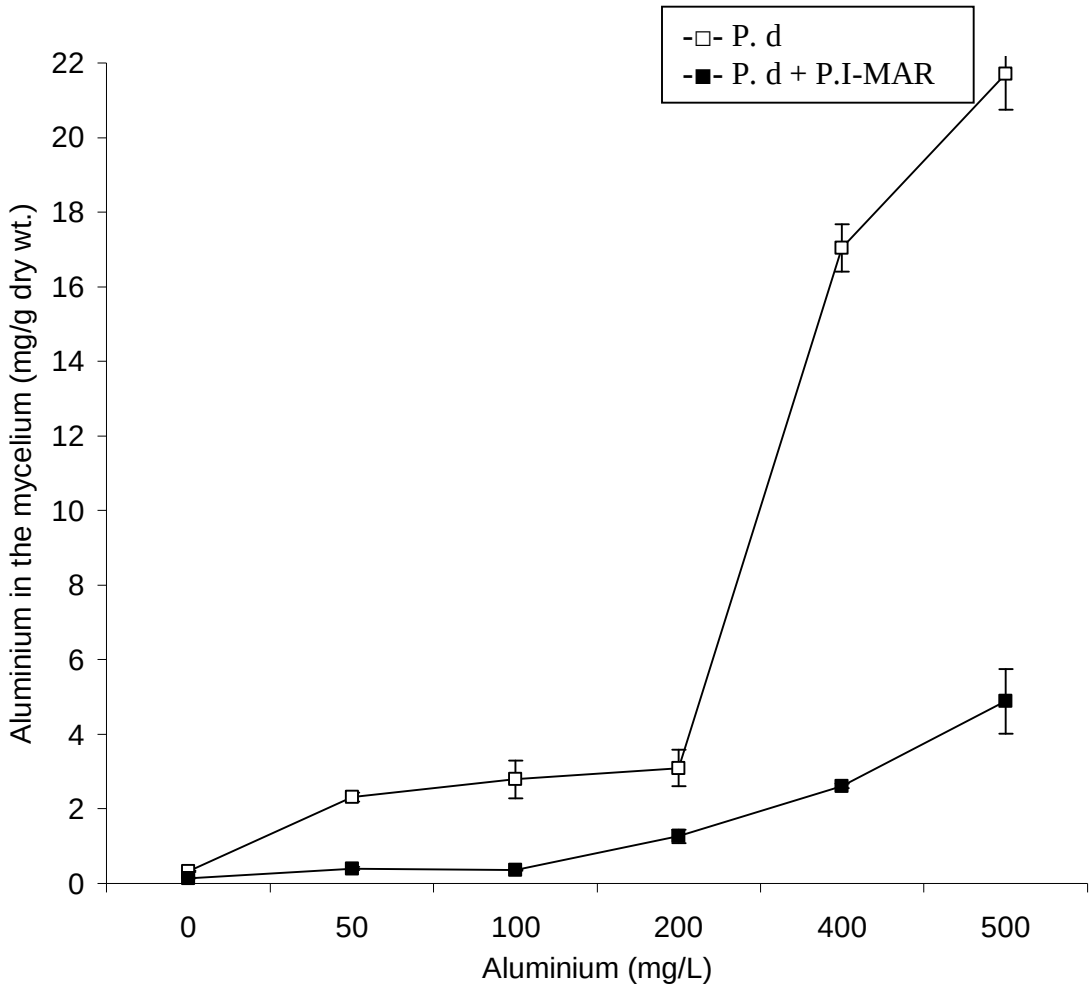

Figure: 6.5 Influence of Aluminium concentrations on the accumulation of Aluminium in

plant shoot of *P. deltoides* plants inoculated with P .I-MAR. Mean ± SE.

6.3 Nutrient element concentration in plant shoots

The uptake of P, Ca, Mg and K was mainly influenced by the presence of aluminium in the medium. The foliar concentrations of P, Ca, Mg and K were significantly higher in ectomycorrhizal plantlets when compared to nonmycorrhizal plantlets at all aluminium concentrations. The uptake of phosphorus was significantly inhibited in plantlets grown at different concentrations of aluminium (Table 6.6 and Figure 6.6). The phosphorus content of the ectomycorrhizal plantlets significantly increased than nonmycorrhizal plantlets at each aluminium concentration tested. The calcium content in the nonmycorrhizal plantlets was significantly lower than the ectomycorrhizal plantlets and there was no significant decrease in the calcium content of nonmycorrhizal and ectomycorrhizal plantlets were found with increased concentrations of aluminium (Table 6.7 and Figure 6.7). The ectomycorrhizal plantlets accumulated significantly more magnesium content in its shoot tissue as compared to nonmycorrhizal plantlets at all aluminium concentrations. There was no significant change in the magnesium content of nonmycorrhizal plantlets while there was increase in the magnesium content at 50 mg/L of Al in the ectomycorrhizal plantlets and no significant change at further higher concentrations of aluminium (Table 6.8 and Figure 6.8). The potassium content increased in the nonmycorrhizal plantlets till 400 mg/L of Al and then decreased at 500 mg/L of aluminium concentration and there was no significant change in the potassium content of ectomycorrhizal plantlets with increasing concentrations of aluminium. The ectomycorrhizal plantlets accumulated significantly more potassium in its shoot as compared to nonmycorrhizal plantlets.

Table: 6.6 Influence of Aluminium concentrations on the Phosphorus content (mg/g dry weight)

in the plant shoot of *P. deltoides* (P.d) plants inoculated with P .I-MAR.

Al (mg/L)	*P. d*	*P. d +P.I-MAR*
0	**13.21 ± 0.55**	**20.70 ± 0.47**
50	**10.75 ± 0.50**	**18.96 ± 1.89**
100	**10.25 ± 0.60**	**16.75 ± 0.88**
200	**9.87 ± 0.56**	**12.71 ± 0.23**
400	**7.47 ± 0.16**	**12.85 ± 0.42**
500	**6.73 ± 0.48**	**11.51 ± 0.88**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**28.70**	**5.739**	**3.256 ***
Treatment	**1**	**309.9**	**309.9**	**175.8 *****
Concentration	**5**	**268.2**	**53.63**	**30.43 *****
Residual	**24**	**42.30**	**1.763**	

* P<0.05

*** P<0.001

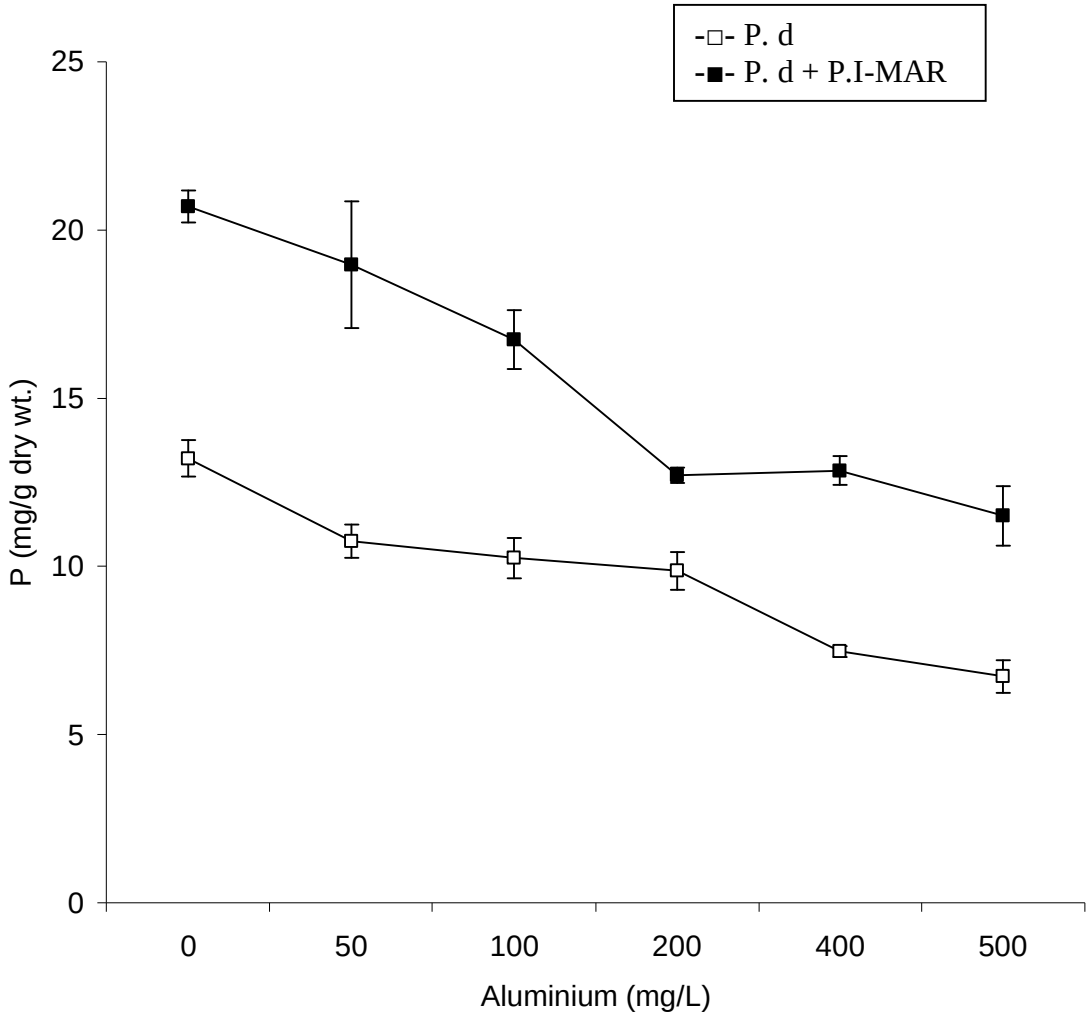

Figure: 6.6 Influence of Aluminium concentrations on the Phosphorus content in the plant shoot

of *P. deltoides* plants inoculated with P .I-MAR. Mean ± SE.

Table: 6.7 Influence of Aluminium concentrations on the Calcium content (mg/g dry weight)

in the plant shoot of *P. deltoides* (P.d) plants inoculated with P .I-MAR.

Al (mg/L)	*P. d*	*P. d + P.I-MAR*
0	**20.14 ± 2.19**	**26.62 ± 0.92**
50	**20.45 ± 1.74**	**32.50 ± 1.27**
100	**20.83 ± 1.14**	**24.91 ± 0.32**
200	**19.28 ± 1.46**	**27.36 ± 0.89**
400	**19.53 ± 0.44**	**28.16 ± 1.89**
500	**19.25 ± 0.94**	**27.08 ± 1.44**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**51.59**	**10.32**	**1.933 ns**
Treatment	**1**	**555.4**	**555.4**	**104.0 *****
Concentration	**5**	**52.88**	**10.58**	**1.981 ns**
Residual	**24**	**128.1**	**5.338**	

*** P<0.001

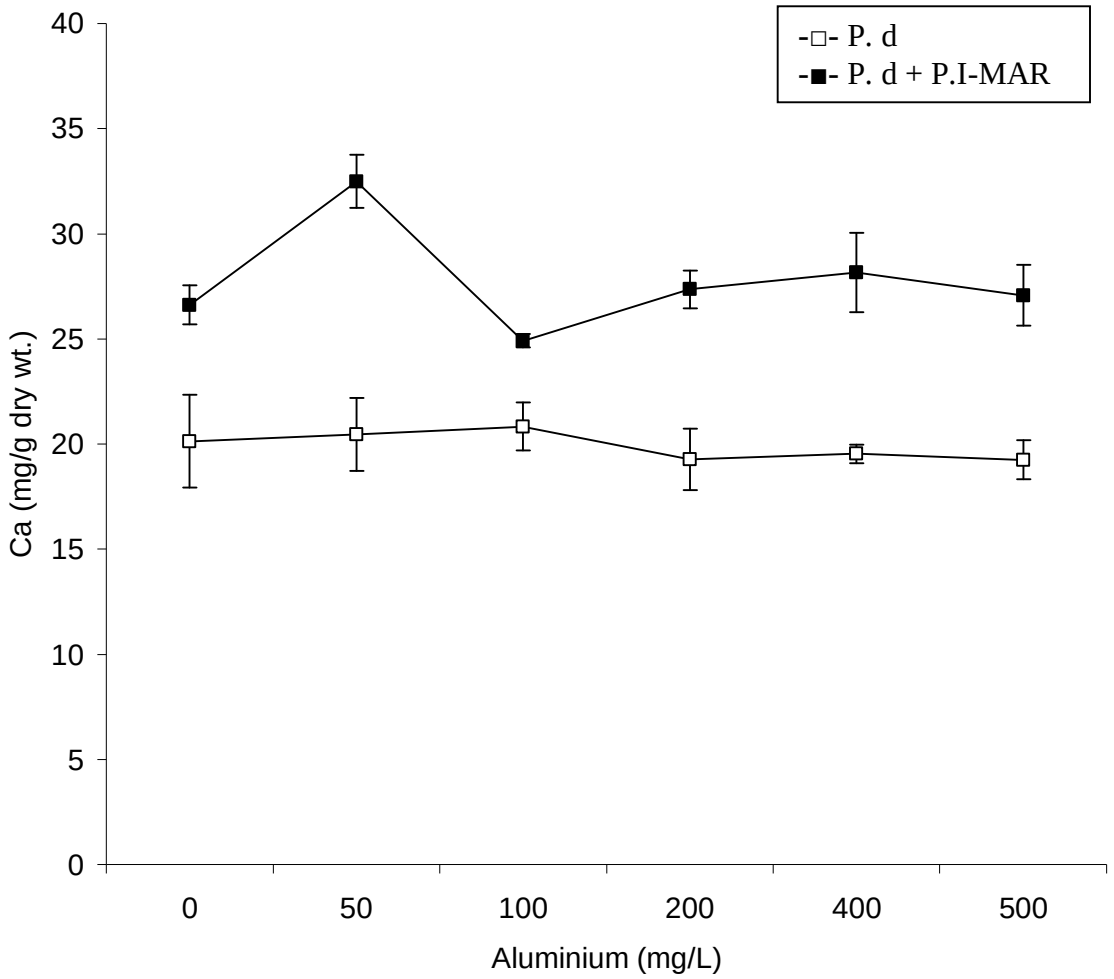

Figure: 6.7 Influence of Aluminium concentrations on the Calcium content in the plant shoot

of *P. deltoides* plants inoculated with P .I-MAR. Mean ± SE.

Table: 6.8 Influence of Aluminium concentrations on the Magnesium content (mg/g dry weight)

in the plant shoot of *P. deltoides* (P.d) plants inoculated with P .I-MAR

Al (mg/L)	*P. d*	*P. d + P. I-MAR*
0	**12.16 ± 0.12**	**18.82 ± 0.27**
50	**12.97 ± 0.40**	**24.55 ± 2.06**
100	**11.51 ± 0.71**	**19.58 ± 0.34**
200	**10.99 ± 0.72**	**18.74 ± 0.44**
400	**11.01 ± 0.76**	**18.77 ± 0.30**
500	**10.79 ± 0.55**	**18.75 ± 0.33**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**21.37**	**4.275**	**2.471 ns**
Treatment	**1**	**618.9**	**618.9**	**357.7 *****
Concentration	**5**	**69.82**	**13.96**	**8.071 *****
Residual	**24**	**41.53**	**1.730**	

*** P<0.001

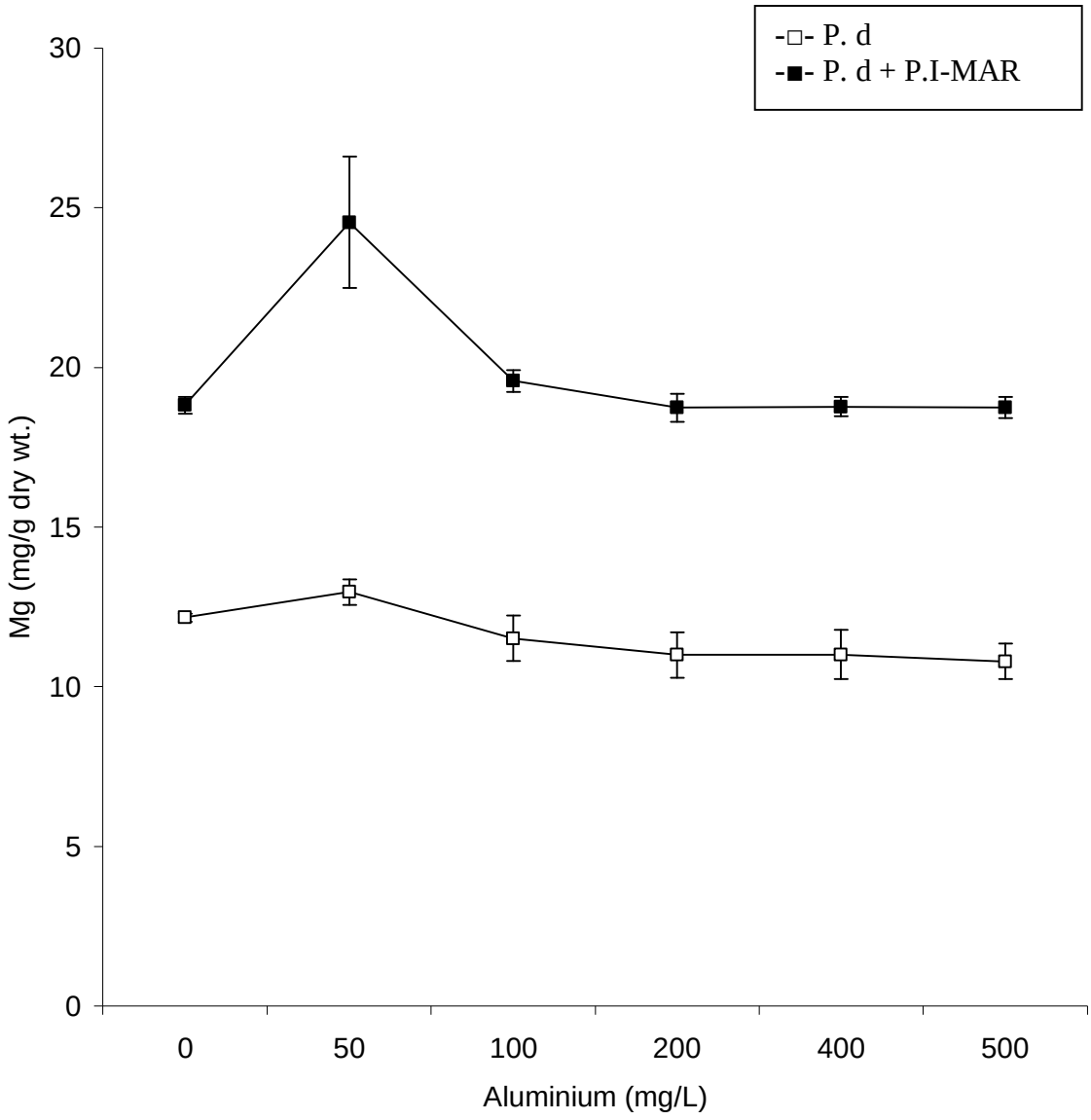

Figure: 6.8 Influence of Aluminium concentrations on the Magnesium content in the plant shoot

of *P. deltoides* plants inoculated with P .I-MAR. Mean ± SE.

Table: 6.9 Influence of Aluminium concentrations on the Potassium content (mg/g dry weight)

in the plant shoot of *P. deltoides* (P.d) plants inoculated with P .I-MAR

Al (mg/L)	*P. d*	*P. d + P. I-MAR*
0	**57.30 ± 0.55**	**90.48 ± 0.58**
50	**62.12 ± 0.89**	**85.93 ± 1.95**
100	**62.32 ± 0.65**	**86.01 ± 3.16**
200	**70.20 ± 0.71**	**86.82 ± 2.97**
400	**62.05 ± 1.73**	**81.77 ± 0.81**
500	**47.07 ± 0.93**	**85.64 ± 0.48**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**520.3**	**104.1**	**14.01 *** **
Treatment	**1**	**6052**	**6052**	**814.9 *** **
Concentration	**5**	**472.6**	**94.53**	**12.73 *** **
Residual	**24**	**178.2**	**7.426**	

*** P<0.001

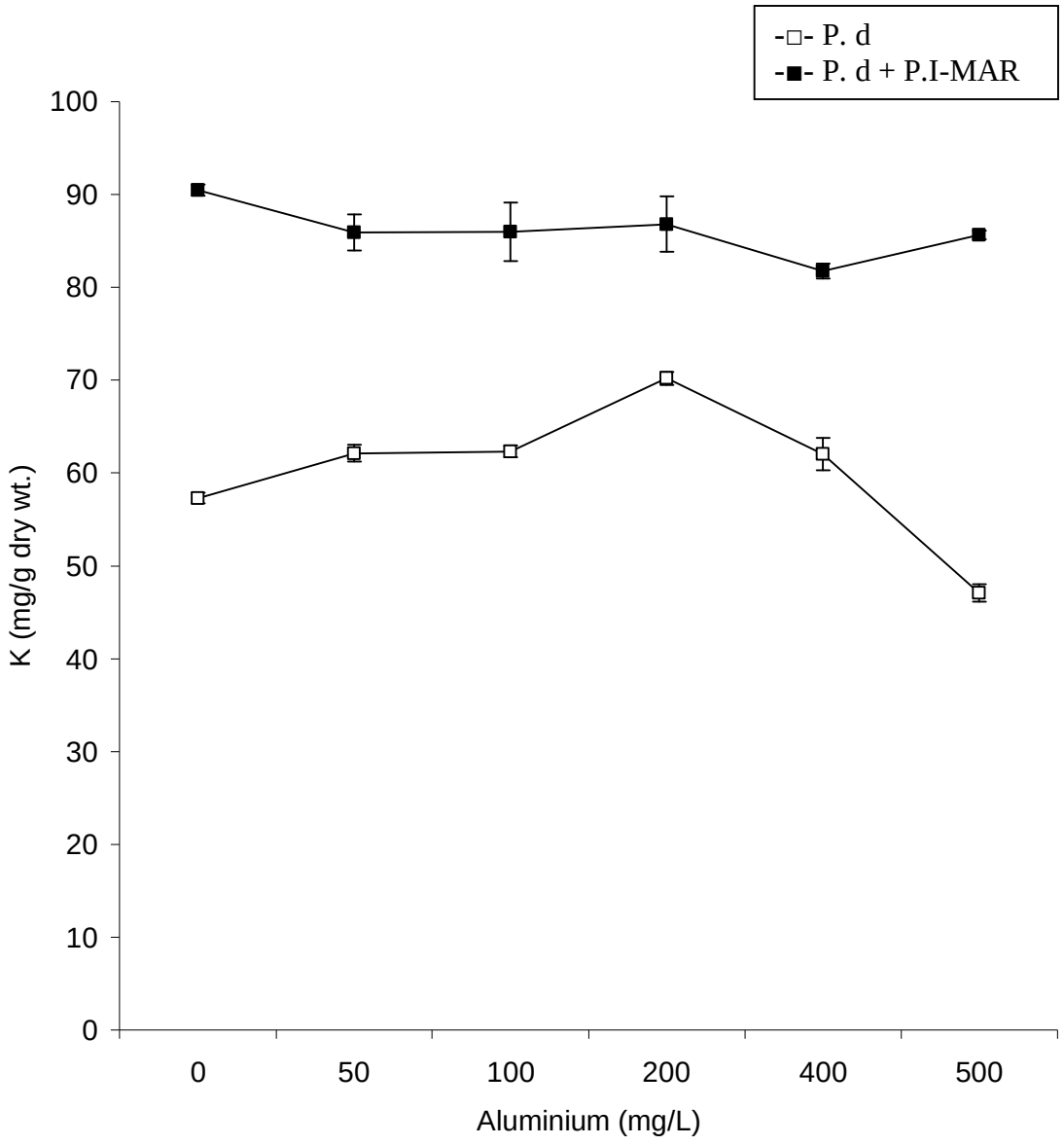

Figure: 6.9 Influence of Aluminium concentrations on the Potassium content in the plant shoot of

P. deltoides plants inoculated with P .I-MAR. Mean ± SE.

6.4 Effect of aluminium on the growth of *E. tereticornis* plantlets *in vitro*

The axenically grown plantlets of *E. tereticornis* were grown in soilrite-vermiculite support moistened with Melin's medium containing different concentrations of aluminium (as 0, 50, 100, 200, 400 and 500 mg/L). The plantlets were inoculated with ectomycorrhizal fungi *P. tinctorius* (Pt-NIC) and *P. albus* (Pt-P) separately and grown for 60 days. The plantlets were harvested after 60 days and were analyzed for growth parameters, such as shoot height, shoot dry weight and root dry weight and % mycorrhization. The results showed that the growth of the plantlets was significantly improved by the presence of ectomycorrhizal fungi. The ectomycorrhizal plantlets showed more tolerance than nonmycorrhizal plantlets to increasing concentrations of aluminium indicating that nonmycorrhizal plantlets were more sensitive to the presence of aluminium. Pt-NIC and Pt-P were able to form ectomycorrhizas with the roots of *E. tereticornis* plants. Pt-NIC colonized 65.6 % and Pt-P colonized 57.2% of lateral roots when grown in absence of aluminium. The percent colonization was decreased as the aluminium concentration increased in the growth medium. Pt-NIC showed more colonization when compaed to Pt-P in all the concentrations of aluminium tested in this study (Table 6.10). The mycorrhizas formed were invariably branched (Figure 6.10, 6.11) and were golden yellow in colour. The lateral roots of the plantlets were completely covered by the fungal hyphae forming mantle. The presence of aluminium significantly reduced the percent colonization of the lateral roots of *E. tereticornis* plantlets by Pt-NIC and Pt-P

isolates. The percent inhibition in colonization was calculated by using following formula.

$$\% \text{ inhibition in colonization} = \frac{\text{Control- Test}}{\text{Control}} \times 100$$

Control meant the plants growing without aluminium

Test meant the plants growing in presence of different concentrations of aluminium.

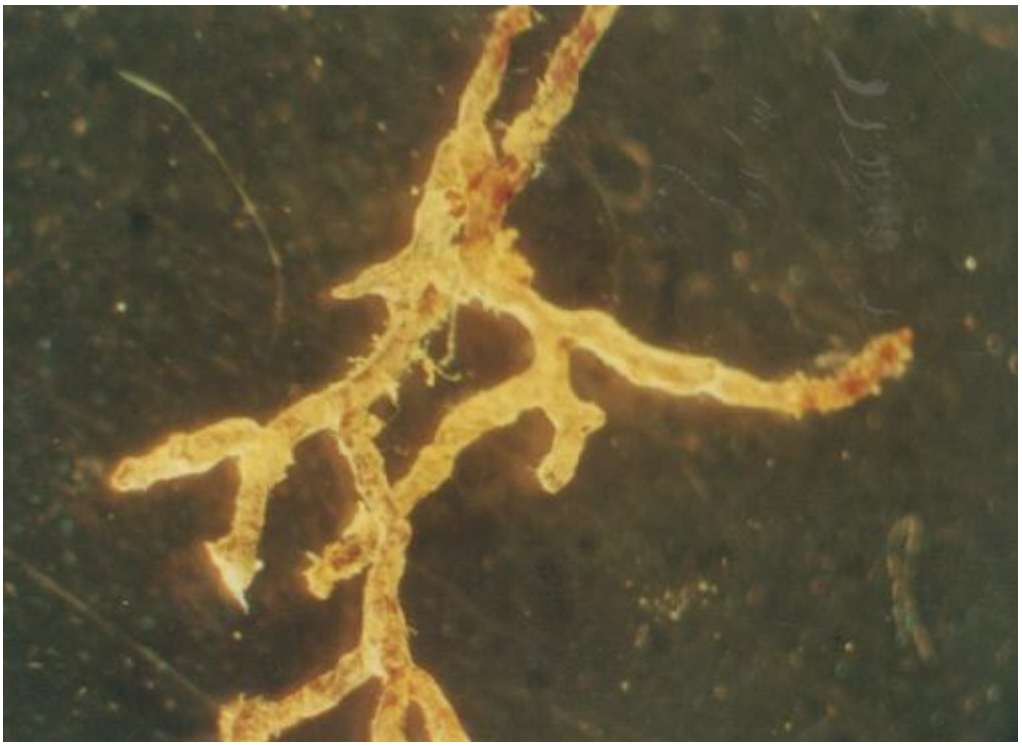

Figure: 6.10 Ectomycorrhizal roots of *E. tereticornis* colonized by *P. albus* (Pt-P)

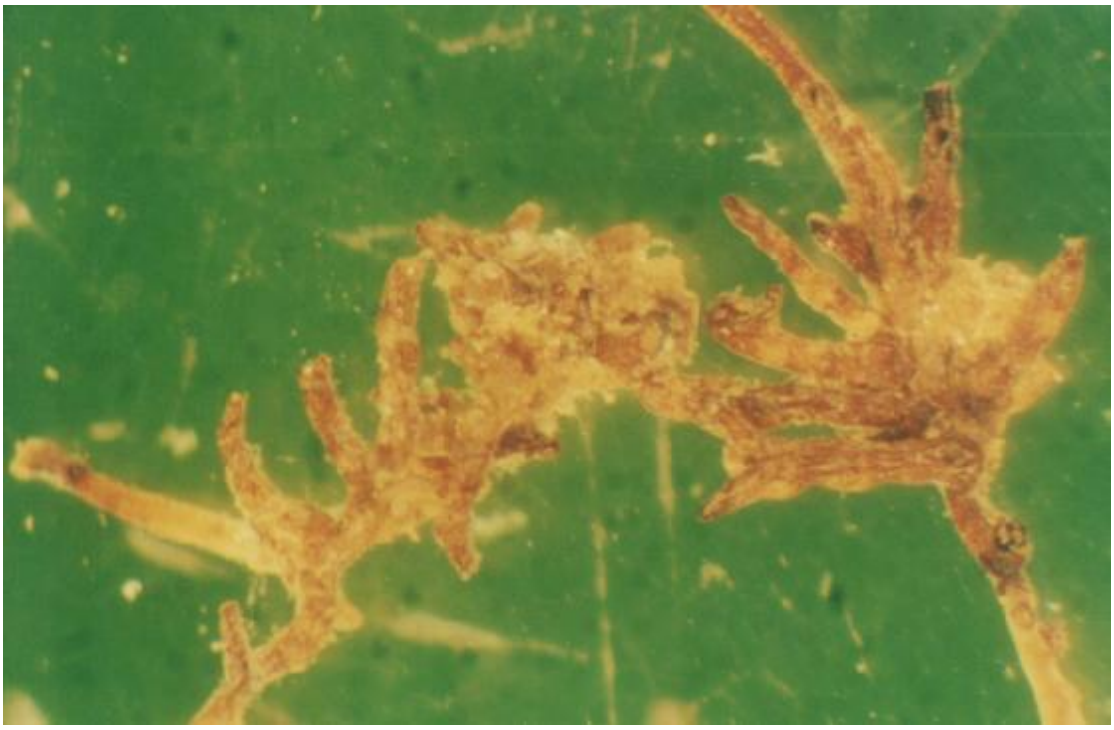

Figure: 6.11 Ectomycorrhizal roots of *E. tereticornis* colonized by *P. tinctorius* (Pt-NIC)

The addition of 50, 100, 200, 400 and 500 mg/L of aluminium inhibited the % colonization of root tips infected with *P. tinctorius* (Pt-NIC) by 17.31, 21.82, 33.57, 35.90 and 37.44 respectively. The same concentrations of aluminium inhibited the % colonization of root tips in case of *P. albus* (Pt-P) by 19.56, 37.44, 42.36, 44.02 and 50.46 respectively (Table 6.10). The shoot height of the plantlets significantly decreased as compared to control plantlets (0 mg/L) with increasing concentrations of aluminium in both ectomycorrhizal and nonmycorrhizal plants. However, inoculation of ectomycorrhizal fungi improved the shoot height as compared to nonmycorrhizal plants (Table 6.11 and Figure 6.12). Compared to the control plantlets (0 mg/L) the shoot dry weight (Table 6.12 and Figure 6.13) and root dry weight (Table 6.13 and Figure 6.14) of the plantlets decreased significantly with increasing concentrations of aluminium, both in mycorrhizal and nonmycorrhizal plantlets. The shoot and root dry weights of ectomycorrhizal plantlets were significantly higher than nonmycorrhizal plantlets at all aluminium concentrations. Among the two *Pisolithus* isolates, Pt-NIC inoculated plantlets showed significantly higher shoot and root dry weights compared to Pt-P inoculated plantlets indicating their better suitability in aluminium contaminated soils.

Table: 6.10 Influence of Aluminium concentrations on the % mycorrhization of *E. tereticornis*

plants inoculated with Pt-NIC and Pt-P**.**

Al ***(mg/L)***	*% mycorrhization*		*% inhibition*	
	Pt-NIC	*Pt-P*	*Pt-NIC*	*Pt-P*
0	**65.57 ± 0.83aA**	**57.25 ± 2.51aB**	**0**	**0**
50	**54.22 ± 2.57bA**	**46.05 ± 2.50bA**	**17.31**	**19.56**
100	**51.27 ± 0.65bA**	**35.82 ± 1.73cB**	**21.82**	**37.44**
200	**43.56 ± 3.90cA**	**33.00 ± 1.66cdA**	**33.57**	**42.36**
400	**42.03 ± 0.90cA**	**32.05 ± 1.22cdB**	**35.90**	**44.02**
500	**41.02 ± 1.38cA**	**28.36 ± 1.39dB**	**37.44**	**50.46**

Values sharing a common letter in the column (lower case) and rows (uppercase) are not significant ($P<0.05$).

Table: 6.11 Influence of Aluminium concentrations on the shoot height (cms) of *E. tereticornis* (E.t) plants inoculated with Pt-NIC and Pt-P**.**

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t + Pt-P*
0	**4.17 ±0.09**	**7.03 ± 0.12**	**6.03 ± 0.12**
50	**3.67 ± 0.12**	**5.90 ± 0.17**	**5.20 ± 0.23**
100	**3.07 ± 0.09**	**4.53 ± 0.18**	**4.43 ± 0.12**
200	**2.67 ± 0.09**	**4.03 ± 0.23**	**3.43 ± 0.12**
400	**2.17 ± 0.03**	**3.80 ± 0.25**	**3.33 ± 0.07**
500	**0.00 ± 0.00**	**3.07 ± 0.07**	**3.00 ± 0.12**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F value*
Interaction	**5**	**4.045**	**0.809**	**13.67 *****
Treatment	**1**	**39.90**	**39.90**	**674.4 *****
Concentration	**5**	**60.72**	**12.14**	**205.3 *****
Residual	**24**	**1.420**	**0.059**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F value*
Interaction	**5**	**4.456**	**0.891**	**23.25 *****
Treatment	**1**	**23.52**	**23.52**	**613.6 *****
Concentration	**5**	**49.38**	**9.876**	**257.6 *****
Residual	**24**	**0.920**	**0.038**	

*** P<0.001

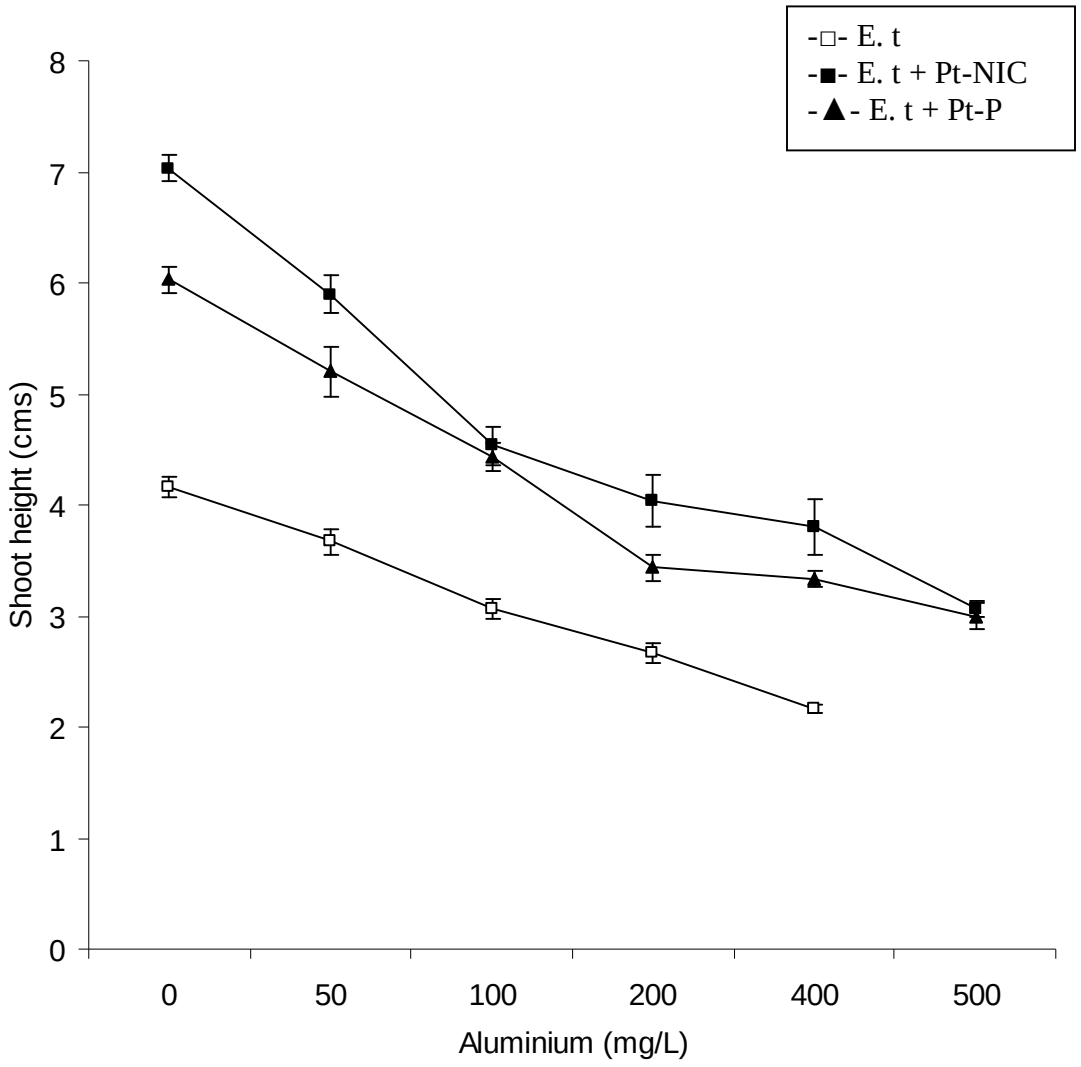

Figure: 6.12 Influence of Aluminium concentrations on the shoot height (cms) of *E. tereticornis*

plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

Table: 6.12 Influence of Aluminium concentrations on the shoot dry weight (mg) of *E. tereticornis*

(E.t) plants inoculated with Pt-NIC and Pt-P**.**

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t +Pt-P*
0	**17.7 ± 0.33**	**31.0 ± 0.58**	**24.0 ± 0.58**
50	**15.0 ± 0.58**	**29.0 ± 0.58**	**21.7 ± 0.88**
100	**12.3 ± 0.34**	**30.0 ± 1.16**	**21.0 ± 0.58**
200	**6.7 ± 0.34**	**21.3 ±0.88**	**16.0 ± 0.58**
400	**2.6 ± 0.34**	**16.0 ± 0.58**	**8.3 ± 0.67**
500	**0.0**	**9.0 ± 0.58**	**6.7 ± 0.33**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**58.67**	**11.73**	**11.12 *****
Treatment	**1**	**1681**	**1681**	**1593 *****
Concentration	**5**	**1877**	**375.3**	**355.6 *****
Residual	**24**	**25.33**	**1.056**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**15.56**	**3.111**	**4.000 *****
Treatment	**1**	**469.4**	**469.4**	**603.6 *****
Concentration	**5**	**1530**	**306.1**	**393.5 *****
Residual	**24**	**18.67**	**0.778**	

*** P<0.001

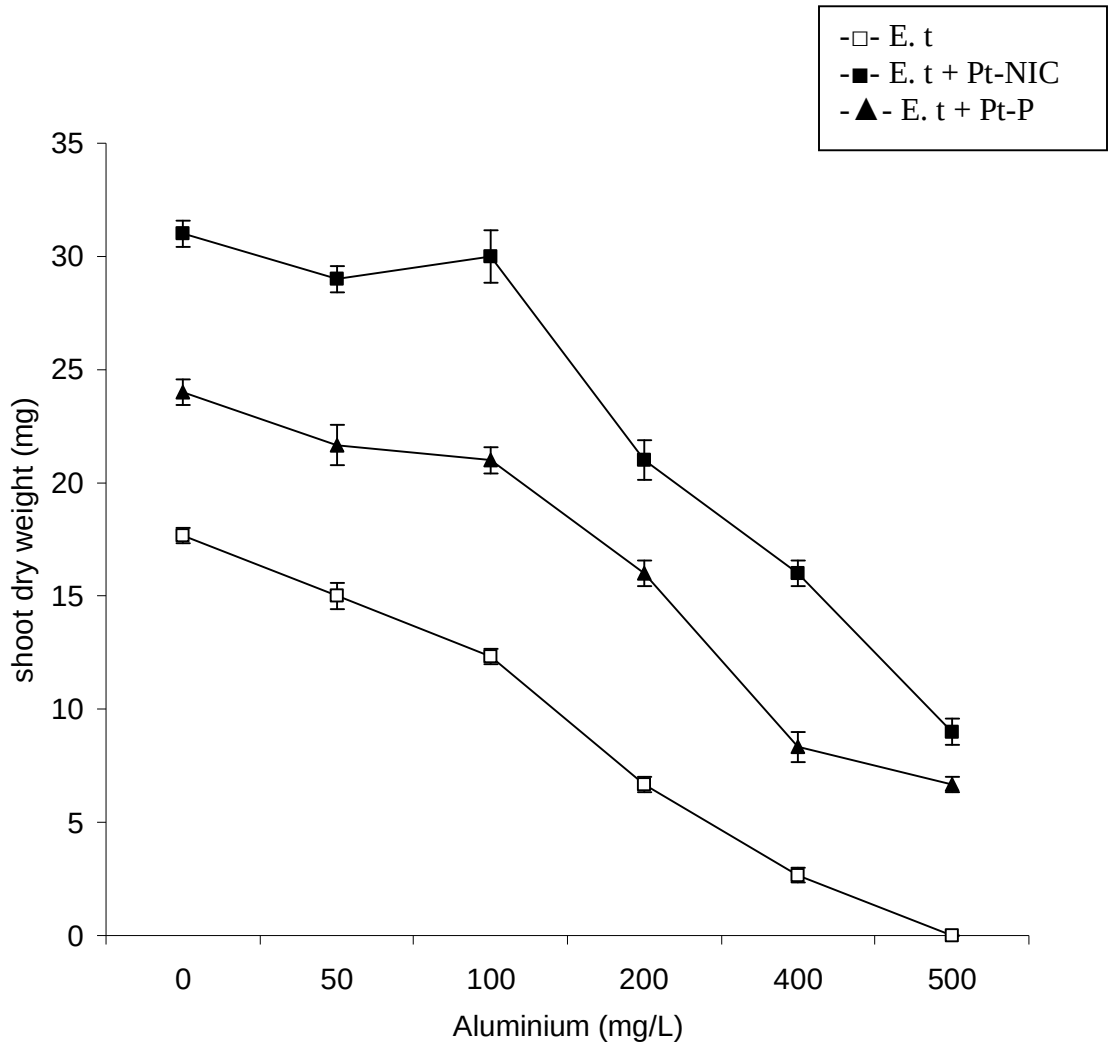

Figure: 6.13 Influence of Aluminium concentrations on the shoot dry weight (mg) of *E .tereticornis* plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

Table: 6.13 Influence of Aluminium concentrations on the root dry weight (mg) of *E. tereticornis*

(E.t) plants inoculated with Pt-NIC and Pt-P.

Al (mg/L)	*E. t*	*E. t + Pt- NIC*	*E. t + Pt-P*
0	**5.15 ± 0.14**	**11.67 ± 0.33**	**9.67 ± 0.33**
50	**3.90 ± 0.06**	**8.00 ± 0.58**	**7.67 ± 0.33**
100	**2.80 ± 0.12**	**6.33 ± 0.33**	**7.00 ± 0.58**
200	**2.57 ± 0.23**	**5.33 ± 0.33**	**5.00 ±0.58**
400	**1.96 ± 0.03**	**4.00 ± 0.58**	**2.33 ± 0.33**
500	**0.00**	**3.33 ± 0.33**	**2.00 ± 0.58**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**17.83**	**3.565**	**11.87 *** **
Treatment	**1**	**124.1**	**124.1**	**413.1 *** **
Concentration	**5**	**167.9**	**33.58**	**111.8 *** **
Residual	**24**	**7.209**	**0.300**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**18.73**	**3.746**	**10.52 *** **
Treatment	**1**	**74.65**	**74.65**	**209.7 *** **
Concentration	**5**	**167.5**	**33.49**	**94.10 *** **
Residual	**24**	**8.542**	**0.356**	

*** P<0.001

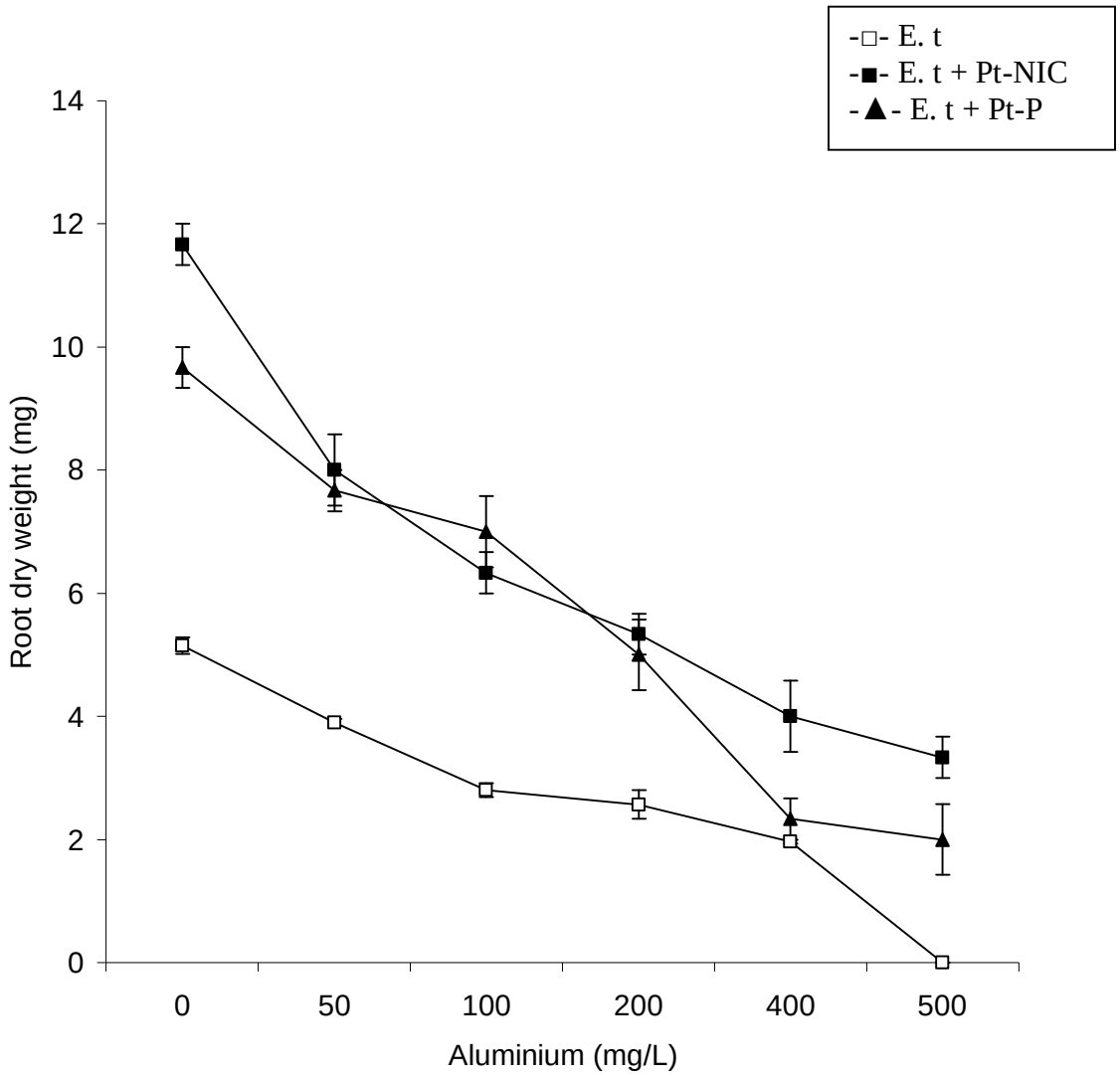

Figure: 6.14 Influence of Aluminium concentrations on the root dry weight (mg) of *E. tereticornis* plants inoculated with Pt-NIC and Pt-P. Mean value ± SE

6.5 Aluminium concentrations in plant shoots

The aluminium content in the plant shoot of ectomycorrhizal and nonmycorrhizal plantlets increased with increasing concentrations of aluminium in the growth medium (Table 6.14 and Figure 6.15). The addition of 50 and 100 mg/L of aluminium did not significantly altered shoot aluminium concentration in Pt-NIC inoculated ectomycorrhizal and further 200, 400 and 500 mg/L of aluminium concentration increased its content significantly in plant shoots whereas tha addition of even 50 mg/L of aluminium significantly increased aluminium content of the Pt-P inoculated plant shoots.

The accumulation of aluminium in the shoots of nonmycorrhizal *E. tereticornis* plantlets was drastically increased with increasing concentrations of aluminium and the nonmycorrhizal plantlets died at 500 mg/L of aluminium concentration. However, the accumulation was significantly lesser in ectomycorrhizal plantlets and this could be the reason of the better suitability of the ectomycorrhizal plants than nonmycorrhizal plants in presence of aluminium. There was no significant difference in the accumulation of aluminium among the two different *Pisolithus* isolates inoculated *E. tereticornis* plantlets.

Table: 6.14 Influence of Aluminium concentrations on the accumulation of Aluminium (mg/g dry weight) in shoot of *E. tereticornis* (E.t) plants inoculated with Pt-NIC and Pt-P

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t + Pt-P*
0	**0.70 ± 0.03**	**0.37 ± 0.04**	**0.23 ± 0.13**
50	**1.87 ± 0.07**	**0.48 ± 0.01**	**0.43 ± 0.04**
100	**6.99 ± 0.27**	**0.51 ± 0.01**	**0.61 ± 0.02**
200	**20.53 ± 0.41**	**1.05 ± 0.05**	**1.06 ± 0.09**
400	**22.34 ± 1.70**	**1.99 ± 0.07**	**2.93 ± 0.15**
500	**0.00**	**5.40 ± 0.19**	**5.09 ± 0.91**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**1094**	**109.4**	**161.6*** **
Treatment	**1**	**597.9**	**298.9**	**441.6*** **
Concentration	**5**	**563.1**	**112.6**	**166.4*** **
Residual	**24**	**24.37**	**0.677**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**845.4**	**169.1**	**213.1*** **
Treatment	**1**	**454.2**	**454.2**	**572.6*** **
Concentration	**5**	**756.7**	**151.3**	**190.8*** **

Residual	**24**	**19.04**	**0.793**

*** P<0.001

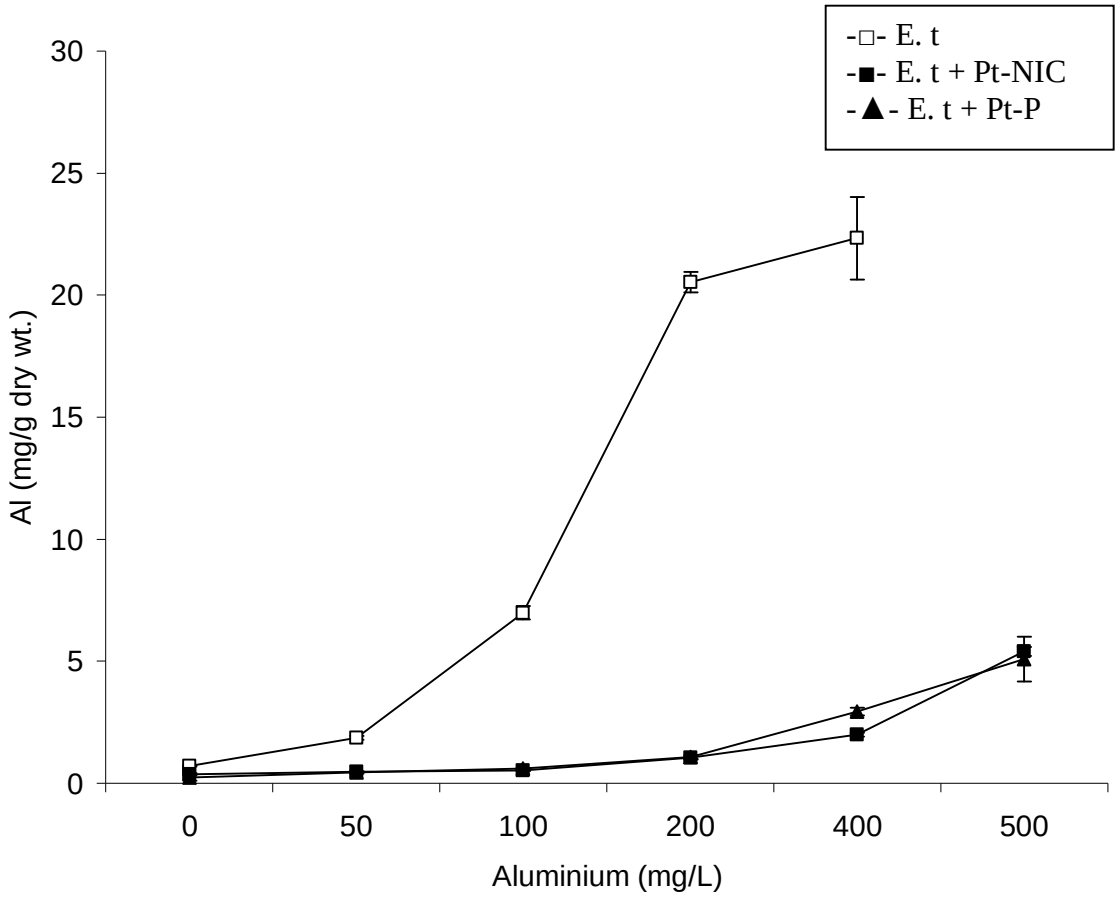

Figure: 6.15 Influence of Aluminium concentrations on the accumulation of Aluminium in

shoot of *E. tereticornis* plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

6.6 Nutrient element concentrations in plant shoots

The ectomycorrhizal plantlets showed significantly higher foliar concentrations of P, Ca, Mg and K in their plant shoot tissue when compared to nonmycorrhizal plantlets at all aluminium levels. The uptake of phosphorus was significantly more in ectomycorrhizal plantlets as compared to nonmycorrhizal plantlets. The phosphorus content in the plant shoot decreased significantly with increasing concentrations of aluminium in nonmycorrhizal plantlets while there was no significant change in phosphorus content of the plant shoot in ectomycorrhizal plantlets (Table 6.15 and Figure 6.16). The Pt-NIC inoculated plantlets accumulated significantly more phosphorus at higher aluminium concentrations than Pt-P inoculated plantlets. The calcium content decreased significantly at higher concentrations of aluminium in nonmycorrhizal plantlets, while the calcium content increased significantly beyond 200 mg/L of aluminium concentration in ectomycorrhizal plantlets. Among the two isolates of *Pisolithus,* Pt-NIC inoculated plantlets had significantly higher calcium content in their shoot as compared to Pt-P (Table 6.16 and Figure 6.17). The magnesium content in the shoot tissue of nonmycorrhizal plantlets was significantly lower than ectomycorrhizal plantlets and Pt-NIC inoculated plantlets showed better uptake of magnesium than Pt-P inoculated plantlets (Table 6.17 and Figure 6.18). Similarly, the potassium content was significantly decreased in the plant shoots with increasing concentrations of aluminium. The potassium content of nonmycorrhizal plantlets decreased with increasing concentrations of aluminium while there was no significant change in potassium

content in the ectomycorrhizal plantlets till 400 mg/L and then decreased at 500 mg/L of aluminium concentration.

Table: 6.15 Influence of Aluminium concentrations on the Phosphorus content (mg/g dry weight) in the plant shoot of *E. tereticornis* (E.t) plants inoculated with Pt-NIC and Pt-P.

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t + Pt-P*
0	**10.64 ±0.46**	**15.65 ± 0.31**	**15.67 ± 0.23**
50	**9.99 ± 0.36**	**15.81 ± 0.30**	**15.93 ± 0.88**
100	**8.56 ± 0.05**	**15.89 ± 0.54**	**14.49 ± 0.78**
200	**8.51 ± 0.43**	**17.15 ± 0.59**	**14.91 ± 0.38**
400	**7.93 ± 0.86**	**16.91 ± 0.55**	**14.31 ± 0.51**
500	**0.00**	**16.01 ± 1.24**	**14.12 ± 0.33**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**115.8**	**23.16**	**23.40*** **
Treatment	**1**	**670.7**	**670.7**	**677.7*** **
Concentration	**5**	**113.8**	**22.76**	**22.99*** **
Residual	**24**	**23.75**	**0.989**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**85.61**	**17.12**	**21.27*** **
Treatment	**1**	**479.9**	**479.9**	**596.1*** **

Concentration	**5**	**146.4**		**29.27**	**36.36*** **
Residual		**24**	**19.32**		**0.805**

*** P<0.001

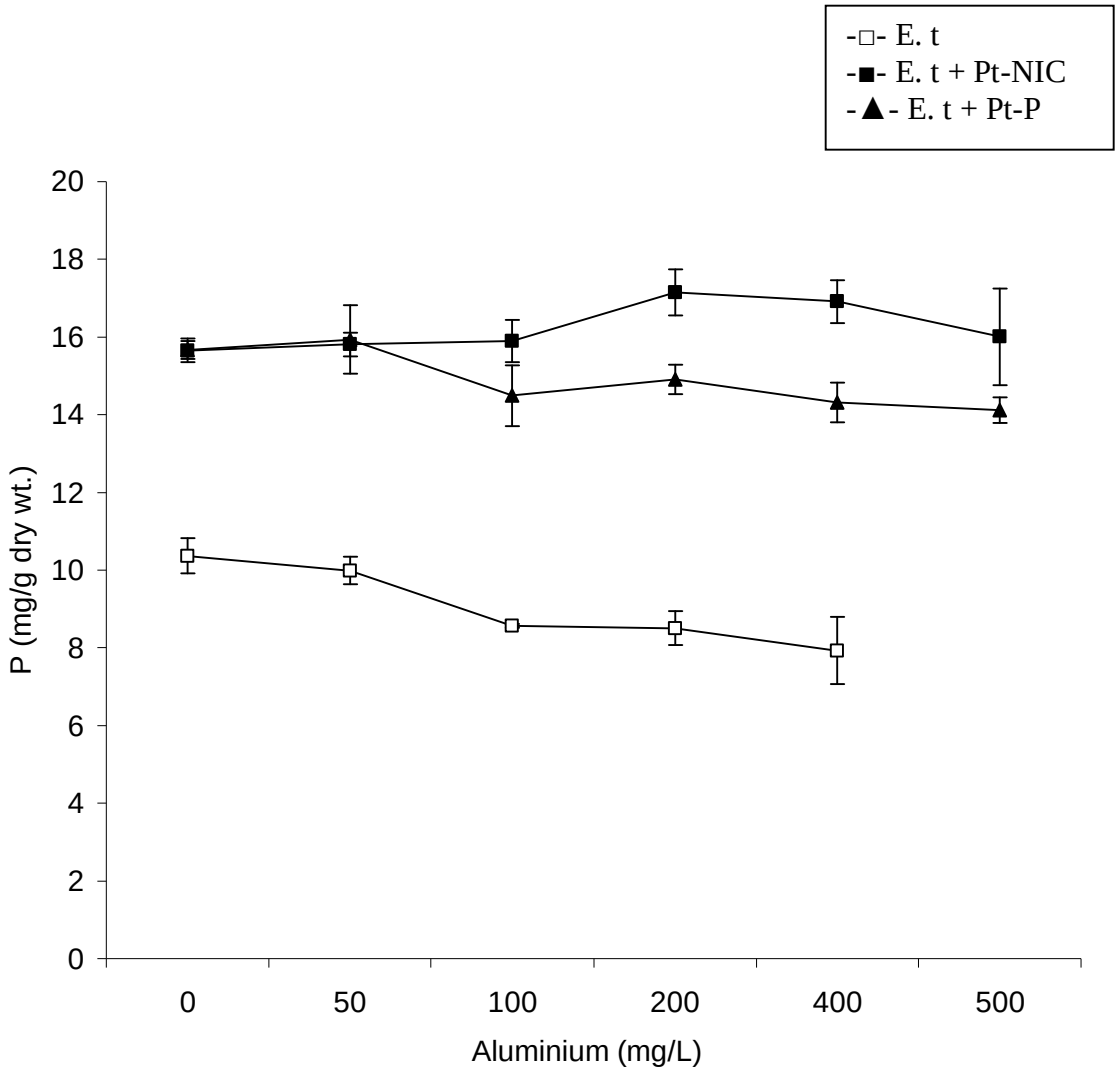

Figure: 6.16 Influence of Aluminium concentrations on the Phosphorus content in the shoot

of *E. tereticornis* plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

Table: 6.16 Influence of Aluminium concentrations on the Calcium content (mg/g dry weight)

in the shoot of *E. tereticornis* (E.t) plants inoculated with Pt-NIC and Pt-P..

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t + Pt-P*
0	**12.99 ± 0.90**	**14.62 ± 1.21**	**12.40 ± 0.38**
50	**11.84 ± 0.75**	**12.90 ± 0.69**	**11.59 ± 0.68**
100	**10.67 ± 0.85**	**12.27 ± 0.58**	**13.33 ± 0.47**
200	**10.02 ± 0.64**	**17.17 ± 0.98**	**14.74 ± 0.45**
400	**10.23 ± 1.10**	**17.37 ± 1.07**	**14.69 ± 0.33**
500	**0.00**	**22.80 ± 1.72**	**14.58 ± 0.39**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean Square*	*F*
Interaction	**5**	**514.4**	**102.9**	**37.14 *** **
Treatment	**1**	**427.8**	**427.8**	**154.4 *** **
Concentration	**5**	**39.5**	**7.899**	**2.852 ***
Residual	**24**	**66.48**	**2.770**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of- square*	*Mean square*	*F*
Interaction	**5**	**229.9**	**45.99**	**36.72 *** **
Treatment	**1**	**163.3**	**163.3**	**130.4 *** **
Concentration	**5**	**126.7**	**25.34**	**20.24 *** **
Residual	**24**	**30.05**	**1.252**	

* P< 0.05
*** P<0.001

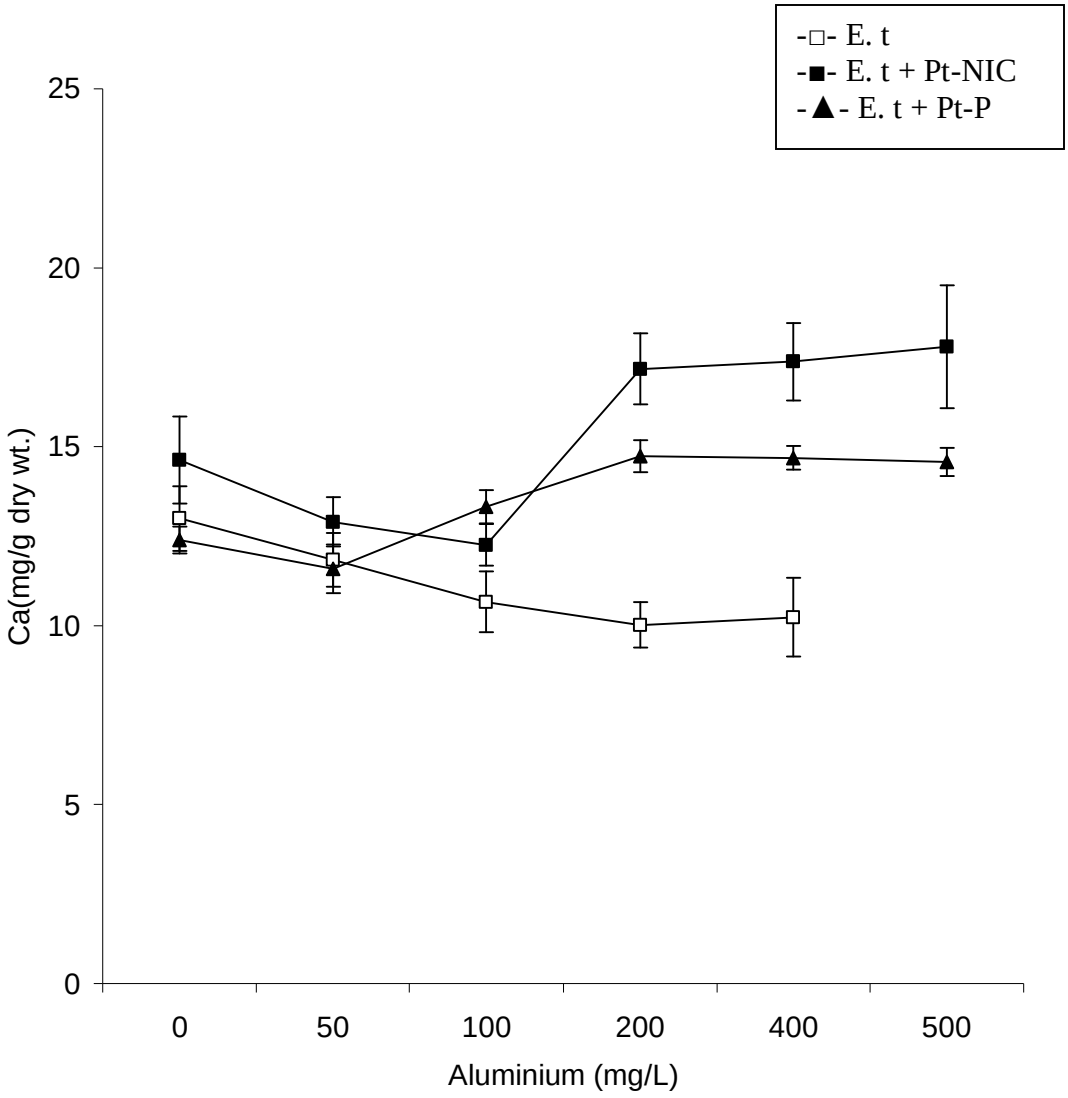

Figure: 6.17 Influence of Aluminium concentrations on the Calcium content in the shoot of

E. tereticornis plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

Table: 6.17 Influence of Aluminium concentrations on the Magnesium content (mg/g dry weight) in the shoot of *E. tereticornis* (E.t) plants inoculated with Pt-NIC and Pt-P.

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t + Pt-P*
0	**4.43 ± 0.02**	**8.04 ± 0.31**	**6.46 ± 0.06**
50	**4.23 ± 0.04**	**7.88 ± 0.19**	**6.36 ± 0.32**
100	**4.19 ± 0.12**	**7.78 ± 0.22**	**7.71 ± 0.16**
200	**3.75 ± 0.26**	**9.22 ± 0.64**	**8.06 ± 0.70**
400	**3.10 ± 0.51**	**8.91 ± 0.52**	**8.36 ± 0.47**
500	**0.00**	**9.65 ± 0.65**	**8.39 ± 0.51**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**41.55**	**8.309**	**20.38*** **
Treatment	**1**	**252.5**	**252.5**	**619.3*** **
Concentration	**5**	**9.898**	**1.980**	**4.855** **
Residual	**24**	**9.786**	**0.408**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**42.27**	**8.454**	**23.28*** **
Treatment	**1**	**164.3**	**164.3**	**452.5*** **
Concentration	**5**	**12.74**	**2.549**	**7.018*** **
Residual	**24**	**8.716**	**0.363**	

*** P<0.001

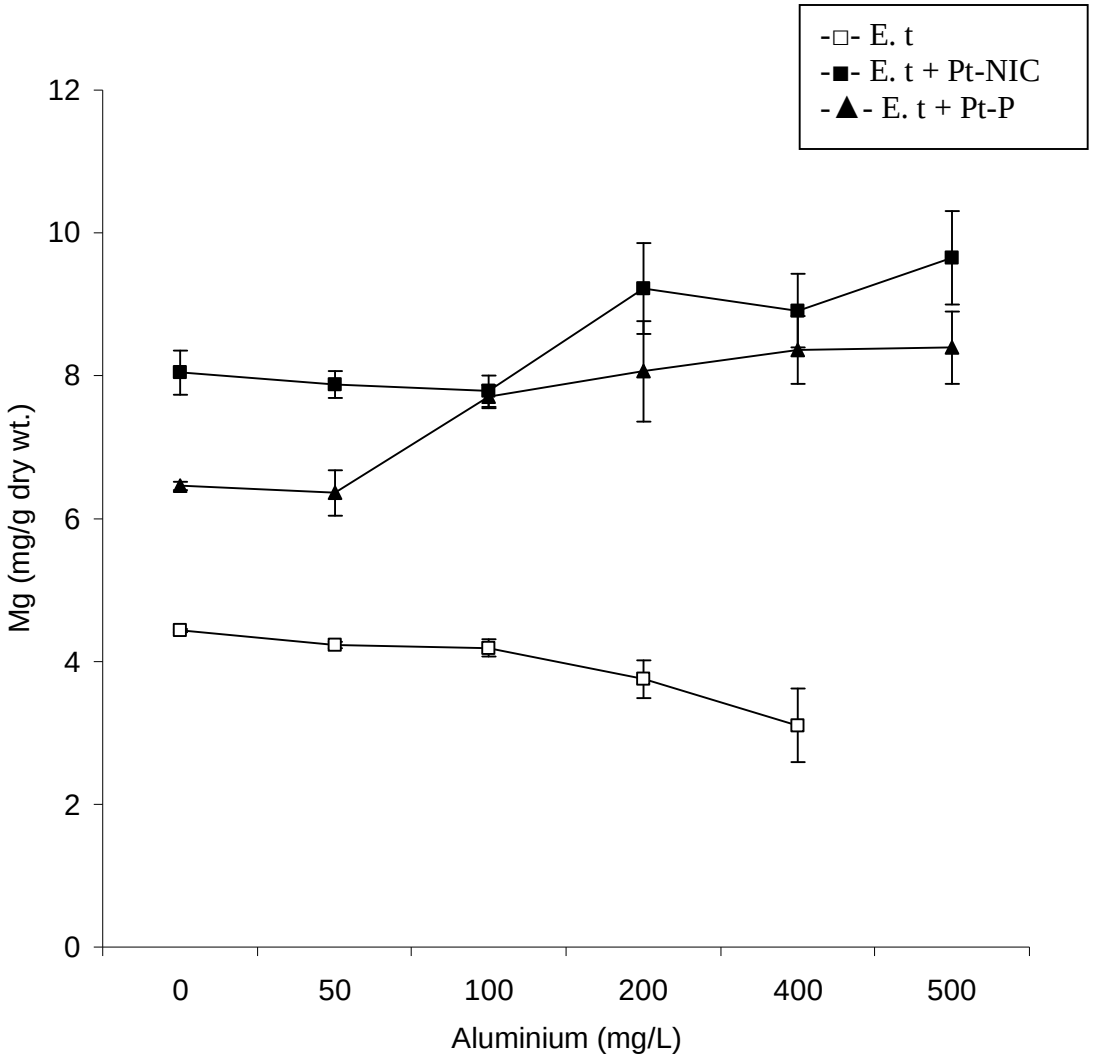

Figure: 6.18 Influence of Aluminium concentrations on the Magnesium content in the shoot

of *E. tereticornis* plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

Table: 6.18 Influence of Aluminium concentrations on the Potassium content (mg/g dry weight) in the shoot of *E. tereticornis* (E.t) plants inoculated with Pt-NIC and Pt-P.

Al (mg/L)	*E. t*	*E. t + Pt-NIC*	*E. t + Pt-P*
0	**31.78 ± 0.19**	**34.13 ± 0.25**	**35.86 ± 0.20**
50	**30.94 ± 0.41**	**34.59 ± 0.68**	**38.04 ± 2.72**
100	**27.99 ± 1.00**	**33.47 ± 0.83**	**33.42 ± 0.48**
200	**26.59 ± 0.91**	**32.93 ± 0.77**	**33.80 ± 2.35**
400	**18.67 ± 0.76**	**32.98 ± 2.77**	**32.15 ± 1.02**
500	**0.00**	**30.59 ± 0.26**	**30.72 ± 0.17**

Analysis of Variance

E. t + Pt-NIC

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**861.0**	**172.2**	**56.77*** **
Treatment	**1**	**982.7**	**982.7**	**324.0*** **
Concentration	**5**	**1343**	**268.7**	**88.58*** **
Residual	**24**	**72.80**	**3.033**	

E. t + Pt-P

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**754.6**	**150.9**	**35.76*** **
Treatment	**1**	**1156**	**1156**	**273.9*** **
Concentration	**5**	**1524**	**304.7**	**72.20*** **
Residual	**24**	**101.3**	**4.221**	

*** P<0.001

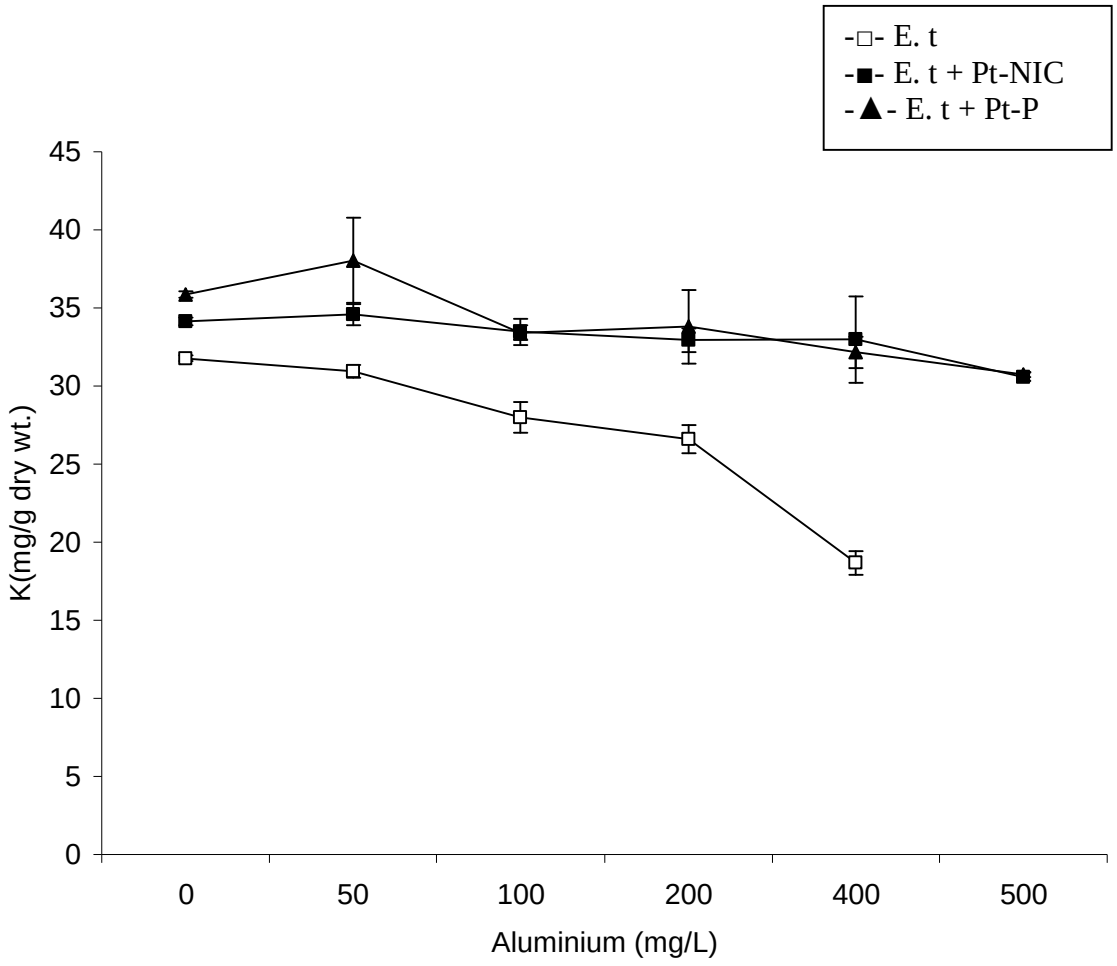

Figure: 6.19 Influence of Aluminium concentrations on the Potassium content in the shoot of

E. tereticornis plants inoculated with Pt-NIC and Pt-P. Mean ± SE.

6.7 Effect of aluminium on the growth of *P. pinaster* seedlings *in vitro*

The axenically germinated seedlings of *P. pinaster* on water agar medium were transferred to soilrite-vermiculite support moistened with Melin's medium containing different concentrations of aluminium. The seedlings were inoculated with ectomycorrhizal fungus *H. cylindrosporum* (D2*) and harvested after 60 days of exposure to aluminium. The seedlings were analyzed for growth parameters, uptake of mineral nutrients and aluminium concentration in the plant shoots. The response of nonmycorrhizal and ectomycorrhizal seedlings to increasing concentrations of aluminium indicated that nonmycorrhizal seedlings were more sensitive to the presence of aluminium. *H. cylindrosporum* formed ectomycorrhizas with the roots of *P. pinaster* and colonized 51.9 % of lateral roots of *P. pinaster* in absence of aluminium. The mycorrhizas formed were bifurcate with numerous irregularly spaced branches (Figure 6.20). The mycorrhizae were white in colour.The addition of aluminium inhibited the % colonization of root tips by *H. cylindrosporum* and the results showed that 50 mg/L of Al inhibited the % colonization of root tips by 7.95 and 72.5 at 500 mg/L of Al concentration (Table 6.19).

Table: 6.19 Influence of Aluminium concentrations on the % mycorrhization of *P. pinaster*

inoculated with D2*.

Al ***(mg/L)***	*% mycorrhization*	*% inhibition*
0	**51.92 ± 1.23a**	**0**
50	**47.79 ± 1.27b**	**7.95**
100	**26.22 ± 1.39c**	**49.5**
200	**21.43 ± 1.73d**	**58.7**

400	**16.67 ± 0.85e**	**67.9**
500	**14.28 ± 1.15e**	**72.5**

Values sharing a common letter within the fungus are not significantly different at P<0.05

Figure: 6.20 Ectomycorrhizal roots of *P. pinaster* colonized by *H. cylindrosporum* (D2*)

The mycorrhizal colonization was significantly decreased as the concentration of aluminium increased in the growth medium (Table 6.19). The growth of the seedlings was significantly improved by the inoculation of ectomycorrhizal fungus in presence of aluminium as compared to nonmycorrhizal seedlings. Compared with the control seedlings, there was significant reduction in shoot height in case of nonmycorrhizal as well as ectomycorrhizal seedlings at higher aluminium concentrations. (Table 6.20 and Figure 6.21). However, the shoot height was significantly more in ectomycorrhizal seedlings than nonmycorrhizal seedlings at each aluminium concentration applied. The shoot dry weight (Table 6.21 and Figure 6.22) and root dry weight (Table 6.22 and Figure 6.23) of the ectomycorrhizal seedlings were significantly higher than nonmycorrhizal seedlings. The growth of nonmycorrhizal seedlings were completely inhibited at 500mg/L of aluminium concentration and the seedlings died while the ectomycorrhizal seedlings of *P. pinaster* survived.

6.8 Aluminium concentrations in plant shoots

The accumulation of aluminium in the plant shoot of ectomycorrhizal and nonmycorrhizal seedlings increased significantly with increasing concentrations of aluminium in the medium (Table 6.23 and Figure 6.24). The results of plant- fungus interaction studies in presence of aluminium indicated that ectomycorrhizal seedlings accumulated less of aluminium in its shoot tissue compared to nonmycorrhizal seedlings at all concentrations of aluminium. The inoculation of ectomycorrhizal fungus protected the seedlings from the detrimental effects of aluminium and the ectomycorrhizal seedlings

survived while the nonmycorrhizal seedlings died at 500 mg/L of aluminium concentration.

Table: 6.20 Influence of Aluminium concentrations on the shoot height (cms) of *P. pinaster*

(P.p) plants inoculated with D2*.

Al (mg/L)	*P. p*	*P. p+ D2**
0	**8.90 ± 0.15**	**11.07 ± 0.27**
50	**7.97 ± 0.23**	**9.90 ± 0.32**
100	**6.87 ± 0.18**	**7.60 ± 0.06**
200	**5.23 ± 0.03**	**6.43 ± 0.09**
400	**2.47 ± 0.03**	**3.47 ± 0.07**
500	**0.00**	**3.43 ± 0.12**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F value*
Interaction	**5**	**7.409**	**1.482**	**18.59 *** **
Treatment	**1**	**27.39**	**27.39**	**343.5 *** **
Concentration	**5**	**321.0**	**64.19**	**805.2 *** **
Residual	**24**	**1.913**	**0.080**	

*** P<0.001

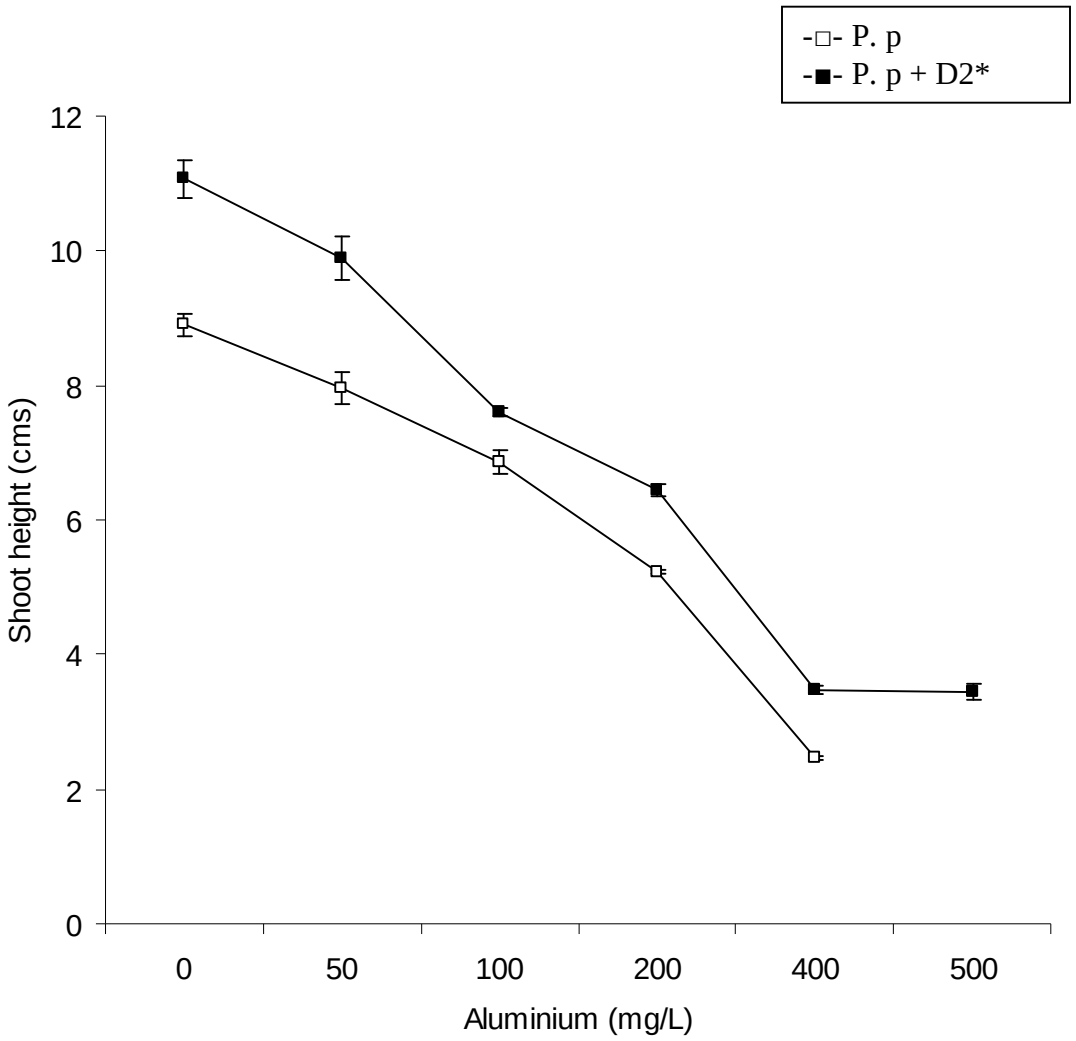

Figure: 6.21 Influence of Aluminium concentrations on the shoot height of *P. pinaster* plants inoculated with D2*. Mean ± SE.

Table: 6.21 Influence of Aluminium concentrations on the shoot dry weight (mg) of *P. pinaster* (P.p) plants inoculated with D2*.

Al (mg/ L)	*P. p*	*P. p+ D2**
0	**16.33 ± 0.88**	**22.33 ± 0.88**
50	**11.00 ± 0.58**	**17.33 ± 0.67**
100	**6.33 ± 0.88**	**13.00 ± 0.58**
200	**4.33 ± 0.33**	**9.00 ± 0.58**
400	**3.33 ± 0.88**	**7.00 ± 0.58**
500	**0.00**	**4.67 ± 0.34**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**10.33**	**2.067**	**1.617 ns**
Treatment	**1**	**256.0**	**256.0**	**200.3 *****
Concentration	**5**	**1180**	**236.0**	**184.7 *****
Residual	**24**	**30.67**	**1.278**	

*** P<0.001

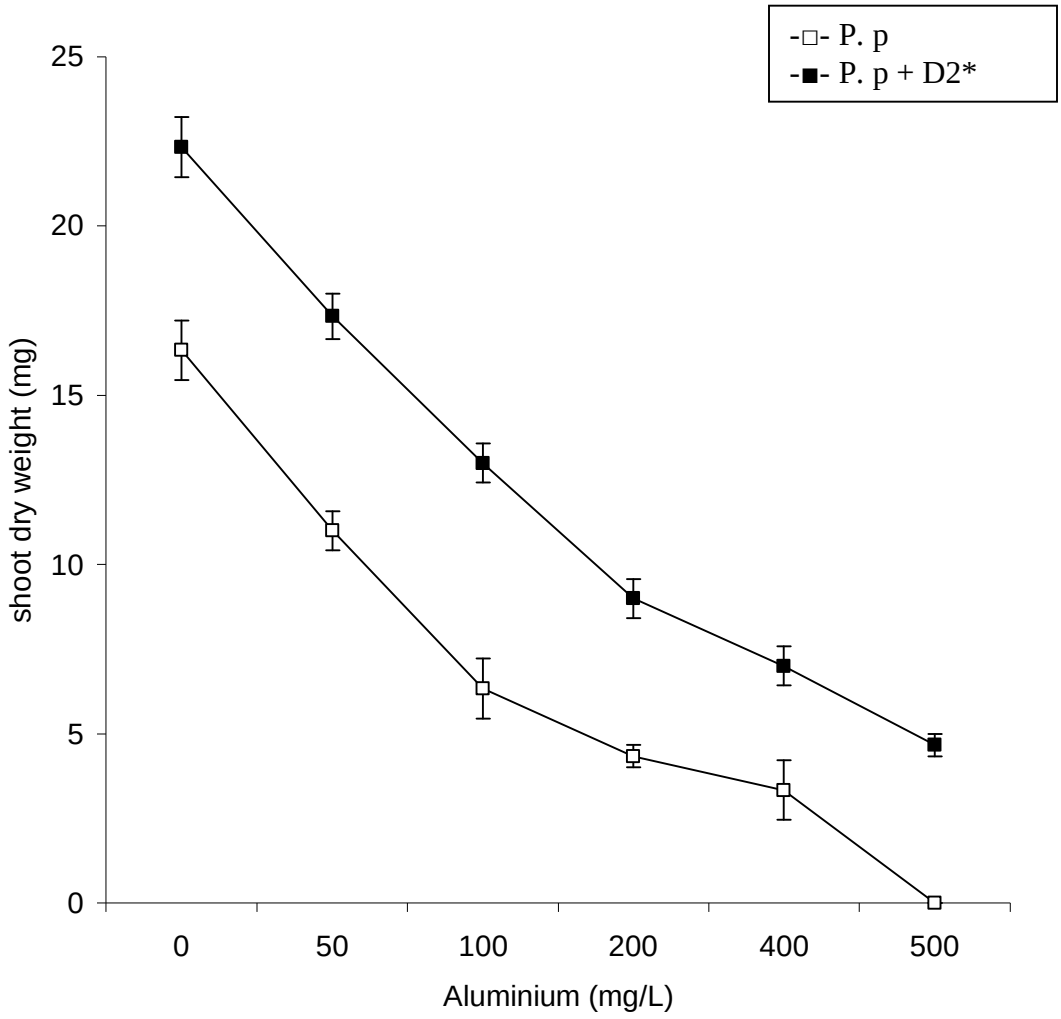

Figure: 6.22 Influence of Aluminium concentrations on the shoot dry weight (mg) of *P. pinaster* plants inoculated with D2*. Mean ± SE

Table: 6.22 Influence of Aluminium concentrations on the root dry weight (mg) of *P. pinaster* (P.p) plants inoculated with D2*.

Al (mg/L)	*P. p*	*P. p+ D2**
0	**4.00 ± 0.58**	**9.67 ± 0.34**
50	**3.33 ± 0.34**	**7.00 ± 0.58**
100	**1.67 ± 0.34**	**5.67 ± 0.34**
200	**1.00 ± 0.01**	**4.33 ± 0.34**
400	**1.00 ± 0.00**	**2.33 ± 0.34**
500	**0.00**	**1.00 ± 0.00**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**22.92**	**4.583**	**13.75*** **
Treatment	**1**	**90.25**	**90.25**	**270.8*** **
Concentration	**5**	**161.6**	**32.32**	**96.95*** **
Residual	**24**	**8.000**	**0.333**	

*** P<0.001

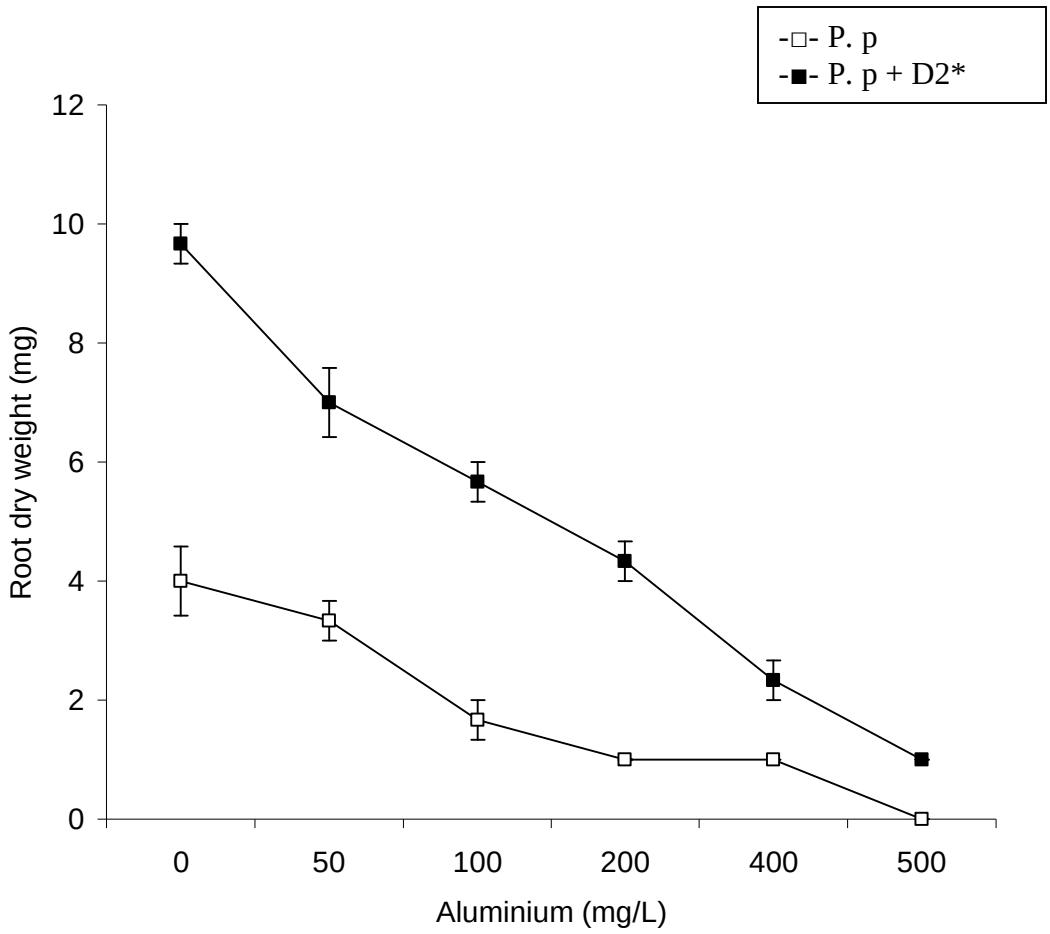

Figure: 6.23 Influence of Aluminium concentrations on the root dry weight (mg) of *P. pinaster* plants inoculated with D2*. Mean value ± SE.

Table: 6.23 Influence of Aluminum concentrations on its accumulation (mg/g dry weight)

in the shoot of *P. pinaster* (P.p) plants inoculated with D2*.

Al (mg/L)	*P. p*	*P.p + D2**
0	**0.62 ± 0.02**	**0.31 ± 0.01**
50	**2.71 ± 0.08**	**0.89 ± 0.03**
100	**9.03 ± 0.07**	**1.34 ± 0.05**
200	**18.55 ± 0.24**	**2.36 ± 0.07**
400	**45.19 ± 1.14**	**9.25 ± 0.19**
500	**0.00**	**16.80 ± 0.29**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**2340**	**467.9**	**1260*** **
Treatment	**1**	**509.3**	**509.3**	**1371*** **
Concentration	**5**	**2842**	**568.4**	**1530*** **
Residual	**24**	**8.915**	**0.372**	

*** P<0.001

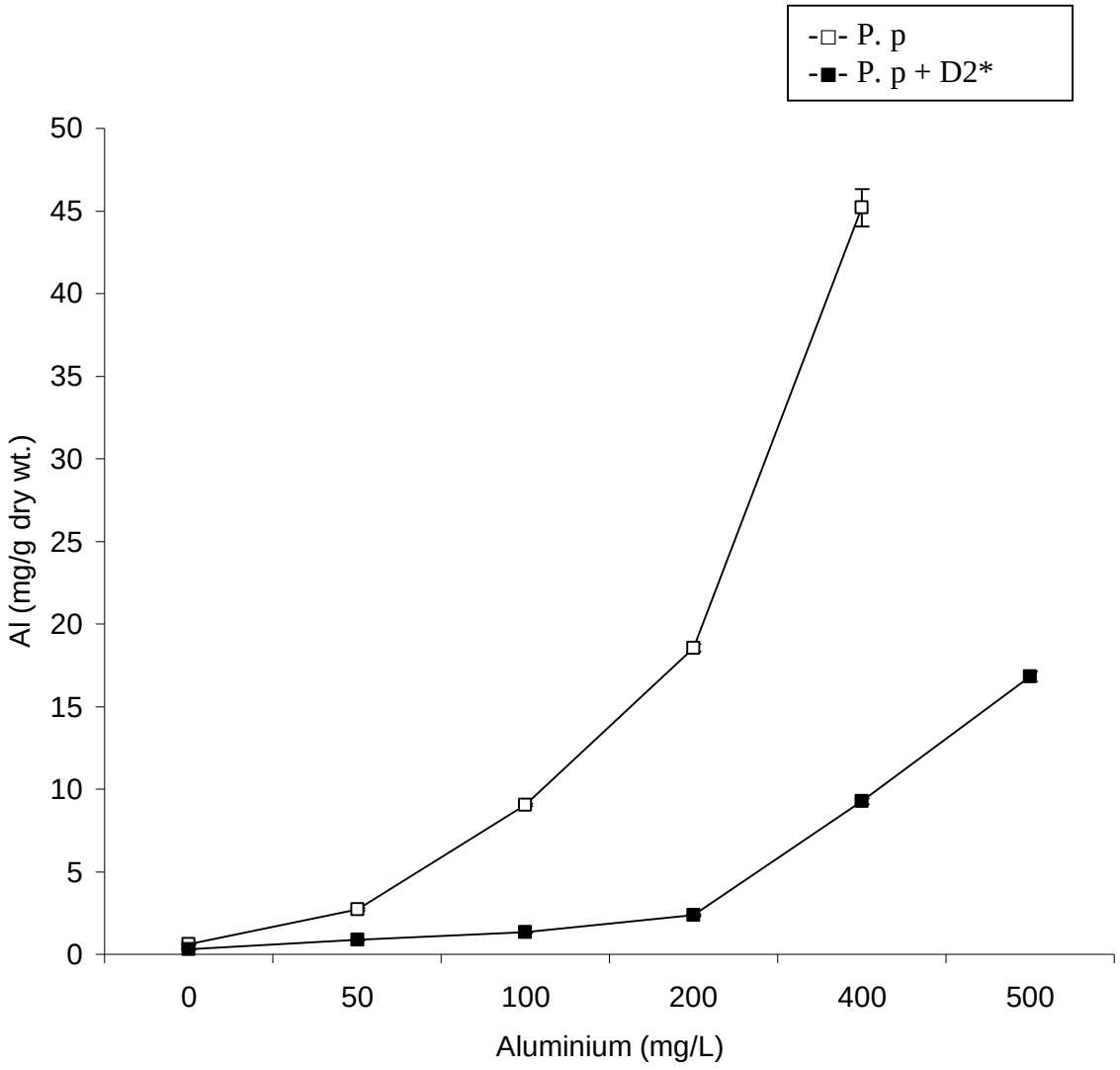

Figure: 6.24 Influence of Aluminum concentrations on its accumulation in the shoot of *P. pinaster* plants inoculated with D2*. Mean± SE.

6.9 Nutrient element concentration in plant shoots

The mineral content (as uptake of Ca, Mg, K and P) was adversely affected by the presence of aluminium in the medium. However, the inoculation of ectomycorrhizal fungus improved the uptake of nutrients as compared to nonmycorrhizal seedlings. The phosphorus content was significantly decreased in the nonmycorrhizal seedlings grown at different concentrations of aluminium, while there was no significant difference in the phosphorus content of ectomycorrhizal seedlings (Table 6.24 and Figure 6.25) and the phosphorus content of the ectomycorrhizal seedlings was significantly higher than nonmycorrhizal seedlings at each aluminium concentration applied.

The calcium content of nonmycorrhizal seedlings decreased with increased concentrations of aluminium. However, the calcium content was significantly improved at higher concentrations of aluminium in ectomycorrhizal seedlings as compared to the control seedlings (Table 6.25 and Figure 6.26). The calcium content of the ectomycorrhizal seedlings was significantly higher than nonmycorrhizal seedlings at each concentration of aluminium. There was significant decrease in the magnesium content of nonmycorrhizal seedlings and ectomycorrhizal seedlings with increasing concentrations of aluminium (Table 6.26 and Figure 6.27), although the ectomycorrhizal seedlingd had higher magnesium content at all aluminium concentrations. The potassium content of both ecto and nonmycorrhizal seedlings was significantly decreased with increased concentrations of aluminium (Table 6.27 and Figure 6.28), but

the ectomycorrhizal seedlings had more of potassium in their shoots as compared to nonmycorrhizal seedlings at all aluminium concentrations.

Table: 6.24 Influence of Aluminium concentrations on the Phosphorus content (mg/g dry wt)

in the shoot of *P. pinaster* (P.p) plants inoculated with D2*.

Al (mg/L)	*P. p*	*P. p +D2**
0	**9.16 ± 0.29**	**10.31 ± 0.10**
50	**8.21 ± 0.29**	**11.92 ± 0.35**
100	**8.38 ± 0.29**	**11.28 ± 0.07**
200	**7.57 ± 0.04**	**10.46 ± 0.24**
400	**6.49 ± 0.03**	**10.66 ± 0.11**
500	**0.00**	**10.55 ± 0.04**

Analysis of Variance

Source of Variation	***Df***	***Sum-of-squares***	***Mean square***	***F***
Interaction	**5**	**79.93**	**15.99**	**140.2*** **
Treatment	**1**	**161.0**	**161.0**	**1412.0*** **
Concentration	**5**	**96.17**	**19.23**	**168.7*** **
Residual	**24**	**2.736**	**0.114**	

*** P<0.001

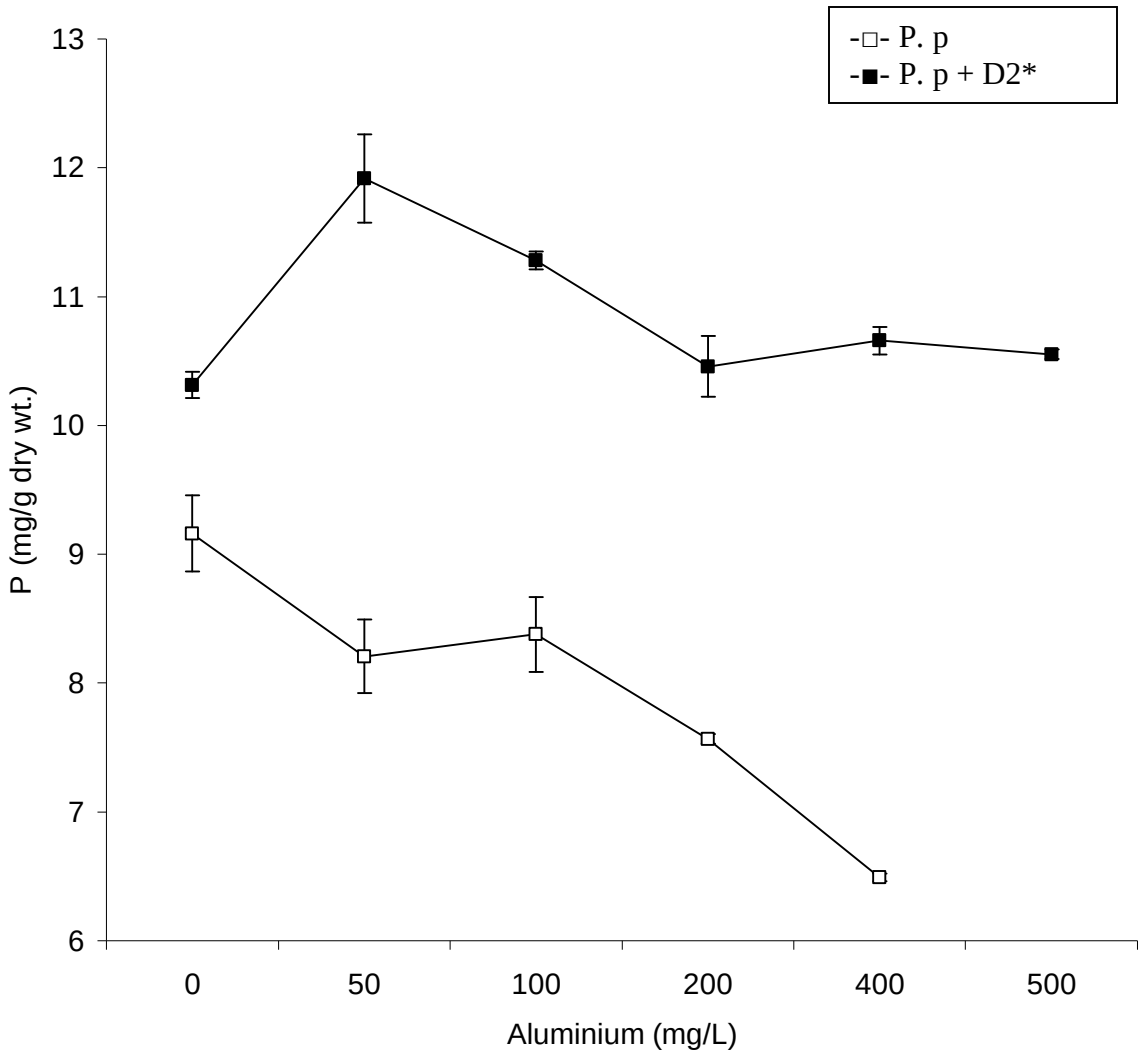

Figure: 6.25 Influence of Aluminium concentrations on the Phosphorus content in the shoot of

P. pinaster plants inoculated with D2*. Mean ± SE.

Table: 6.25 Influence of Aluminium concentrations on the Calcium content (mg/g dry weight)

in shoot of *P. pinaster* (P.p) plants inoculated with D2*.

Al (mg/L)	*P. p*	*P.p +D2**
0	**8.89 ± 0.44**	**10.92 ± 0.34**
50	**8.86 ± 0.49**	**10.67 ± 0.29**
100	**8.06 ± 0.37**	**10.51 ± 0.03**
200	**7.07 ± 0.39**	**12.09 ± 0.42**
400	**6.60 ± 0.40**	**11.17 ± 0.34**
500	**0.00**	**10.73 ± 0.14**

Analysis of Variance

Source of Variation	*Df*	*Sum-of-squares*	*Mean square*	*F*
Interaction	**5**	**84.96**	**16.99**	**48.44*** **
Treatment	**1**	**177.0**	**177.0**	**504.5*** **
Concentration	**5**	**88.76**	**17.75**	**50.61*** **
Residual	**24**	**8.419**	**0.3508**	

*** P<0.001

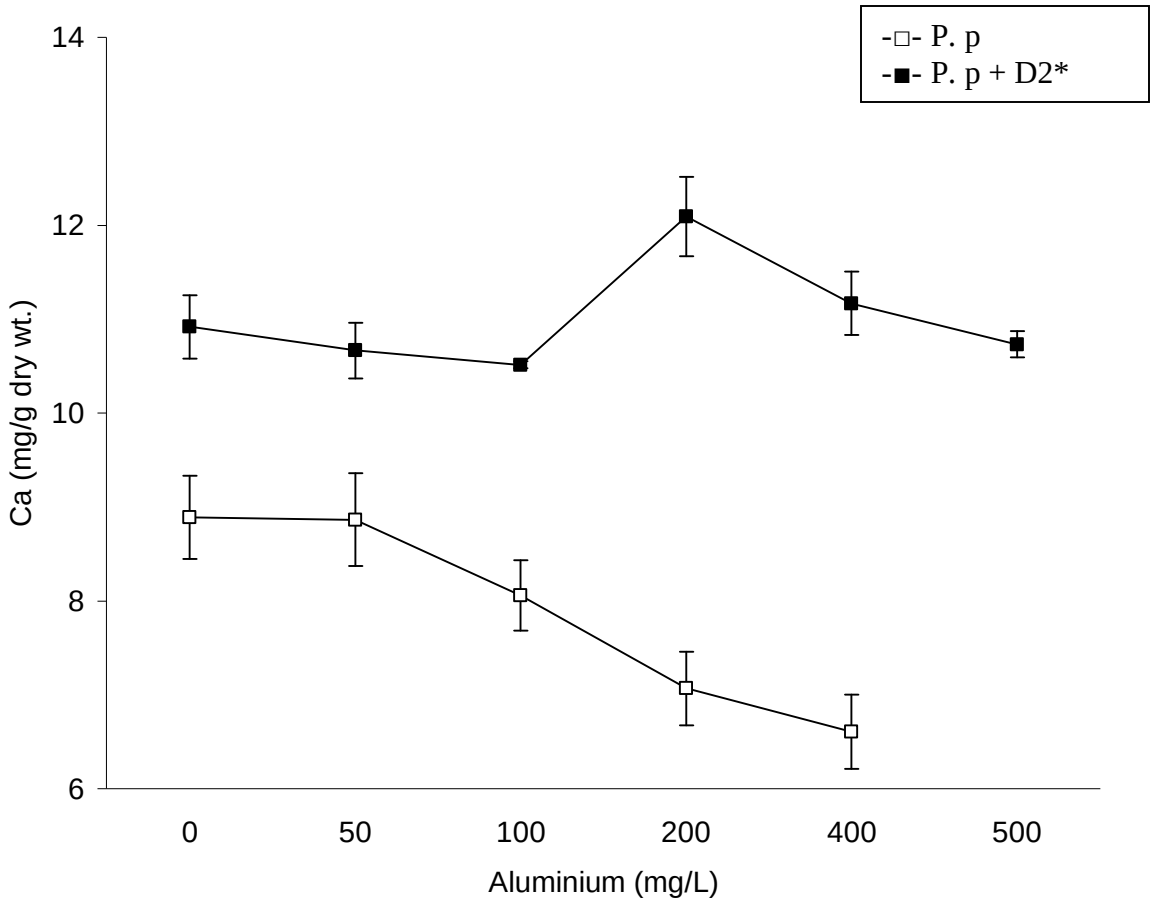

Figure: 6.26 Influence of Aluminium concentrations on the Calcium content in shoot of *P. pinaster* plants inoculated with D2*. Mean ± SE.

Table: 6.26 Influence of Aluminium concentrations on the Magnesium content (mg/g dry weight)

in shoot of *P. pinaster* (P.p) plants inoculated with D2*.

Al (mg/L)	*P. p*	*P.p +D2**
0	**3.04 ± 0.06**	**7.01 ± 0.09**
50	**3.18 ± 0.04**	**5.84 ± 0.34**
100	**2.61 ± 0.20**	**5.93 ± 0.06**
200	**2.57 ± 0.25**	**4.94 ± 0.23**
400	**2.27 ± 0.11**	**4.56 ± 0.23**
500	**0.00**	**4.06 ± 0.02**

Analysis of Variance

Source of Variation	*Df*	*Sum of squares*	*Mean Square*	*F*
Interaction	**5**	**4.665**	**0.9331**	**10.71*** **
Treatment	**1**	**87.11**	**87.11**	**999.6*** **
Concentration	**5**	**33.05**	**6.611**	**75.86*** **
Residual	**24**	**2.092**	**0.087**	

*** P<0.001

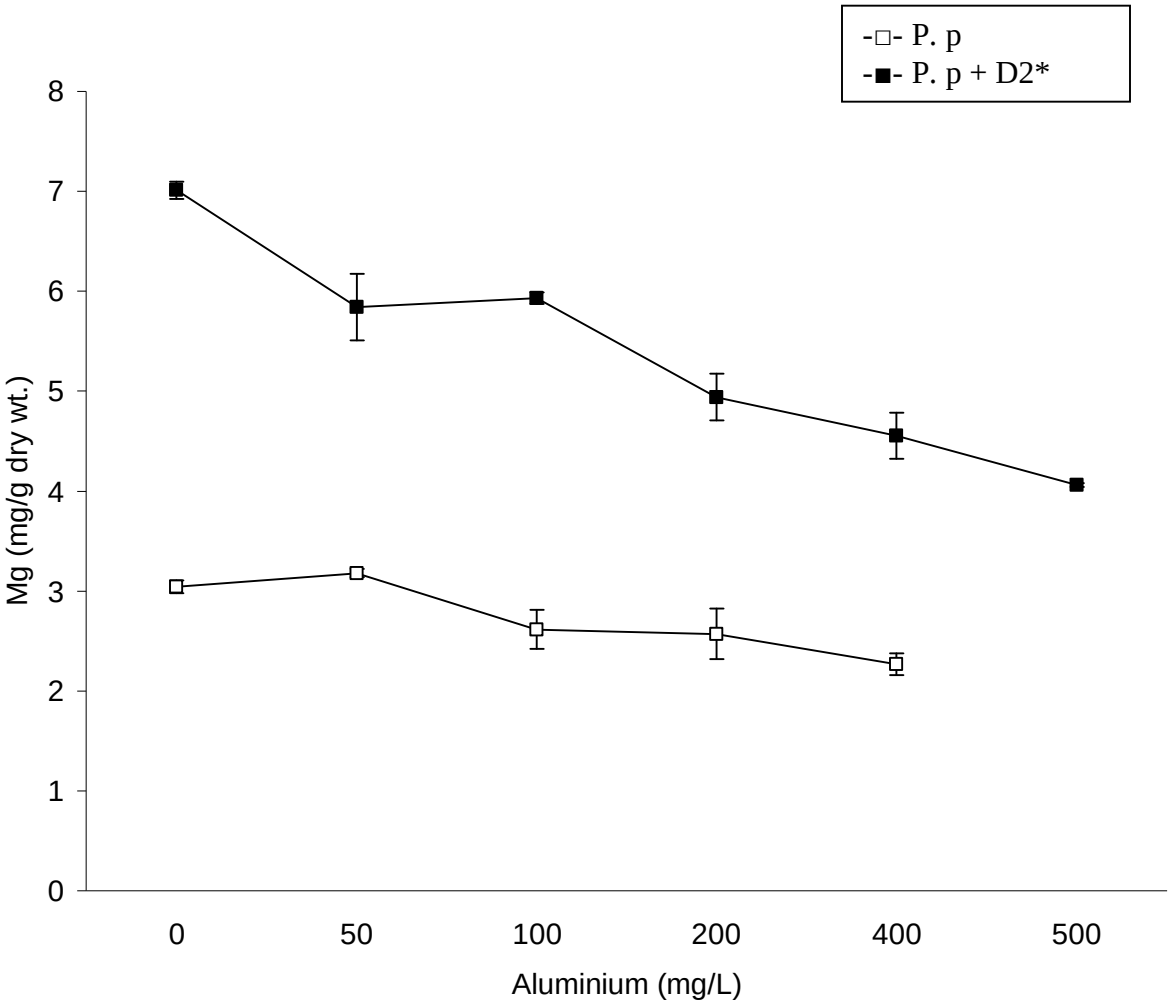

Figure: 6.27 Influence of Aluminium concentrations on the Magnesium content in shoot of

P. pinaster plants inoculated with D2*. Mean ± SE.

Table: 6.27 Influence of Aluminium concentrations on the Potassium content (mg/g dry weight)

in shoot of *P. pinaster* (P.p) plants inoculated with D2*.

Al ***(mg/L)***	*P. p*	*P.p +D2**
0	**21.71 ± 0.73**	**32.16 ± 0.88**
50	**19.46 ± 0.64**	**29.05 ± 1.35**
100	**18.29 ± 0.60**	**27.62 ± 1.85**
200	**17.45 ± 0.72**	**24.72 ± 2.93**
400	**16.75 ± 1.48**	**23.94 ± 2.60**
500	**0.00**	**16.16 ± 1.22**

Analysis of Variance

Source of Variation	***Df***	***Sum-of-squares***	***Mean square***	***F***
Interaction	**5**	**81.22**	**16.24**	**2.419 ns**
Treatment	**1**	**899.8**	**899.8**	**134.0*** **
Concentration	**5**	**1297**	**259.3**	**38.62*** **
Residual	**24**	**161.2**	**6.715**	

*** P<0.001

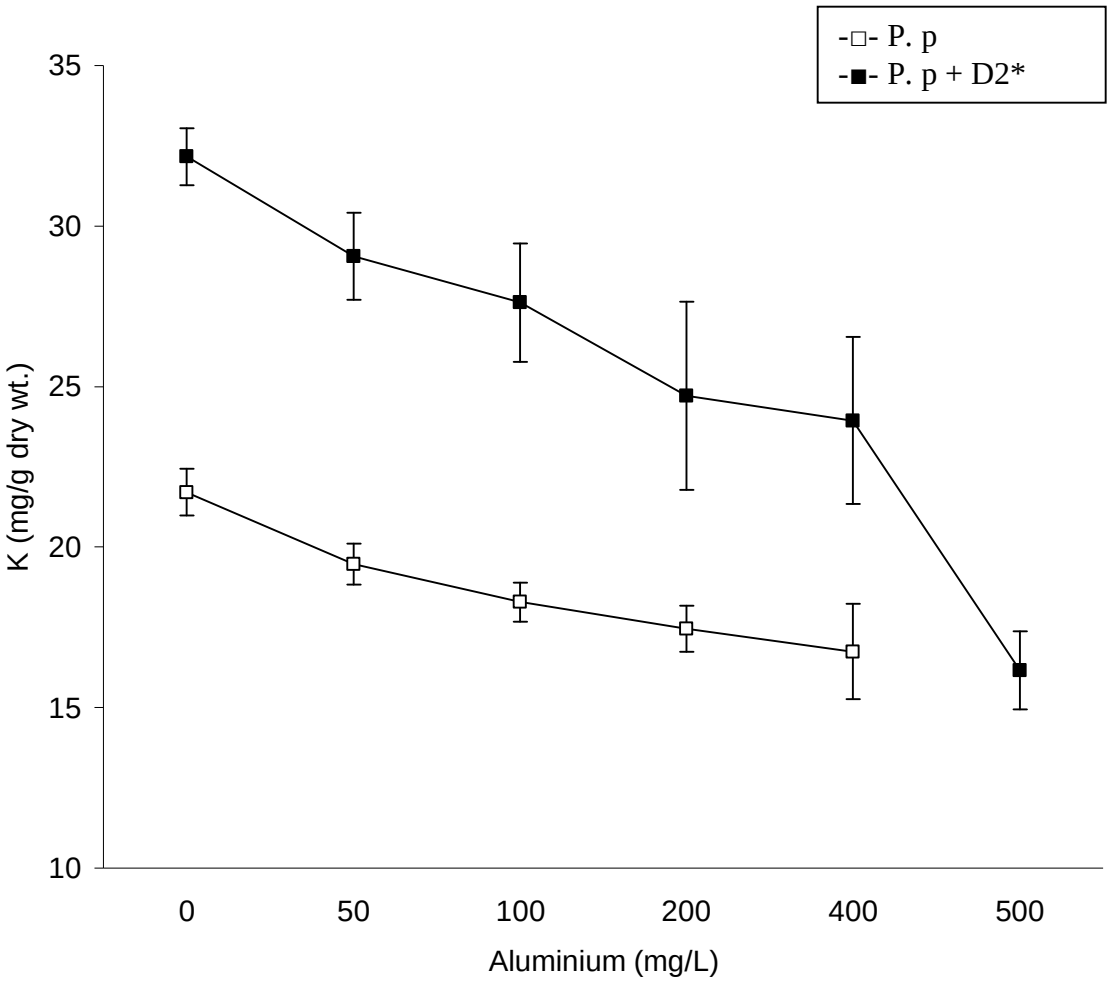

Figure: 6.28 Influence of Aluminium concentrations on the Potassium content in shoot of *P. pinaster* plants inoculated with D2*. Mean ± SE.

The results of *in vitro* plant- fungus interaction studies at different concentrations of aluminium suggested that the inoculation of ectomycorrhizal fungus significantly improved the growth and mineral uptake of *P. deltoides*, *E. tereticornis* and *P. pinaster* plants compared to the nonmycorrhizal plants in the same conditions. The results showed significant increase in shoot height, shoot and root dry weight of ectomycorrhizal plants than nonmycorrhizal plants. The ectomycorrhizal plants had significantly higher mineral content (Ca, Mg, K and P) in their shoots compared to nonmycorrhizal plants at the same aluminium concentration and accumulated less amount of aluminium in its plant tissues.

The lesser accumulation of aluminium and improved mineral uptake could be the reason of better adaptability of ectomycorrhizal plants as compared to nonmycorrhizal plants when grown in presence of aluminium.

Chapter VII

Influence of *P. tinctorius* (Pt-NIC) on the growth and mineral nutrition of *E. tereticornis* seedlings grown in bauxite mined out soils.

In order to select a fungus for high soluble aluminium conditions, the fungus must show rapid growth and root colonization so that the toxic effects brought about by aluminium may be ameliorated quickly. The results of the *in vitro* experiments showed that *P. tinctorius* (Pt-NIC) was the most aluminium tolerant ectomycorrhizal fungus with no apparent decrease in biomass till 5000 mg/L of aluminium concentration and was capable of forming ectomycorrhizas with *E. tereticornis* plants. The Pt-NIC isolate also colonized 65.6% of the lateral roots of *E. tereticornis* plants *in vitro*. So, Pt-NIC isolate along with its host plant *E tereticornis* was selected for the nursery experiment. The fungus was inoculated to *E. tereticornis* plants grown in bauxite mined out soil. The plants were grown in bauxite mined out soil and inoculated with ectomycorrhizal fungus Pt-NIC (Test) (Figure 7.1 and 7.2). The plants were harvested after 15 weeks of growth in nursery (Figure 7.3) and were assessed for its growth parameters such as plant height, shoot and root dry weights and mycorrhizal colonization. The shoots and the roots of the plant were separately analyzed for their mineral contents.

7.1 Plant growth

The ectomycorrhizal plants showed better growth and survival when compared to uninoculated plants in bauxite mined out soil. Pt-NIC formed ectomycorrhizas (Figure 7.4) with the roots of *E. tereticornis* plants and

colonized 57.8% of the lateral roots. After 15 weeks of plant growth the mean plant height of ectomycorrhizal plants was 26.61 cm which was significantly higher than the mean plant height of nonmycorrhizal plants i.e. 17.37 cm (Table 7.1).

Figure 7.1: *Eucalyptus tereticornis* plants in nursery condition at initial stage.

Figure: 7.2 *Eucalyptus tereticornis* plants inoculated with *Pisolithus tinctorius* (Pt-NIC) at

initial stage.

Figure: 7.3 Effect of Pt-NIC on the growth of *E .tereticornis* plantlets grown for 15 weeks in

bauxite mined out soil.

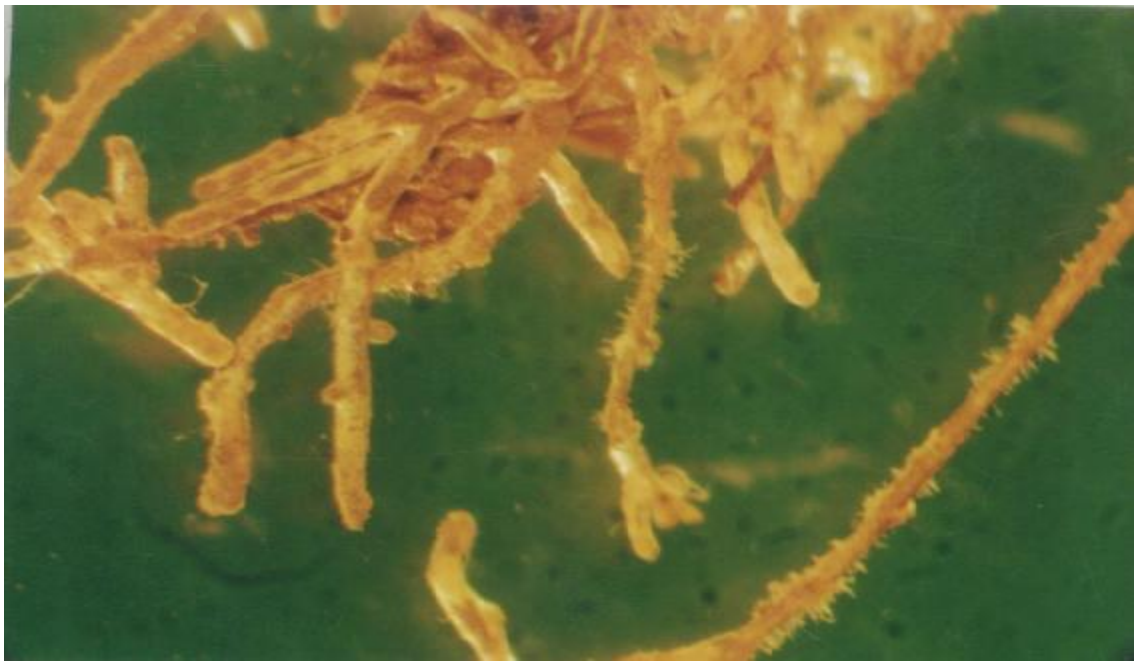

Figure: 7.4 Ectomycorrhizas of *E. tereticornis* colonized by *Pisolithus tinctorius* (Pt-NIC)

Table 7.1 Effect of Pt-NIC on the growth of *E .tereticornis* plants grown in bauxite mined out soil. Mean value ± SE

	Nonmycorrhizal Plants	*Mycorrhizal Plants*
Shoot height **(in cms)**	**17.37**	**26.61 ***
Shoot dry weight **(in gm)**	**0.56**	**1.10 ***
Root dry weight **(in gm)**	**0.13**	**0.33 ***
S/R ratio	**4.38 ***	**3.31**
% mycorrhization	**0.0**	**57.80 ***

* significant at $P<0.05$ as determined by t- test.

The mean dry weights of ectomycorrhizal plants were 1.10 g per plant for the shoot and 0.33 g per plant for the root which was significantly more than 0.56 g per plant for the shoot and 0.13 g per plant for the root in case of nonmycorrhizal plants (Table 7.1). The shoot/ root ratio was significantly lower for the ectomycorrhizal plants (3.31) than for nonmycorrhizal plants (4.38) suggesting the better growth of Pt-NIC inoculated plants of *E. tereticornis* (Table 7.1). The root development was significantly more in ectomycorrhizal plants than for nonmycorrhizal plants as the roots of nonmycorrhizal plants showed very poor growth.

7.2 Metal concentration in plant tissues

7.2.1 Shoots

The ectomycorrhizal plants had lesser shoot aluminium concentration when compared to nonmycorrhizal plants (Table 7.2). The ectomycorrhizal plants had 0.21 mg Al/ g shoot dry weight which was significantly less as compared to 0.31 mg Al/ g shoot dry weight in nonmycorrhizal plants.

7.2.2 Roots

The presence of ectomycorrhizal fungi significantly altered aluminium concentrations in the roots (Table 7.2). The roots of ectomycorrhizal plants accumulated less of aluminium (9.43 mg/ g dry weight) than the roots of nonmycorrhizal plants (24.99 mg/g dry weight). The lesser accumulation of aluminium was probably due to the secretion of organic acids by the ectomycorrhizal fungus to chelate free aluminium ions present in the soil

rather than accumulating it in the plant root as evident from the results of the root analysis.

7.3 Nutrient element concentrations in plant tissues:

7.3.1 Shoots

The inoculation of ectomycorrhizal fungus led to the alterations in the contents of nutrient elements (Table 7.2). Uptake of calcium and potassium was significantly enhanced in the shoots of ectomycorrhizal plants compared to nonmycorrhizal plants while there was no significant change observed in the magnesium and phosphorus content of the plant shoots.

7.3.2 Roots

The roots of ectomycorrhizal plants showed increased uptake of calcium, magnesium, potassium and phosphorus than nonmycorrhizal plants but the results were not significant (Table 7.2).

Table 7.2 Effect of Pt-NIC on the mineral content of *E .tereticornis* plants grown in bauxite mined out soil. Mean value ± SE

	Nonmycorrhizal Plants		*Mycorrhizal Plants*	
	Shoot	Root	Shoot	Root

Ca **(mg/g dry wt.)**	**2.46**	**2.54**	**3.48 ***	**3.61**
Mg **(mg/g dry wt.)**	**1.50**	**1.58**	**1.46**	**2.18**
K **(mg/g dry wt.)**	**7.37**	**3.80**	**8.46 ***	**4.21**
P **(mg/g dry wt.)**	**0.37**	**0.34**	**0.37**	**0.41**
Al **(mg/g dry wt.)**	**0.31***	**24.99 ***	**0.21**	**9.43**

The astriek (*) showed the significant difference at $P<0.05$ as determined by t- test.

Table 7.3 Effect of Pt-NIC on the soil physico-chemical properties after harvesting the plants grown for 15 weeks.

	Initial Soil	*Soil after harvesting plants*	
		Nonmycorrhizal	***Mycorrhizal***
pH of soil	**5.50c**	**5.85a**	**5.62b**
Total Organic C (%)	**0.40c**	**2.72b**	**4.19a**
Total N (%)	**0.06a**	**0.07a**	**0.06a**
Total P **(mg/kg)**	**469.3a**	**457.2a**	**458.6a**
Available P **(mg/kg)**	**0.49c**	**1.74b**	**2.54a**
Mg (**mg/g)**	**0.88b**	**1.99a**	**3.34a**
K **(mg/g)**	**1.40a**	**0.99b**	**1.24ab**
Ca **(mg/g)**	**0.62b**	**1.41a**	**1.68a**
Al **(mg/g)**	**168.2a**	**109.8c**	**125.6b**

Values sharing a common letter in the row are not significant at $P<0.05$

Elemental analysis of soil

The soil collected after harvesting the plants was analyzed for its elemental composition (Table 7.3). The total organic carbon content of the soil increased to 4.19 % (approx 10 times as compared to mined out soil) in ectomycorrhiza plants. Similarly, ectomycorrhizal fungi inoculated soil was found to be rich in available phosphorus content in soil. The available phosphorus increased significantly from 0.493 mg/kg soil to 2.543 mg/kg soil in ectomycorrhiza soil and to the extent of 1.394 mg/kg soil in nonmycorrhizal soil. There was no significant change observed in total Nitrogen, total Phosphorus, Calcium, Magnesium and Potassium content of the soil initially and also after harvesting the plants. The aluminium content was more in ectomycorrhiza inoculated soil 125.605 mg/g soil than uninoculated soil 109.775 mg/g soil but was less than initially found in bauxite mined out soil. These results indicate that ectomycorrhizal fungi play an important role in protecting the plants from its detrimental effects in aluminium contaminated soils. The presence of ectomycorrhizal fungi increased root branching and consequently, the plant root absorbing area to improve the nutritional status of the plant and could increase the availability of limiting nutrients. The soil become rich in the key nutrients which are lacking in bauxite mined out soils such as total organic carbon and available phosphorus.

Overall the results of nursery experiment showed the improvement of plant growth with the inoculation of Pt-NIC which could be used for the successful establishment of *E. tereticornis* plants in bauxite mined out soil.

Chapter VIII
DISCUSSION

Ectomycorrhizal fungi play an important role in enhancing uptake of mineral nutrients for many plant species (Smith and Read, 1997). Accumulation of heavy metals in soils could adversely affect formation and development of ectomycorrhizas of tree species growing in contaminated soils (McCreight and Schroeder, 1982). Soil acidification and aluminium toxicity may be important factors for the decline in the vitality of many forest trees and associated ectomycorrhizal fungal flora. Some ectomycorrhizal fungi can occupy industrial degraded habitats, which are often contaminated by heavy metals such as lead, zinc, cadmium, copper, nickel and aluminium. The isolation of metal tolerant ectomycorrhizal fungi from polluted sites has been well documented (Brown and Wilkins, 1985; Jones and Hutchinson, 1986; Colpaert and van Assche, 1987; Egerton-Warburton and Griffin, 1995). It is uncertain whether the presence of ectomycorrhizal fungi is related to their capacity to evolve metal tolerance when growing on metal contaminated soils (Colpaert and van Assche, 1992, 1993; Egerton-Warburton and Griffin, 1995) or whether they have an innate metal tolerance through out their different ecotypes (Denny and Wilkins, 1987). To confer an increase in metal tolerance to the host, screening of ectomycorrhizas prior to inoculation may be advantageous in selecting isolates with high tolerance to specific metals. Axenic screening provides a rapid evaluation of metal tolerance in ectomycorrhizal fungi and has demonstrated differential tolerances to metals

(McCreight and Schroeder, 1982; Jones and Hutchinson, 1986; Reddy *et al*., 2002) There are evidences that significant intra and interspecific variations exists in the metal sensitivity of ectomycorrhizal fungi (Brown and Wilkins, 1985; Jones and Hutchinson, 1988; Egerton-Warburton and Griffin, 1995; Hartley *et al*., 1997). There may, however be no correlation between metal tolerance of mycorrhizal fungi and the toxicity of the soil origin (Jones and Hutchinson, 1988). Thus it appears that, in many cases soil fungi have not developed metal tolerant ecotypes and any screening programmes should test ectomycorrhizal fungi from a range of sites.

Responses of ectomycorrhizal fungi to toxic metals are of importance in view of interest in the reclamation of polluted sites and influence on plant growth and productivity. Although there appears to be a wide diversity in response between plant- fungal combinations, it has been suggested that tolerance of the mycobiont may be an important factor in conferring plant tolerance (Colpaert and van Assche, 1987). Though the effects of aluminium on plants are well known, data on the toxicity of aluminium to mycorrhizal fungi are scarce. Ectomycorrhizal fungi exhibit differential response to aluminium (Thompson and Medve, 1984; Zel and Bevc, 1993; Jones and Muehlchen, 1994; Tam, 1995; Egerton-Warburton and Griffin, 1995; Marschner *et al*., 1999; Reddy *et al*., 2002). Different ectomycorrhizal fungi were tested for their ability to tolerate various concentrations of aluminium in this study. Gadd (1983) reported the disadvantages of using agar media to assess the sensitivity of ectomycorrhizal fungi to metals, as it forms complexation with

metals and alter the metal availability to fungi. Hartley *et al*. (1997) suggested that fungal biomass production determined in liquid media provide a more accurate assessment of metal sensitivity, as it is independent of growth form. Hence, in the present study, the fungi were grown in liquid medium amended with different concentrations of aluminium. The addition of aluminium reduced the pH of the medium drastically from 5.5 to 3.17. To check whether the reduction in growth of ectomycorrhizal fungi in aluminium amended medium is due to the decline of pH or presence of aluminium, the fungi were grown in the medium having the pH of 3.5 and compared with the growth of ectomycorrhizal fungi at pH 5.5. The results showed that the growth was not inhibited at lower pH as compared to pH 5.5, indicating the fact that inhibition of growth was due to the presence of aluminium. Jongbloed and Borst-Pauwels (1992) studied the influence of aluminium and pH on growth of ectomycorrhizal fungi in liquid culture and found that *Lactarius rufus* and *L. hepaticus* are tolerant to low pH but sensitive to aluminium and *Laccaria bicolor* exhibited high aluminium tolerance and high sensitivity to acidification. The growth of ectomycorrhizal fungi decreased as the concentration of aluminium increased in the growth medium in many ectomycorrhizal fungi in the present study. The growth of Pt-KN6, Pt-P and h-1 isolates was inhibited more than 50% at 200 mg/L of aluminium, whereas the growth of Pt-NIC was not significantly affected.even at 5000 mg/L. P.I-MAR and Pt-N showed tolerance with no significant reduction in growth till 2000 mg/L of aluminium concentration. Reports are available on different

ectomycorrhizal fungi such as *Coenococcum geophilum, Pisolithus tinctorius, Suillus luteus, S. variegates, Thelephora terrestris, Laccaria laccata* and *Canthrellus cibarius* on aluminium tolerance (Thompson and Medve, 1984; Jones and Muehlchen, 1994; Tam, 1995; Egerton-Warburton and Griffin, 1995; Reddy *et al*., 2002). Among these fungi *P. tinctorius* isolated from old coal mining area has been reported to grow upto 2000 mg/L of aluminium (Egerton-Warburton and Griffin, 1995). In the present study Pt-NIC is able to grow upto 5000 mg/L without any reduction in the growth. Infact the growth is stimulated till 750 mg/L of aluminium as compared to non-aluminium amended medium. These results indicate that Pt-NIC showed high tolerance followed by Pt-N and P.I-MAR. The other ectomycorrhizal fungi Pt-P, Pt-KN6 and h-1 seemed to be very sensitive to aluminium.

Mycorrhizal fungi are known as great accumulators of heavy metals (Byrne *et al*., 1976). Reports are available indicating that the increase of aluminium concentration in the growth medium increased its accumulation in the mycelium of ectomycorrhizal fungi. In the present study, the accumulation of aluminium in the mycelium increased with increasing concentrations of aluminium in the culture medium. Pt-NIC was more tolerant to aluminium than Pt-P, Pt-N, Pt-KN6, P.I-MAR and h-1 and accumulated maximum aluminium in its mycelium. Although there was no statistically significant correlation between the growth inhibition and aluminium accumulation in mycelia observed, these results suggest that the higher resistance to aluminium of Pt-NIC in pure culture might be ascribed to a better ability to

regulate aluminium absorption and/or accumulation whereas other fungi would have a lower ability to regulate these processes. These results were in accordance with the findings of Egerton-Warburton and Griffin (1995) who reported that rehabilitated and forest site isolates accumulated aluminium at substrate concentration greater than 1 mg/L Al, with highest accumulation between 12 and 22 mg/L of aluminium (these substrate concentrations corresponded with the limits of mycelial growth). In contrast, the aluminium tolerant mine site isolate only accumulated aluminium when substrate concentrations exceeded 90 mg/L of aluminium. Zel and Bevc (1993) also reported the high accumulation of aluminium in *Lactarius piperatus* compared to *Amanita muscaria* when grown in presence of different concentrations of aluminium. Cumming *et al*. (2001) reported the accumulation of aluminium increased with media aluminium concentration and *P. tinctorius* accumulating four times more aluminium in its mycelium as compared to *Laccaria bicolor*. Reddy *et al*., (2002) reported *Canthrellus cibarius* was more tolerant to aluminium than *P. tinctorius* but accumulating much less quantity of aluminium in its mycelium.

Aluminium influences the mineral nutrition of ectomycorrhizal fungi, generally manifested by imbalances or deficiencies of phosphorus, calcium, magnesium and potassium (Zel and Bevc, 1993; Jongbloed- Borst-Pauwels, 1992; Egerton-Warburton and Griffin, 1995; Reddy *et al*., 2002). In assessment of aluminium tolerance, the response of the fungus may not always occur as a direct effect of the metal, but as a consequence of

aluminium related impairment of biochemical and physiological functions (Egerton-Warburton and Griffin, 1995). In all the ectomycorrhizal fungi, aluminium toxicity was associated with impaired mineral nutrition but the effect varied depending on the fungus. In the present study, we found that at lower concentrations of aluminium, the phosphorus, calcium, magnesium and potassium levels was either increased or not significantly affected while there was significant decrease in phosphorus, calcium, magnesium and potassium content at higher concentrations of aluminium. Zel and Bevc (1993) reported high aluminium concentrations increased accumulation of calcium but induced a reduction of potassium, phosphorus and magnesium level in the mycelium of *Amanita muscaria* and *Lactarius piperatus.* Jongbloed and Borst-Pauwels (1992) reported that potassium uptake was not affected in *Laccaria bicolor* and *Lactarius rufus*, whereas it was reduced by aluminium in *Lactarius hepaticus*. The latter may point to the reduction in negative surface potential by aluminium of the fungus. Egerton-Warburton and Griffin (1995) also detected increased levels of magnesium and calcium in aluminium tolerant isolates and reduced levels in less tolerant isolates of *Pisolithus.* It has been suggested that the toxic effects of aluminium are associated with the Al/Ca ratio, since this ratio governs the binding of aluminium to membranes and subsequently, the toxic effects of this metal on cell function (Rengel, 1992). According to Alva *et al*. (1986) and Kinraide and Parker (1987), magnesium may function in a manner analogue to calcium. This was confirmed by Jongbloed and Borst-Pauwels (1992) who reported an

improvement in aluminium tolerance of *L. rufus* when grown in a medium supplemented with additional magnesium. They also reported that phosphorus uptake was greatly reduced by aluminium due to complexation of aluminium with phosphates and the toxic effects of aluminium were alleviated by the addition of phosphorus. Cumming *et al.*, (2001) reported that the aluminium affected the accumulation of calcium, magnesium and iron in *Pisolithus tinctorius* and *Laccaria bicolor* at concentrations as low as 50 µM. Reduction in calcium and magnesium accumulation may be the result of Donnan interactions in which aluminium binding limits their adsorption to cell walls (Kinraide *et al.*, 1994). The uptake of these cations into the cytoplasm may also be reduced by aluminium, since aluminium blocks Ca^{2+} channels and affects Mg^{2+} transport proteins in the plasma membrane (Rengel and Robinson, 1989; Huang *et al.*, 1992).

In the present study, fungal acid phosphatase activity increased in aluminium treated mycelia of ectomycorrhizal fungi. Reddy *et al.* (2002) reported increased acid phosphatase activity of *Pisolithus sp.* and *Canthrellus cibarius* in presence of aluminium at lower concentration (100 mg/L) and decreased level at high aluminium concentration (200 mg/L) in the medium. Since one of the main effects of aluminium on phosphate in the soil solution is that the freely available phosphate concentration will decrease due to the complexing of phosphate with Al^{3+}, this may be due to the Pi limitation in the presence of aluminium. It may also correspond to a more general stress resistance process, since acid phosphatase of ectomycorrhizal fungi can also be induced by

cadmium (Turnau and Dexheimer, 1995) and can play a role in heavy metal detoxification (Turnau *et al*., 1994). The results recorded in the present study where increased acid phosphatase activity was associated with phosphorus accumulation in the mycelia are in line with the above hypothesis. This hypothesis is in agreement with reports by Gerlitz (1996) who detected higher levels of phosphorus uptake and mobile polyphosphate (a strong aluminium chelator) concentration in *Suillus bovinus* in the presence of aluminium and suggested that Al-'stressed' fungal cells produce polyphosphate to counteract mobile Al^{3+} ions within hyphae. Egerton-Warburton *et al*. (1995) also noted elevated aluminium and phosphorus levels in *Pisolithus tinctorius* sheaths that were associated with limited transfer of aluminium into the cortex and stele of *Eucalyptus rudis*. This pattern may also reflect the formation of Al-polyphosphate complexes in fungal tissues as noticed by Gerlitz (1996). The results recorded in the present study showed that aluminium tolerance of ectomycorrhizal fungi in pure culture involves complex nutritional interactions, which are probably variable depending on the fungus. Pt-NIC is the highly aluminium tolerant ectomycorrhizal fungi and accumulated maximum of aluminium and potassium in its mycelium.

8.2 Isolation of aluminium tolerant mutants and their mineral nutrition study in pure culture

Ectomycorrhizas have the potential to be used as bioindicators of pollution in forest monitoring programs because they are the organs of nutrient uptake and

integral part of the forest ecosystem that react sensitively to environmental changes. Alterations in ectomycorrhizas for their sensitivity to adverse conditions are important for the vitality and health of the trees. In the present study, to increase the efficacy of ectomycorrhizal fungi to tolerate high concentrations of aluminium, the mutants were developed by exposing the mycelia/protoplasts to physical mutagen as U.V light. The mutants through protoplasts were carried out only for the ectomycorrhizal fungus *H. cylindrosporum* (h-1) as the regeneration was reported only in this genus where as the other fungi used in this study (*Pisolithus tinctorius, P. albus* and *Paxillus involutus*) failed to regenerate from the protoplasts. Barrett *et al*. (1989) reported that the protoplasts produced from *Coenococcum geophilum* and *Pisolithus tinctorius* could not be regenerated because of the absence of nuclei. The mycelia of both fungi were composed of predominantly wide hyphae (>3 μm) with thickened and often pigmented and/or encrusted cell walls. The production of viable protoplasts has been reported for a limited number of ectomycorrhizal species such as *Laccaria bicolor* (Kropp and Fortin, 1986), *Hebeloma cylindrosporum*, *H. edurum*, *H. sinapizans*, *Suillus bellinii* (Hebraud and Fevre, 1988), *H. circinans* and *Laccaria laccata* (Barrett et al., 1989). In the present work, the protoplasts from *H. cylindrosporum* were isolated using cell wall lyting enzymes as Cellulase and Driselase and the protoplasts yield of 156 x $10^6/cm^3$ wet mycelium was recorded between 95-110 minutes of incubation in the lytic enzyme solution. The maximum regeneration frequency was recorded 1.2% for *H. cylindrosporum* as was also

reported by Barett *et al.* (1989). The protoplasts were irradiated for 60 seconds (corresponds to 90% killing of living protoplasts) and plated on aluminium amended regeneration medium. The emerging hyphae were dikaryotized with compatible monokaryon h-7 and the resultant aluminium tolerant isolate D2* was selected. The D2* showed increased tolerance to aluminium with Al-EC_{50} value < 750 mg/L, while the wild type D2 had Al-EC_{50} value

< 200 mg/L. Among different ectomycorrhizal fungi, only mutants of *H. cylindrosporum* have been reported and all mutations were induced only by U.V irradiation of protoplasts prepared from homokaryotic strains (Barrett *et al.*, 1989). There were not many reports available in the literature on the production of ectomycorrhizal mutants. The first report of mutant selection was on fungicide resistant mutant of *H. cylindrosporum* (Hebraud and Fevre, 1988). These mutations conferred resistance to fungicides such as benomyl, benodanil, carboxine or oxycarboxine. The second report available was on Indole acetic acid (IAA) overproducer mutant of *H. cylindrosporum* isolated and characterized by Durand *et al.* (1992). Till now, no attempts were made to develop the mutants which have high tolerance to heavy metals.

The fungal mycelium was irradiated in case of *Pisolithus tinctorius, P. albus* and *Paxillus involutus* for six minutes to U.V light and transferred to aluminium amended medium as the selection medium for the production of mutants. The irradiated mycelium of Pt-NIC*, Pt-N* and P.I-MAR* showed more sensitivity to aluminium after irradiation as compared to their wild type

isolates. Only, irradiated Pt-P* showed increased aluminium tolerance after irradiation as wild type Pt-P had Al-EC_{50} value < 200 mg/L , while the irradiated Pt-P* had Al-EC_{50} value < 3000 mg/L. The mutants developed by these procedures were compared with their respective wild type fungi for aluminium tolerance. Among the different irradiated ectomycorrhizal fungi (mutants), only Pt-P* and h-1* showed an increased tolerance to aluminium when compared to their wild type isolates. Comparative analysis of mineral uptake (Ca, Mg, K and P) did not show any significant difference. The uptake of aluminium by Pt-P* and D2* was lower when compared to its wild type isolate at higher concentrations of aluminium and in other fungi there was no significant difference in aluminium accumulation between irradiated and the respective wild type isolate of ectomycorrhizal fungi. The lesser accumulation of aluminium by irradiated Pt-P* and D2* could be the reason of thir higher tolerance to aluminium as compared to their wild type isolates. The production of metal tolerant mutants of ectomycorrhizal fungi in this study is the first attempt and this method could serve as a biological model to develop mutants for a particular metal or multimetal tolerant isolates, which could be used for the remediation of metal contaminated sites.

8.3 Influence of aluminium on plant mineral nutrition in presence of mycorrhizae *in vitro*

Ectomycorrhizal fungi are not present in the environment in a free-living state, but exist in a symbiosis with a host plant. The investigations of ectomycorrhizal fungal metal sensitivity using synthesized ectomycorrhizal

seedlings may provide a more realistic picture of the physiological responses. The majority of symbiosis studies however, have been carried out using artificial substrates such as peat+ vermiculite, soilrite, perlite or acid washed sand. Such studies are very useful for comparing the sensitivity of different species and isolates (Hartley, 1997). Ectomycorrhizal fungi can increase plant tolerance to toxic metals (Jones and Hutchinson, 1986; Dixon, 1988; Dixon and Buschena, 1988) by accumulating metals in the extrametrical hyphae (Galli *et al.*, 1994) and extrahyphal slime (Tam, 1995) thereby reducing uptake into the plant (Marschner and Dell, 1994). In the present study three host plants *Eucalyptus tereticornis, Populus deltoides* and *Pinus pinaster* were used along with the ectomycorrhizal fungi *Pisolithus* species, *Paxillus involutus* and *Hebeloma cylindrosporum* respectively. Reddy and Satyanarayana (1998a, b) reported the ectomycorrhizal formation in micropropagated plantlets of *P. deltoides* and *E. tereticornis* and found that *P. involutus* and *P. tinctorius* respectively formed significantly more mycorrhizas than the other ectomycorrhizal fungal isolates. The ectomycorrhizal and nonmycorrhizal plants were exposed to the different concentrations of aluminium in the soilrite - vermiculite substrate. The results showed that aluminium adversely affected the growth of both mycorrhizal and nonmycorrhizal seedlings. But inoculation with ectomycorrhizal fungi improved the growth of the plantlets when compared to uninoculated plantlets in presence of different concentrations of aluminium. Cumming and Weinstein (1990a) investigated the ability of mycorrhizal fungus *P. tinctorius*

to modulate aluminium toxicity in Pitch pine (*Pinus rigida*) and Hentschel *et al.* (1993), investigated the ability of Norway Spruce (*Picea abies*) colonized by *Paxillus involutus* and concluded that mycorrhizae increase aluminium tolerance. Research in the response of trees to heavy metals indicate that mycorrhizas can protect trees from the detrimental effects of these metals (Brown and Wilkins, 1985; Dixon and Buschena, 1988; Jones and Hutchinson, 1988). Schier and McQuattie (1995) reported that aluminium significantly decreased shoot and root growth of *Pinus strobus* seedlings but had less effect on *Pisolithus tinctorius* inoculated ectomycorrhizal seedlings of white Pine (*Pinus strobus*). Further, foliar symptoms of aluminium toxicity (chlorosis and tip necrosis) were more pronounced in nonmycorrhizal seedlings than ectomycorrhizal seedlings of *P. strobus*. The same results were also obtained by Aggangan *et al.* (1998) that *Pisolithus sp.* isolated from heavy metal contaminated site was effective in promoting *Eucalyptus urophylla* seedling total biomass in nickel amended soils. Improved shoot growth and lower shoot/root ratios were known responses to mycorrhizal colonization (Cline and Reid, 1982; Cumming and Weinstein, 1990a,b; Shafer *et al*., 1985; Shaw *et al*., 1982). In this study, plant roots were susceptible to aluminium damage, the inoculation of ectomycorrhizal fungi enabled the roots to grow normally when compared to short and stubby roots observed in nonmycorrhizal plants. Percent mycorrhizas fluctuated with increasing concentrations of aluminium. There was a significant decrease in the mycorrhizal colonization with increasing concentrations of aluminium in

inoculated plantlets as also reported by Kasuya *et al.* (1990) in *Pinus caribea* inoculated with *Pisolithus tinctorius, Suillus sp., Rhizopogon sp.* and *Hebeloma sp.* The presence of growing fungus appeared to ameliorate aluminium toxicity and with time it appeared that either plant itself or the growing fungus was able to bind Al^{3+} ions, mediate the rhizospheric pH or the substrate or the root exudates complexed the available aluminium so that the element was no longer a problem.

Ectomycorrhizal fungi confer metal tolerance by binding metals to electronegative sites on the cell walls of the hyphae or binding to phosphates and sulfhydral compounds within the cells (Galli *et al*., 1994; Godbold *et al*., 1988). The ability of an ectomycorrhizal fungus to restrict uptake of aluminium by the host plant appears to have a strong bearing on whether or not mycorrhizal colonization results in aluminium tolerance. Amelioration of aluminium toxicity by mycorrhizal colonization of seedlings was greater in shoots than in roots. Hentschel *et al*. (1993) also observed this response to aluminium in Norway spruce (*Picea abies*) colonized by *P. involutus*. In our study the aluminium content in the shoots was significantly less in inoculated plantlets when compared to uninoculated plantlets in presence of different concentrations of aluminium. Aluminium in the control plantlets might have originated from the soilrite or/and Heller's micronutrient solution which contained aluminium as a micronutrient. These results are in line with the results of Schier and McQuattie (1995) who detected significantly lower aluminium accumulation in ectomycorrhizal seedlings of *Pinus strobus* as

compared to nonmycorrhizal seedlings. Brunner and Frey (2000) also reported the distinct aluminium accumulation in the Hartig net and cell wall of the cortex of ectomycorrhizal roots of Norway spruce seedlings colonized by *Hebeloma crustiliniforme* exposed to high levels of aluminium (10mM of Al^{3+}) suggesting its reduced translocation to the shoot portion of the plant. Kieliszewska-Rokicka *et al*. (1998) reported that *Suillus luteus* caused lower Al^{3+} translocation to the upper part of the tested seedling of *Pinus sylvestris* compared with the nonmycorrhizal control and there was no growth reduction of mycorrhizal seedlings by the Al^{3+} concentration of 4.0 mM used. Amelioration of aluminium-induced reduction in growth of seedlings by mycorrhizal colonization may have resulted from reduced uptake of aluminium or to the enhanced uptake of nutrients (Hentschel *et al.,* 1993; Wilkins, 1991). Mycorrhizal fungi influence the uptake of mineral elements from the soil (Harley and Smith, 1983) and the possible mechanisms by which aluminium might disrupt cellular functions is Al-induced mineral deficiencies. In the present study the results showed that the presence of aluminium significantly reduced the uptake of different mineral nutrients such as P, Ca, Mg and K in both ectomycorrhizal and nonmycorrhizal plantlets, but the inoculation of ectomycorrhizal fungi to plantlets significantly improved its mineral content in presence of different concentrations of aluminium. These results were in accordance with the results of Schier and McQuattie (1995) who reported the concentration of phosphorus was more than twice in needles of ectomycorrhizal *Pinus strobus* seedlings than in nonmycorrhizal seedlings

at each aluminium treatment level. In the present study, significantly decreased levels of calcium and magnesium were observed in aluminium treated plantlets. The results were in favour of the hypothesis that aluminium displaces calcium and magnesium from the apoplast of root cortex cells (Godbold *et al.*, 1988).

The calcium and magnesium uptake was most frequently reduced in different woody species under the influence of aluminium (Godbold *et al.*, 1988). From these results, it was concluded that aluminium drastically affected the mineral content of the plants. Mineral nutrition is one of the main functions of the fungal partner in mycorrhizal association so, the inoculation of ectomycorrhizal fungi could alleviate the Al-induced mineral impairment and thus protect the plant from the aluminium toxicity.

8.4 Influence of *P. tinctorius* (Pt-NIC) on the growth and mineral nutrition of *E. tereticornis* plants grown in bauxite mined out soils

The ectomycorrhizas can reduce metal concentrations in shoot tissues, although some fungi are inefficient and other affect growth or uptake alone. It has been suggested that tolerant behaviour of the mycobiont may be an important factor in conferring plant tolerance. Although there appeared to be a wide diversity in responses between different plant-fungus combinations, the amelioration of metal phytotoxicity by ectomycorrhizal fungi has been widely demonstrated. Most published reports suggested that the cell walls of extramatrical hyphae are the main binding sites for heavy metals in ectomycorrhizal fungi (Galli *et al.*, 1994). Other hypothesis for plant

protection against metal toxicity by ectomycorrhizal fungi concern the modification of the rhizosphere by the mycorrhizal fungus, the interactions between the heavy metals and anions, the precipitation of metal oxalates (Harley and Smith, 1983). Much work had been done on ameliorating the toxicity of aluminium by amending the different concentrations of aluminium in peat- vermiculite or acid washed sand/ silica but there was no report in the literature where the problem was actually dealt in contaminated soils. In the present study the most aluminium tolerant isolate *P. tinctorius* (Pt-NIC) was tested for its ability to improve the growth and survival of *E. tereticornis* plants grown in bauxite mined out soil. The plants were grown for 15 weeks in nursery conditions. The results showed better growth and survival of Pt-NIC inoculated *E. tereticornis* plants when compared to uninoculated plants. The shoot height, shoot and root dry biomass significantly increased in inoculated plants compared to uninoculated plants. The level of calcium and potassium increased significantly in shoot tissues of inoculated plants compared to uninoculated plants. The level of aluminium present in uninoculated plant roots was significantly higher than inoculated plant roots indicating that mycorrhizal fungi protected the plants from aluminium toxicity. Ectomycorrhizal fungus *P. tinctorius* is known for its wide distribution and broad host range (Marx, 1991). Strains of *Pisolithus* can tolerate a wide range of environmental stresses, such as high soil temperature (Marx, 1991), very acidic pH (Willenborg *et al*., 1990), low phosphorus soils

(Bougher *et al.*, 1990), drought resistance (Werener, 1992) and can increase plant tolerance to high levels of heavy metals.

In the present study, the physico-chemical properties of the soil after harvesting the plants were compared with the initial physico-chemical properties of the bauxite mined out soil and results showed the improvement of organic carbon and available phosphorous status of the soil in Pt-NIC inoculated soil than uninoculated soil. The soil inoculated with Pt-NIC had more aluminium than uninoculated soil probably the fungus had chelated aluminium in the soil by secreting organic acids rather than accumulating it in the plant roots as evident from the root analysis that nonmycorrhizal plant roots accumulated more aluminium than ectomycorrhizal roots. These results are in accordance with the hypothesis that the ectomycorrhizal fungus modified the rhizosphere by complexing the heavy metals and thus the plant is protected from the detrimental effects of the toxic metal. In this respect, it can be considered that an ectomycorrhizal pioneering fungus in the mycorrhizal colonization of the host plant at the first stages of growth is the most critical in afforestation and reclamation of contaminated soils.

From these results it was concluded that inoculation of Pt-NIC to *E. tereticornis* plants grown in bauxite mined out soil improved the growth and survival of plants. The fungus also improved the mineral nutrition of the plants grown in bauxite mined out soil compared to uninoculated plants. These results suggest that Pt-NIC could serve as a fungus of choice in reclamation of bauxite mined out/ aluminium contaminated soils.

SUMMARY

Mycorrhizae are symbiotic associations that form between the roots of most plant species and fungi. Bi-directional movement of nutrients characterizes these symbioses where carbon flows to the fungus and inorganic nutrients move to the plant, thereby providing a critical linkage between the plant root and soil. Ectomycorrhiza are of great significance to forestry species and the host derives many benefits from symbiotic association as increased rate of nutrient uptake from soil, resistance to feeder root pathogens, increased resistance to soil toxins and adverse soil conditions.

In recent decades, great attention has been paid to the problem of heavy metals that severely contaminate the soil leading to wide spread seedling mortality and several decades of delay in revegetation schemes. In contrast to the plants, some fungi exhibit heavy metal tolerance to high concentrations of metals, which normally cause severe toxicity symptoms in higher plants. Tolerance of vascular plants to aluminium is now a major area of research worldwide because, aluminium is abundant and occasionally toxic component of acidic soils. Low pH and nutrient availability enhance the competitive adsorption of aluminium over calcium and magnesium by their displacement from apoplast exchange sites and also forms complexes with inorganic phosphorus and inhibit the uptake of phosphorus and potassium in the apoplast. Ectomycorrhizal fungi exhibit varying degree of aluminium tolerance and improve metal tolerance of their host plant by primarily

accumulating metals in walls of extramatrical hyphae and extrahyphal slime and thus the passage of metal to shoots is restricted.

In the present study, different ectomycorrhizal fungi were screened for their tolerance to aluminium and the mutants were developed which can tolerate high concentration of aluminium. The influence of aluminium tolerant isolates on the growth and mineral nutrition of the host plants was studied *in vitro*. A study was also conducted to see the efficacy of Pt-NIC (aluminium tolerant isolate of *P. tinctorius*) on the growth of *E. tereticornis* plants grown in bauxite mined out soil.

Screening of different ectomycorrhizal fungi for their tolerance to Aluminium

The different ectomycorrhizal fungi were screened for their tolerance to aluminium in Melin's medium. Aluminium was added (as $Al_2 (SO_4)_3 .16H_2O$) in the medium after 3 days of inoculation to have a final concentrations of 0, 200, 500, 750, 1000, 2000, 3000 and 5000 mg/L. After 3 weeks of fungal growth, the biomass was harvested by filtration and the parameters such as pH of filtrate, dry weight of mycelia and phosphatase activity were determined. The mineral content (uptake of Ca, Mg, K, Al and P) of mycelia was estimated by inductively coupled plasma emission spectrophotometer.

The results showed that with increasing concentration of aluminium in the culture medium reduction in growth was observed in all the fungi. The growth of Pt-P, Pt-KN6 and h-1 was inhibited more than 50% at 200 mg/L of aluminium, whereas the growth of Pt-N and Pt-NIC was not significantly

affected. The *P. involutus* (P.I-MAR) also showed tolerance to aluminium with no significant reduction in growth till 1000 mg/L of Al and the growth was decreased at higher concentrations. The presence of aluminium in the culture medium affected the mineral nutrition of all the ectomycorrhizal fungi. At lower concentrations, the calcium and magnesium content in the mycelium was either increased or not affected in all the fungi tested. At higher concentrations, the calcium content was decreased significantly in all the fungi except in h-1 where the level of calcium in the mycelium increased significantly. The potassium content in the mycelium of all fungi was not affected at lower concentrations and at higher concentrations the potassium level was decreased. At lower concentrations of aluminium, the phosphorus level was increased and was significantly affected at higher concentrations of aluminium except in case of Pt-NIC and Pt-KN6, where the phosphorus level was significantly increased at higher concentrations in the mycelia. The phosphatase activity of many fungi also increased as the concentration of aluminium increased in the culture medium except in Pt-NIC where the activity was significantly decreased with increased aluminium concentration.

Isolation of Aluminium tolerant mutants of Ectomycorrhizal fungi

To increase the efficacy of ectomycorrhizal fungi for their tolerance to aluminium, these fungi were irradiated with U.V light. Two approaches were followed to develop the mutants, one by directly irradiating the young mycelium with U.V. light and the other by irradiating the protoplasts. In both approaches, the mutants were isolated under selection pressure of aluminium.

Isolation of mutants through protoplasts were carried out only for the ectomycorrhizal fungus *H. cylindrosporum* (h-1) as the regeneration of protoplasts was achieved only in h-1. The mutants developed by these procedures were compared with their respective wild type fungi for aluminium tolerance. Among the different irradiated ectomycorrhizal fungi (mutants), only Pt-P* and h-1* showed an increased tolerance to aluminium when compared to their wild type isolates. The irradiated h-1* and wild type h-1 (monokaryon) were dikaryotized with the compatible monokaryon h-7 and the resultant D2* (irradiated) and D2 (wild type) were used for further study. The other irradiated fungi (P.I-MAR*, Pt-N* and Pt-NIC*) showed less growth and more sensitivity towards aluminium compared to their wild type and irradiated Pt-KN6* failed to grow after irradiating with U.V light. The mineral nutrition of ectomycorrhizal fungi irradiated with U.V. light was studied and compared with the respective wild type isolates. Comparative analysis of mineral uptake (Ca, Mg, K and P) did not show any significant difference. The uptake of aluminium by Pt-P* was lower when compared to its wild type isolate and in other fungi there was no significant difference in aluminium accumulation between irradiated and the respective wild type isolate of ectomycorrhizal fungi. The phosphatase activity of irradiated mycelium was significantly decreased in all fungi except in Pt-P* where it showed higher activity in presence of aluminium compared to their respective wild type isolates.

Influence of ectomycorrhizal fungi on plant mineral nutrition in presence of

Aluminium (*in vitro*)

The ectomycorrhizal fungus P.I-MAR was tested with *Populus deltoides*, Pt-NIC and Pt-P* with *Eucalyptus tereticornis* and D2* with *Pinus pinaster* seedlings in this study. The seeds of *P. pinaster* were surface sterilized and aseptically germinated. The micro- propagated plantlets of *P. deltoides* and *E. tereticornis* were used to study the mycorrhizal association. The plantlets/seedlings were grown in soilrite: vermiculite moistened with Melin's medium containing different concentrations of aluminium (0, 50, 100, 200, 400 and 500 mg/L), and one half of the plantlets/seedlings were inoculated with respective ectomycorrhizal fungi. The plantlets/seedlings were harvested after 60 days and studied for various growth parameters and also the mineral nutrient levels.

P. deltoides : The presence of aluminium in soilrite- vermiculite adversely affected the growth; the plantlets showed the aluminium toxicity symptoms such as short, stubby roots and pale patches on the distal part of the leaves. The shoot height, shoot and root dry weights were decreased with increasing concentrations of aluminium. The ectomycorrhizal plantlets showed improved growth in presence of different concentrations of aluminium as compared to nonmycorrhizal plantlets. The concentrations of aluminium in the plant shoot of ectomycorrhizal and nonmycorrhizal plantlets increased with increasing concentrations of aluminium in the medium. The aluminium content was

increased in nonmycorrhizal plantlets compared to ectomycorrhizal plantlets. The phosphorus content decreased significantly with increasing concentrations of aluminium in both ecto and nonmycorrhizal plantlets, but the phosphorus content was significantly more in ectomycorrhizal plantlets. The potassium, calcium and magnesium content in the nonmycorrhizal plantlets was significantly lower than the ectomycorrhizal plantlets and there was no significant decrease in the potassium, calcium and magnesium content of nonmycorrhizal and ectomycorrhizal plantlets were found with increased concentrations of aluminium.

E. tereticornis : The growth of *E. tereticornis* plantlets was significantly inhibited with increasing concentrations of aluminium but the presence of ectomycorrhizal fungi improved the growth of ectomycorrhizal plantlets as compared to nonmycorrhizal plantlets. At 500 mg/L of aluminium concentration the uninoculated plantlets failed to survive. The shoot height, shoot and root dry weights were significantly decreased with increasing concentration of aluminium and the inoculated seedlings showed better survival than uninoculated plantlets. The aluminium content in the plant shoot of ectomycorrhizal and nonmycorrhizal plantlets increased with increasing concentrations of aluminium in the growth medium. The ectomycorrhizal plantlets accumulated less of aluminium as compared to nonmycorrhizal plantlets. There was no significant difference in aluminium accumulation among the two isolates of *Pisolithus* species. The ectomycorrhizal plantlets showed significantly higher foliar concentrations of phosphorus, calcium,

magnesium and potassium in their plant shoot tissue when compared to nonmycorrhizal plantlets at all aluminium levels. The phosphorus and potassium content decreased in uninoculated plantlets but did not change significantly in inoculated plantlets with increasing concentrations of aluminium. The calcium content decreased significantly at higher concentrations of aluminium in nonmycorrhizal plantlets when compared to control plantlets, while the calcium content increased significantly beyond 200 mg/L of Al concentration in ectomycorrhizal plantlets. The magnesium content decreased in nonmycorrhizal plantlets and increased significantly in ectomycorrhizal plantlets with increasing concentrations of aluminium. Among the two isolates of *Pisolithus* species Pt-NIC showed better uptake of mineral ions as compared to Pt-P.

***P. pinaster* :** The response of nonmycorrhizal and ectomycorrhizal seedlings to increasing concentrations of aluminium indicated that nonmycorrhizal seedlings were more sensitive to the presence of aluminium. The growth was significantly inhibited in presence of different concentrations of aluminium in both ecto and nonmycorrhizal seedlings. The accumulation of aluminium in the plant shoot of ectomycorrhizal and nonmycorrhizal seedlings increased significantly with increasing concentrations of aluminium but the ectomycorrhizal seedlings accumulated less aluminium as compared to nonmycorrhizal seedlings. The phosphorus content significantly decreased in the nonmycorrhizal seedlings, while there was no significant difference in the phosphorus content of ectomycorrhizal seedlings. The calcium content of

nonmycorrhizal seedlings decreased with increased concentrations of aluminium and was significantly improved in ectomycorrhizal seedlings. There was significant decrease in the magnesium and potassium content of nonmycorrhizal seedlings and ectomycorrhizal seedlings with increasing concentrations of aluminium. The results showed that mineral content was adversely affected by the presence of aluminium in the medium, but the inoculation of ectomycorrhizal fungus improved the uptake of nutrients as compared to nonmycorrhizal seedlings.

Influence of *P. tinctorius* (Pt-NIC) on the growth and mineral nutrition of *E. tereticornis* seedlings grown in bauxite mined out soils.

A nursery experiment was conducted with *E. tereticornis* plants inoculated with *P. tinctorius* (Pt-NIC) and grown in bauxite mined out soil collected from National Aluminium Company Ltd., Damanjodi, Orissa, India. The level of aluminium and other physico-chemical properties of the soil were analyzed. The plants inoculated with Pt-NIC and uninoculated seedlings were grown for 15 weeks in a nursery. After 15 weeks of growth in nursery, the plants were harvested and studied for various growth parameters along with the mineral contents in the plant tissues. The results of nursery trial showed better survival of *P. tinctorius* (Pt-NIC) inoculated plants of *E. tereticornis* in bauxite mined out soil as compared to uninoculated plants. The inoculated plants accumulated significantly less of aluminium in their roots thus protecting the plants from its detrimental effects. The soil after harvesting the plants was found rich in organic carbon (increased approx 10 times as compared to mined

out soil) in ectomycorrhizal plants. Similarly, ectomycorrhizal plant soil was found to be rich in available phosphorus content in soil. There was no significant change observed in total nitrogen, total phosphorus, calcium, magnesium and potassium content of the soil initially and after harvesting the plants. The aluminium content was more in ectomycorrhizal fungi inoculated soil than uninoculated soil but was less than initially found in bauxite mined out soil.

From these results, it was concluded that Pt-NIC was the most aluminium tolerant isolate of *P. tinctorius* among the different ectomycorrhizal fungi used in the study. P.I-MAR and Pt-N showed moderate tolerance to aluminium and Pt-KN6, Pt-P and h-1 were aluminium sensitive isolates. To increase the tolerance of these isolates, the fungal mycelium /protoplasts (in case of *H. cylindrosporum*) were irradiated with U.V light. After irradiation only Pt-P* and D2* showed increased tolerance to aluminium as compared to their wild type isolate. Pt-N*, P.I-MAR* and Pt-NIC* showed decreased tolerance after irradiation with U.V light as compared to their wild type isolates. The aluminium tolerant wild type Pt-NIC, P.I-MAR and the irradiated Pt-P* and D2* were used for further plant-fungal interaction studies *in vitro*. The results showed that ectomycorrhizal plantlets/seedlings showed improved growth, better uptake of mineral nutrients and lesser accumulation of aluminium in plant shoots as compared to nonmycorrhizal plantlets/seedlings when grown at different concentrations of aluminium. The efficacy of most tolerant Pt-NIC isolate was also checked *ex vitro* in bauxite

mined out soil and the results showed better suitability of Pt-NIC inoculated ectomycorrhizal *E. tereticornis* plants as compared to nonmycorrhizal plants in nursery conditions. Overall, the present work serves as a biological model to develop the mutants of ectomycorrhizal fungi to be used for the reclamation of bauxite mined out soils.

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APPENDIX I

Modified Melin's medium (Melin, 1921)

$CaCl_2.2H_2O$	50 mg/L
NaCl	25 mg/L
KH_2PO_4	500 mg/L
$(NH_4)_2HPO_4$	250 mg/L
$MgSO_4.7\ H_2O$	150 mg/L
Ferric citrate (1%)	1.2 ml/L
Glucose	2500 mg/L
Biotin	0.4 mg/L
Thiamine HCl	1.0 mg
Heller's Micronutrients (100X)	10.0 ml/L
pH	5.5

Heller's micronutrients (100X) (Heller, 1953)

$FeCl_3$	0.37 mM
$ZnSO_4$	0.35 mM
Boric acid	0.16 mM
$MnSO_4$	0.05 mM
$CuSO_4.5H_2O$	0.012 mM
$AlCl_3$	0.022 mM
$NiCl_2$	0.013 mM
KI	0.0063 mM

Autoclave 20 minutes at 121^oC

Modified Melin Norkarn's medium (Marx and Bryan, 1975)

$CaCl_2.2H_2O$	50 mg/L
NaCl	25 mg/L
KH_2PO_4	500 mg/L
$(NH_4)_2HPO_4$	250 mg/L
$MgSO_4.7\ H_2O$	150 mg/L
Ferric citrate (1%)	1.2 ml/L
Glucose	10.0 g/L
Malt extract	3.0 g/L
Biotin	0.4 mg/L
Thiamine HCl	1.0 mg
pH	5.5

Autoclave 20 minutes at 121^oC

YMG medium

Yeast extract	4.0 g/L
Malt extract	10.0 g/L
Glucose	4.0 g/L

Autoclave 20 minutes at 121^oC