ORGANIC ACID EXUDATION BY ECTOMYCORRHIZAL FUNGI IN RESPONSE TO ALUMINIUM

A
DISSERTATION

Submitted in partial fulfillment of the requirement
For the award of the degree of
Masters of Science
in
Biotechnology

UNDER THE GUIDANCE OF

Dr. M. S. REDDY

BY
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Candidate's Declaration

I, hereby, declare that the work presented in the dissertation entitled **"Organic acid exudation by ectomycorrhizal fungi in response to Aluminium"** in partial fulfillment of the requirement for the award of the degree of **Masters in Biotechnology,** Department of Biotechnology and Environmental Sciences, Thapar Institute of Engineering and Technology, Patiala, is an authentic record of my work during the period of five months from January 2006 to May 2006, under the supervision of Dr. M. Sudhakara Reddy, Coordinator, TIFAC-CORE, Thapar Institute of Engineering and Technology, Patiala. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Place: Patiala

Date: **(Harpreet Kaur Kanwal)**

This is to certify that the above statement made by the candidate is correct and true to the best of our knowledge.

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Date: **(Harpreet Kaur Kanwal)**

Place:

Abstract

Different ectomycorrhizal fungi (MAR, h1, Pt-P and Pt-N) were screened for their tolerance to Aluminium. The fungal cultures were grown in MMN media (pH 4.5) having different concentrations of Al (0, 50, 100, 150, 200 and 250 μg/ml of Al). The growth pattern of fungi, pH of culture filtrate, acid phosphatase activity and organic acid exudation were studied. The growth of fungi decreased with an increase in Al concentration. The maximum growth was observed in case of *Paxillus involutus* (MAR), followed by Pt-N. Pt-P and h1 were found to be sensitive to Aluminium. The acid phosphatase activity increased as the concentration of Al increased in the medium. A negative correlation between pH of filtrate and Al concentration was observed, which concluded that acids were formed during the period of exposure of fungi to Aluminium. Oxalic acid was the main acid that was produced in large amounts by these ectomycorrhizal fungi in response to Al. Organic acid tolerance mechanism was studied in these cultures through the amounts of organic acids produced in these fungal cultures by RP-HPLC. It was found that organic acid exudation mechanism operates in h1, while in Pt-P, Pt-N it is absent. In MAR, the mechanism is operative only upto a limited extent. Also, a cultivation method was developed to enable exposure of ectomycorrhizal plants with intact extramatrical mycelium to media solutions containing different concentrations of aluminium. *Populus deltoides* was micropropagated through tissue culture and micropropagated plants were colonized with *Paxillus involutus* (MAR). The plants were transferred to test tubes containing glass beads along with MMN media (pH 4.5). *Paxillus involutus* (MAR) was inoculated near the roots. Different concentration of Al (0, 100, 150, 200 and 250μg/ml) in the form of $Al_2(SO_4)_3.16H_2O$ were added afterwards and the plantlets were grown for 30 days. The amount of organic acids in mycorrhizal and non-mycorrhizal systems was measured by withdrawing filtrates from the solution and analyzing by RP-HPLC. It was observed that levels of oxalic acid were significantly higher in mycorrhizal treatments than in non-mycorrhizal controls. Thus, the results obtained suggest that production of oxalic acid is stimulated by exposure to elevated Al in case of mycorrhizal plants colonized by MAR. Thus, the tolerance mechanism of organic acid exudation operates in case of mycorrhizal *Populus deltoides*

CONTENTS

Chapter I

1. INTRODUCTION

1.1 Mycorrhiza and its types

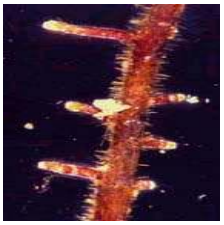
The roots of almost all higher plants are known to form mutualistic symbiosis with fungi. These are termed mycorrhizas (fungus roots, from the Greek: *mykes* = mushroom or fungus and *rhiza* = 45

, as shown in figure 1a). The term mycorrhiza was first applied to fungus-tree associations described in 1885 by the German forest pathologist A. B. Frank. In older literature, these combined Greek works are often incorrectly pluralised as mycorrhizae.

Allen (1991) defined a mycorrhiza as:

> *"A mutualistic symbiosis between plant and fungus localized in a root or root-like structure in which energy moves primarily from plant to fungus and inorganic resources move from fungus to plant*."

mycos (fungus) +



rhiza (roots) =

mycorrhiza

Figure 1a: Symbiotic association of the fungus and the roots of the plant showing the peculiar features of mycorrhiza.

1.2 Functioning of Mycorrhiza:

The main mycorrhizal response is one of increased efficiency of mineral uptake especially of poorly mobile ions and there is a voluminous literature dealing with the nutritional benefits that plants derive from mycorrhizal associations. Mycorrhizas are also involved in the transfer of nutrients from components of soil minerals and organic residues to solution, and in nutrient cycling in an ecosystem.

In all types of mycorrhiza, fungal hyphae permeate soil and litter beyond the depletion zones developed around non-mycorrhizal roots. Thus, mycorrhizal roots explore a larger

soil volume and have a greater absorptive area than non-mycorrhizal roots. The fungal partners in some mycorrhizas may also have chemical mechanisms to liberate fixed inorganic ions or minerals incorporated in organic matter in the soil.

About 95% of the world's land plants form the mycorrhizal relationship in their native habitats. The mycorrhizal condition is the rule among plants, not the exception (Harley and Smith, 1983). It is estimated that mycorrhizal fungal filaments explore hundreds to thousands more soil volume compared to roots alone.

Benefits include:

- Improved nutrient and water uptake
- Improved root growth
- Improved plant growth and yield
- Reduced transplant shock
- Reduced drought stress
- Parasitic nematode control

1.3 Types of Mycorrhiza

Fungi of very diverse kinds, belonging to all the major groups, take part in mycorrhizas. Based on morphology and the species involved, several different types are recognized. The types can be distinguished by their positions along two gradients, from a lack of penetration of cortical cells (ectomycorrhizas) to penetration (vesicular-arbuscular and arbuscular mycorrhizas) and from an enclosed (ectomycorrhizas) to open root (vesicular-arbuscular and arbuscular mycorrhizas). Any natural ecosystem normally contains a mixture of types of mycorrhizal associations.

There are several different types of mycorrhiza.

- Arbutoid Mycorrhizas
- Ectomycorrhizas (EM)
- Ectendomycorrhizas
- Ericoid Mycorrhizas
- Monotropoid Mycorrhizas
- Orchid Mycorrhizas
- Vesicular-Arbuscular and Arbuscular Mycorrhizas (VAM)

About two-third of the plants are symbiotic with VAM glomalean fungi, while a relatively small number of plants develop EM and they dominate forest ecosystems.

1.4 Ectomycorrhizas

EM are common in both Gymnosperms (Pinaceace, Cupressaceae) and Angiosperms (Juglandaceae, Fagaceae, Betulaceae, Tiliaceae, Salicaceae, Ulmaceae, Rosaceae, Leguminosae, Sapindaceae, Aceraceae, Myrtaceae, Ericaceae and Dipterocarpaceae) (Smith and Read, 1997).

The fungi involved in the formation of ectomycorrhizas are mostly Basidiomycetes and are often species that have quite large and conspicuous fruiting bodies. Toadstools and earthballs are conspicuous examples. Among the basidiomycetes there are Hymenomycetes such as *Boletus, Cortinaris, 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 EM with forest trees.

Most ectomycorrhizal fungi can synthesize plant growth substances, which cause the morphology of the root to be changed. The changes induced differ between species. At one extreme, branching is totally suppressed and, at the other, prolific branching takes place, with a single root having a dense aggregate of as many as 2000 mycorrhizal tips.

Most ectomycorrhizas are enveloped in a dense weft of hyphae while others appear smooth and seem to lack radiating hyphae. All gradations between these two forms can be found.

The first sign of mycorrhiza formation is the development of fungal mycelium around a short root. This gradually thickens over the root surface developing into a sheath (sometimes called the mantle). The sheath may be only one or two cells thick but can exceed 100 μm. The entire root, including the apex, is often enclosed by the sheath so that there is no direct contact between younger roots and the soil. Root hair development is normally totally suppressed. Thus, all nutrients and water absorbed by the roots have to pass through the fungal sheath. Depending on the fungal species, various secondary structures in addition to radiating adsorptive hyphae may be found associated with the sheath.

Behind the zone of cell division, hyphae from the innerside of the sheath penetrate between the root cortical cells producing a network of hyphae called the Hartig net (as shown in Figure 1b). The size and shape of the cells of the Hartig net differ greatly depending on the EM fungal species as does the depth of penetration, which is never beyond the endodermis. The Hartig net (Figure1b) is a three-dimensional branching structure that provides a large area of contact with the cortical cells allowing efficient transfer of mineral nutrients to the plant and metabolites to the endophyte. Hyphal penetration of cortical cells does not normally occur and is limited to the cell wall.

EM hyphae may completely enclose the lateral roots and thus prevent direct acquisition of mineral nutrients by these roots.

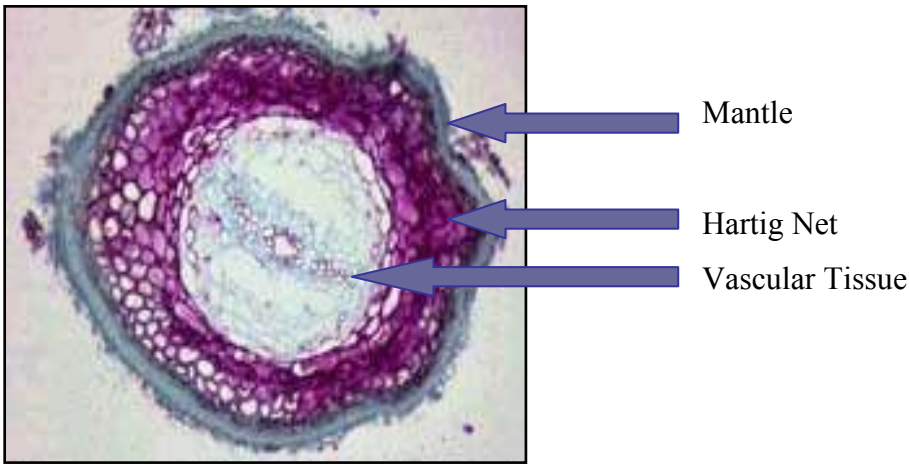


Figure 1b: Transverse section of Root showing Mantle, Hartig net (formed by ectomycorrhiza) and Vascular tissue.

EM fungi can be found on about 90% of the trees in temperate forests and each tree can be associated with many fungal species concurrently. Despite the general view that ectomycorrhizas are rare in the tropics, species within at least 14 families are known to form ectomycorrhizas. This number is likely to be an underestimate since knowledge of the mycorrhizal status of angiospermous trees in the tropics is extremely limited.

In most communities where EM fungi are present there appears a marked succession of species with increasing age of the trees and the pattern of fungal species is similar for a range of tree hosts.

1.5 Extracellular and cellular mechanisms sustaining metal tolerance in ectomycorrhizal fungi

Exposure to heavy metals, whether of natural origin, such as metalliferous rocks, or of anthropic activity origin such as pollution, may be toxic for soil organisms. The degree of toxicity depends mainly on the metallic element and its bioavailability in the soil. Metal bioavailability is a function of abiotic factors such as metal concentration, humidity and soil pH values but also on biotic factors such as the presence of metal liberating soil bacteria. Various metals e.g. Zn, Cu and Mn, are essential at low concentrations but become toxic at increasing concentrations, other metals have never been shown to be essential for the development of living organisms and are toxic even at very low concentratioons e.g. Hg, Cd, Pb (Trevors *et al*., 1986).

Mycorrhizal fungi participate in crucial symbiotic association with plants that grow on contaminated sites, and alleviate metal toxicity for their host plants (Godbold *et al*., 1988; Jentschke and Godbold, 2000; Schutzendubel and Polle, 2002). Various mechanisms involved in metal tolerance have been characterized in ectomycorrhizal fungi and can be described as extracellular that includes chelation and cell wall binding or intracellular that includes binding to nonprotein thiols and transport into intracellular compartments and the last one is detoxification mechanism.

(1) Extracellular Mechanisms:

These are mainly implied in avoidance of metal entry

Chelation: 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 mobilization of nutrients. Increasing evidence suggests that exudation of organic acids (Jones, 1998) plays a major role in Al tolerance of higher plants. Lapeyrie *et al*., (1987) has reported that as mycorrhizal fungi exude a range of organic acids and produce slime capable of binding metals, so organic acids released by mycorrhizal fungi may be responsible for the amelioration of metal toxicity in mycorrhizal fungi.

Cell wall binding: The binding properties of the cell wall and its role as a mechanism of metal tolerance have been a controversial one. Although the root cell wall is directly in contact with metals in the soil solution, adsorption onto the cell wall must be of limited

capacity and thus have a limited effect on metal activity at the surface of the plasma membrane. According to Ernst *et al*., (1992), it is difficult to explain metal-specific tolerance by such a mechanism.

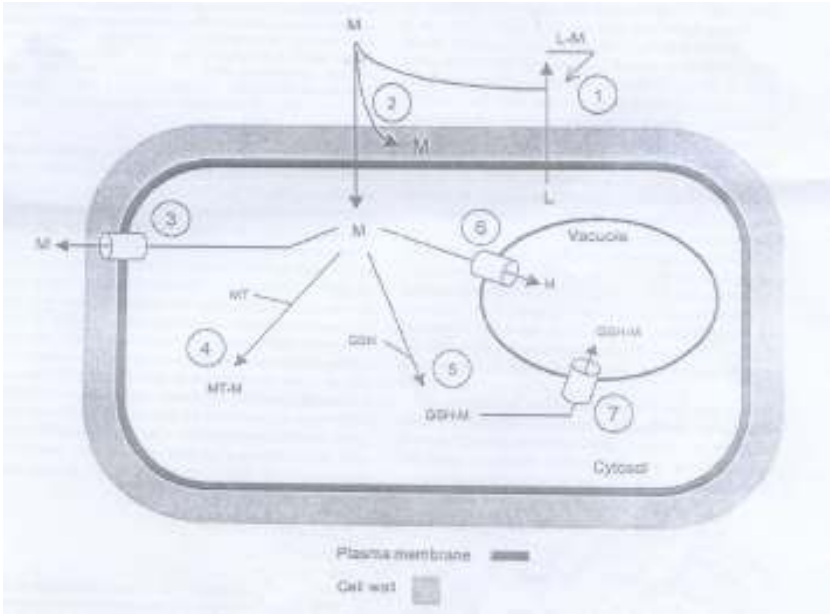


Figure 2: Schematic representation of cellular mechanisms potentially involved in metal tolerance in ectomycorrhizal fungi.

1. Extracellular chelation
2. Cell wall binding
3. Enhanced Efflux
4. Intracellular chelation by metallothionine
5. Intracellular chelation by Glutathione
6. Subcellular compartmentation
7. Vacuolar compartmentation of GSH-M complex

(2) Intracellular mechanism: This system aims to reduce metal burden in the cytosol. Large amounts of metals may enter into the cells despite extracellular and cell wall-binding capacities of ectomycorrhizal fungi. Morselt *et al*., (1986) first observed that tolerance to metals in the ectomycorrhizal fungus *Pisolithus tinctorius* was based on the presence of 'metallothionein (MT)- like peptides. Metallothioneins are a class of ubiquitously occuring low molecular weight cysteine and metal rich proteins containing sulfur based metal clusters. Crucial roles for this protein result in its involvement in homeostasis of essential trace metals, Zn and Cu, or sequestration of the environmentally toxic metals, Cd and Hg. More recently, glutathione was found to be increased under Cd exposure in *Paxillus involutus* (Ott *et al*., 2002; Courbot *et al*., 2004) as well as γ-glutamylcysteine and a compound mostly related to an metalliothionein (Courbot *et al*., 2004).

Transport mechanisms:

Metal transport proteins may be involved in metal tolerance either by extruding toxic metal ions from the cytosol of the cell or by allowing metal sequestration into intracellular compartments. Blaudez *et al.*, (2000) suggested the significant accumulation of Cd in the vacuolar compartment as an essential Cd detoxification mechanism in the ectomycorrhizal fungus *Paxillus involutus*. A crucial step in Cd detoxification, certainly in fission yeasts and probably in higher plants involves the accumulation of Cd-conjugated phytochelatins in the vacoule. Based on the same fact, Adriaensen (2005) found that an enhanced Zn efflux may act as a potential tolerance mechanism in the ectomycorrhizal fungus *Suillus bovinus*.

(3) Antioxidative mechanisms: These allow the fungus to counteract the accumulation of reactive oxygen species directly or indirectly, initiated by metals, may be part of tolerance mechanisms. The formation of free radical species, which can be initiated directly or indirectly by metals, can cause severe damage to different cellular components. Formation of metal-induced reactive oxygen species could occur *via* several mechanisms. According to Halliwell and Gutteridge (1999), the Fenton or Habber-Weiss reactions are catalyzed by redox active metals (e.g. Cu, Fe, Cr, V) and these generate the highly reactive hydroxyl (OH*) radical from H_2O_2 and superoxide (O_2^-) substrates. Redox-inactive metals such as Cd, Ni, Hg and Zn deplete glutathione and protein bound

sulphydryl groups, resulting in the production of reactive oxygen species. Several indirect mechanisms are considered to account for the action of redox-inactive metals, for example, Avery *et al*., (2001) suggested that these metals might displace redox active metals from cellular binding sites. Evidence for a role of reactive oxygen species in metal induced damage to yeast cells includes increased metal tolerance during anerobicity, protection exerted by certain free radical scavengers, and the many overlaps in the molecular mechanisms used by yeasts to cope with oxidative and metal stress (Avery *et al*., 2001). Thioredoxins are small heat stable oxidoreductases, which contain two conserved cystein domains in their active sites. Trotter and Grannt (2002) reported that they are required to maintain redox homeostasis in response to both oxidative and reductive stress conditions.

1.6 Organic acid production as a mechanism of tolerance in fungi:

Low molecular weight (LMW) organic acids are common substances in nature, playing a role in many metabolic reactions. In soils, they are released by plant roots, fungi and bacteria and are produced during microbial decomposition of organic material (Fox and Comerford, 1990). They are also used as nutrients by microorganisms (Lundstrom, 1994; Jones and Darrah, 1994). Organic acids can bind elements such as metals. They also might play a role in weathering processes (Lundstrom, 1994).

Different organic molecules, particularly, di- and tricarboxylic acids that do not belong to the matrix of cell wall, are excreted by fungal cells to chelate metal ions (as shown in figure 3), among other functions. In particular, citrate has been shown to be the most important Al^{+3} complex former in soil solution from podzolized forest soils (Landeweert *et al*., 2001; Van Hees *et al*., 2001). The induction of oxalic acid efflux correlated strongly with Cu tolerance in brown rot fungi (Green and Clausen, 2003), and oversecretion of oxalic acid probably contribuited to metal tolerance exhibited by *Beauveria caledonica* (Foramina *et al*., 2005a). Similarly, ectomycorrhizal fungi also often respond to metal exposure by increased oxalate exudation (Ahonen-Jonnarth *et al*., 2000; Cumming *et al*., 2001).

Exudation of organic acids may provide a source of protons for metal solubilisation from metal containing minerals, often resulting in soil acidification (Devevre *et al.,* 1996; Foramina *et al.,* 2005b). The recent findings that metal tolerant ectomycorrhizal fungi

grew and solubilized metal containing minerals better than non-tolerant species (Foramina *et al.,* 2005b) confirm a possible relationship between tolerance to metals and extracellular chelation by extruded ligands.

The metal sensitive ectomycorrhizal isolates do not dissolve as much metal as tolerant isolates because their growth and metabolism are more quickly affected because of metal toxicity. However, molecular mechanisms involved in the synthesis and release of organic compounds, even in the well-studied model organisms *Saccharomyces cerevisiae* and *Arabidopsis thaliana* are still poorly understood. There is no doubt that the understanding of extracellular complexation mechanism will greatly benefit from advances in molecular studies in this area.

Ahonen-Jonnarth *et al*., (2000) showed that in constrast to nonmycorrhizal pine seedlings, seedlings colonized with *Suillus variegates* or *Rhizopogon roseolus* responded to Al exposure with a strongly increased exudation of oxalic acid, which is an efficient Al 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 the most efficient in mineral weathering because of its complaxant as well as its acid properties. Cromack *et al*., (1979) reported that by forming complexes wih metal ions, such as Ca, Al and Fe, oxalate would release phosphate from insoluble phosphates.

1.7 Aluminium toxicity

Aluminium toxicity is one of the major factors that limit plant growth and development in many acid soils. Excess Al^{3+} concentration in soil solution is caused by low soil pH (<5.0). The concentration of Al in soil solution depends on soil pH as well as the concentration of organic and inorganic compounds that can form complexes with Al. Al toxicity rarely occurs in some soils where soil reduction after flooding proceeds very slowly. Aluminum toxicity is one of the major factors limiting crop production on acid upland soils, and is often associated with strong P fixation and P deficiency.

Al toxicity occurs on the following soils:

- Acid upland soils with large exchangeable Al content. Al toxicity often occurs together with Mn toxicity.
- Acid sulfate soils, particularly when rice is grown as upland crop for a few weeks before flooding (e.g., Thailand).
- Flooded soils with pH < 4.0 before Fe toxicity symptoms appear.

Although aluminiium is the third most element in the earth's crust, it lacks biological functions and shows a low bioavailability. Acid rain, however, solubilizes Al to toxic levels. Most research on the biological effects of Al has been centred on the analysis of Al tolerant plants and also several studies have been reported concerning Al effects on microorganisms, with more interest to mycorrhizal fungi and soil bacteria.
Aluminum accumulates preferentially in the root tips at sites of cell division and cell elongation. The most important symptom of Al toxicity is the inhibition of root growth. This can be due to the effect of Al on cell walls, as well as the toxic effects of Al on the plasma membrane of younger and outer cells in roots or on the root symplasm. Al affects plasma-membrane functions and decreases the influx of Ca^{2+} and Mg^{2+}.

Effects on plants include root growth inhibition and shoot growth inhibition by inducing nutrient (Mg, Ca and P) deficiencies, drought stress, and phytohormone imbalances. The symptoms include: orange-yellow to white interveinal chlorosis on leaves, poor growth or stunted growth, yellow to white mottling of interveins which is followed by leaf tip death and leaf margin scorch, necrosis of chlorotic areas during severe Al toxicity, stunted and deformed roots in susceptible cultivars.

1.8 Aim of the study:

There are many potential extracellular and intracellular mechanisms that may be involved in the tolerance of ectomycorrhizal fungi to excess metals in the environment. These include mechanisms that reduce the uptake into cytosol by extracellular chelation or binding to cell wall or efflux from the cytosol. Low molecular weight organic acids are common substances in nature that play a role in many metabolic reactions. Organic acids can bind elements such as metals, and their role as detoxification agents has been known

widely. Aluminium has been found to increase production of organic acids by roots, especially oxalic acid. In the present study, different ectomycorrhizal fungi were screened for their tolerance to Al and the organic acids (exuded by fungi in Al stress) were quantified by reverse phase high performance liquid chromatography (RP-HPLC). Also, organic acid exudation by nonmycorrhizal as well as mycorrhizal *Populus deltoides* (inoculated with *Paxillus involutus*) in Al stress were quantified through RP-HPLC. The main aim was to study the organic acid exudation (tolerance mechanism) in ectomycorrhizal fungi.

1.9 Objectives:

- Screening of ectomycorrhizal fungi for their tolerance to Aluminium
- Exudation of organic acids by ectomycorrhizal fungi in response to Al stress.
- Exudation of organic acids by mycorrhizal as well as nonmycorrhizal *Populus deltoides* in response to Al stress.

Chapter II

2. Review of Literature

2.1 Aluminium toxicity

Aluminium toxicity is one of the major factors that limit plant growth and development in many acid soils. Root cells plasma membrane, particularly of the root apex, seems to be a major target of Al toxicity. However, strong interaction of Al^{3+}, the main Al toxic form, with oxygen donor ligands (proteins, nucleic acids, polysaccharides) results in the inhibition of cell division, cell extension, and transport (Wallace and Anderson, 1984; Horst, 1995; Frntzios *et al*., 2001). These effects are further complicated by interactions of Al with other ions in different plant genotypes and under stress conditions (Foy, 1978). Cytotoxicity of Al has been well documented in plants (Delhaize and Ryan, 1995; Horst *et al*., 1995; Kollmeier *et al*., 2000; Marienfeld *et al*., 2000). It is generally known that plants grown in acid soils due to Al solubility at low pH have reduced root systems and exhibit a variety of nutrient-deficiency symptoms, with a consequent decrease in yield. It has been reported by LeNoble *et al*., (1996) that in many countries with naturally acid soils, which constitute about 40% of world arable soil, Al toxicity is a major agricultural problem and is intensively studied in plant systems.

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, 1990). However aluminium becomes increasingly soluble as the soil pH decreases below 4.5.

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, 1991); Excess uptake of cations over anions (Tang and Rengel, 2003); continuous use of ammonia and amide containing fertilizers and nitrogen fixation by legumes (Bolan and Hedley, 1991; Coventry and Slattery, 1991; Tang and Rengel, 2003).

Ryan *et al*., (1993); Blancaflor *et al*., (1998); Zhang *et al*., (1998); Vazquez *et al.,* (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.

Lazof and Holland (1992) and Silva *et al*., (2000) reported that the disruption of cell division due to Al binding to nuclei of root tip cells, leading to decreased cell production, is responsible for impedence of root growth during prolonged exposure to Al.

Ryan *et al*., (1993) and Kollmeimer *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.

2.2 Effect of Aluminium toxicity on plant growth and metabolism

Aluminum accumulates preferentially in the root tips at sites of cell division and cell elongation. Inhibition of root and shoot growth is a visible symptom of Al toxicity. The earliest symptoms concern roots. Shoots in contrast to the situation observed for Mn toxicity are less affected (Chang *et al*., 1999). Root stunting is a consequence of Al-induced inhibition of root elongation. Roots are usually stubby and brittle and root tips and lateral roots become thick and may turn brown (Mossor-Pietra-szewska *et al*., 1997). Such roots are inefficient in absorbing both nutrients and water. Young seedlings are more susceptible than older plants. Al apparently does not interfere with seed germination, but does impair the growth of new roots and seedling establishment. Long-term exposure of plants to Al also inhibits shoot growth by inducing nutrient (Mg, Ca and P) deficiencies, drought stress and phytohormone imbalances.

The Signs of Al toxicity include: orange-yellow to white interveinal chlorosis on leaves, poor with or stunted growth, yellow to white mottling of interveins which is followed by leaf tip death and leaf margin scorch, necrosis of chlorotic areas during severe Al toxicity, stunted and deformed roots in susceptible cultivars. Amongst the earliest symptoms of Al toxicity in different plant systems, reduction of net Ca^{+2} and Mg^{+2} uptake (Huang *et al*; 1993; Rengel, 1992; Jones *et al.,* 1994), reductions in P uptake (Huang *et al*., 1993; Mugwira, 1980), blockage of plasma membrane embedded Ca^{+2} channel (Ding

et al., 1993; Huang *et al*. 1993; Rengel *et al.,* 1996), interfers in active ion uptake processes functioning across the root cell plasma membrane (Kochian, 1995; Wright, 1989), reduction in K^+ efflux (Horst, 1995), accumulation of callose (Weissemeir and Horst,1995) and extrusion of malate (Delhaize *et al*.,1995) have been shown to occur within the first 30 minutes of exposure to Al.

Thornton *et a*l., (1986) reported that the common responses of shoots to Al include: cellular and ultrastructural changes in leaves, increased rates of diffusion resistance, reduction of stomatal aperture, decreased photosynthetic activity leading to chlorosis and necrosis of leaves, total decrease in leaf number and size, and a decrease in shoot biomass.

Blancaflor *et al*. (1998) have studied Al-induced effects on microtubules and actin microfilaments in elongating cells of maize root apices, and related the Al-induced growth inhibition to stabilization of microtubules in the central elongation zone. With respect to growth determinants (auxin, gibberellic acid and ethylene), Al apparently interacts directly or indirectly with the factors that influence organization of the cytoskeleton, such as cytosolic levels of Ca^{2+} (Jones *et al*., 1998), Mg^{2+} and calmodulin (Grabski *et al*., 1998), cell-surface electrical potential, callose formation (Horst *et al*., 1995), and lipid composition of the plasmamembrane (Zhang *et al*., 1999). Yamamoto *et al*., (2001) have shown that peroxidation of lipids is a relatively early event following Al exposure and appears to partly influence the Al-induced production of callose, but not the Al-induced inhibition of root elongation. By comparison, the loss of plasma membrane integrity is a relatively late event and seems to be a consequence of the cracks in the root formed by the inhibition of root elongation.

2.3 Uptake and distribution of aluminium

Al ions are taken up by plants mostly through the root system and only small amounts penetrate the leaves. According to Takabatake and Shimmen (1997), plasma membrane represents the primary target of Al toxicity. The primary effects of Al on root membrane permeability may appear only after a few minutes or even hours after exposure to Al. It is likely that these effects are mediated by Al ability to bind to the carboxyl and phosphate groups of the cell wall and membrane, respectively (Gunsé *et al*., 1997).

Although a primary response to Al has been localized to root apex (Kochian, 1995; Taylor, 1988; Sivaguru *et al.,* 1999), the mechanism of the Al-induced growth inhibition remains poorly understood and controversial. Lazof *et al.,* (1992) reported that Al entrance to root symplast in considerable quantities possibly affects the growth of the membrane from the cytosolic side. However, Horst (1995) and Rengel (1996) focused their attention on the apoplast. According to Rengel (1996), the major portion of absorbed Al is localised in apoplast ranging from 30.9 % of the total tissue Al content.

Reid (1995) reported using giant cells of the alga *Chara corallina* that 99.99% of the total cellular Al accumulates in the cell wall and according to Chang *et al*., (1999) this concerns mainly the part of cell wall pectin which remains in the protoplast even after enzymatic digestion of the wall. These authors even hypothesize that Al may bind to the pectin, newly produced during Al treatment. Quantitative information on the uptake and cellular distribution of Al is required to understand the mechanisms of Al toxicity. Induction of callose (b-1, 3-glucan) formation is a sensitive marker for genotypic Al toxicity (Horst *et al.,* 1995). Mossor-Pietraszewska (2001) reported that callose is accumulated in the cell wall around plasmodesmata in response to the damage caused by Al in the roots of various plants.

Larson *et al*., (1996) observed increasing callose deposition in wild-type *Arabidopsis* seedling roots with increasing Al concentrations over the range of 0 to 100 mM $AlCl_3$. Callose may cause the blockage of cell to cell transport by blocking plasmodesmata (Sivaguru *et al*., 2000). Ectomycorrhizal fungi may influence seedling absorption and tolerance to Al and heavymetals in soils. Turnau (1996) repoted that both the cell walls and the cytoplasm of fungal tissue are the main accumulation sites for metal ions and this results in decreased metal transfer from the fungus to the root.

2.4 Mycorrhiza and their multifunctional Roles:

The effects of mycorrhizal fungi have traditionally been considered within the rather narrow perspective of their effects on the mineral nutrition of individual plants. Most biologists are familiar with the idea that these symbiotic fungi may improve plant uptake of dissolved mineral nutrients. Research during the past 20 years has increasingly viewed symbiotic mycorrhizal associations between plants and fungi within a wider, multifunctional perspective. New molecular methods have been applied to investigate

mycorrhizal fungal communities (Bruns and Bidartondo, 2002) and greater attention has been paid to their possible effects at the level of the plant community. Finlay (2004) reported the new multifunctional perspective of mycorrhiza that includes mobilisation of N and P from organic polymers, possible release of nutrients from mineral particles or rock surfaces *via* weathering, effects on carbon cycling, interactions with mycoheterotrophic plants, mediation of plant responses to stress factors such as drought, soil acidification, toxic metals and plant pathogens, as well as a range of possible interactions with groups of other soil microorganisms

There is clearly a large potential for interaction between mycorrhizal hyphae and mineral surfaces through pores and recent research by Rosling *et al*. (2003) revealed that at least 50% of the mycorrhizal fungal taxa found in a podzol soil in the north of Sweden were exclusively associated with the mineral soil horizons. Further studies of different fungi and their responses to different mineral substrates by Mahmood *et al*., (2001); Rosling *et al*., (2003) have revealed significant variation in the responses of different fungi. Studies by Wallander *et al*. (1999) using particle-induced X-ray emission (PIXE) analysis of element contents of fungal rhizomorphs also suggested that an ectomycorrhizal *Rhizopogon* species had the ability to mobilise significant amounts of P and K from the minerals apatite and biotite and probably plays a significant role in transporting these to trees. During recent years increasing emphasis has been placed upon the ability of mycorrhizal fungi to mobilise N and P from organic polymers (Read, 1991).

2.5 Tolerance of mycorrhiza towards aluminium toxicity

The advantage of a diverse and healthy mycorrhizal community includes better survival and nutrition of plants in stressed environments. Mycorrhizas in forests are almost universal in vascular plants and occur in almost all soils. The hyphae of both ectotrophic and endotrophic mycorrhizas extend from the root surface to much greater distances through the soils than through root hairs and act as a secondary root system that facilitates the uptake of certain nutrients (P, Zn and S) by the host. Rousseau *et al*., (1994) found that while extramatrical mycelia accounted for less than 20% of the total nutrient absorbing surface mass, they contribuited nearly 80% of the absorbing surface area of pine seedlings.

Blaudez *et al*., (2001) studied 39 ectomycorrhizal fungal isolates of *Paxillus involutus, Pisolithus tinctorius, Suillus bovinus, S. luteus* and *S. variegatus* on multimetal amended medium to determine their *invitro* tolerance and found strong interspecific variation in metal tolerance. *S. luteus* and *P. tinctorius* were more tolerant to Cu, Cd and Zn than *Paxillus involutus*, while the reverse was true for Nickel.

Wit *et al., (*2001) reported Al, a key element in critical load calculations for forest and studied the effect of two levels (100 and 200 μmol L^{-1}) of enhanced Al 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 Al till concentrations of 200 μmol L^{-1} was reduced uptake of Mg. Fine root production was not affected by Al concentration. However, similar concentrations were also reported to reduce root growth and Ca and Mg uptake for spruce seedlings in nutrient solution studies. Possibly factors like mycorrhiza and rhizosphere processes (exudation of organic acids) play an important role in protecting the root from exposure to Al in the field.

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

Sverdrup and Warfvinge (1993); and Cronan and Grigal (1995) reported that the Al contents or the Ca/Al ratio in roots or in ectomycorrhizas are valuable indicators in the assesment 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.

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 P concentration under Al exposure whereas uninoculated seedlings exhibited altered patterns of foliar P accumulation because Al complexes inorganic P thus reducing its availability for plant uptake.

Hartley *et al*., (1997) studied the effects of multiple metal contaminations on ectomycorrhizal seedlings (*Pinus sylvestris*) 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 Al and found that the presence of *Paxillus involutus* in the rhizosphere (no proper infection) reduced the negative effects of Al on plant growth. They also reported large decrease in root Ca and Mg concentration in Al treated plants.

Zel and Bevc (1993) reported the drastic effect in the mineral content of *Lactarius piperatus* and *Amanita muscaria* mycelia when exposed to elevated concentrations of Al. They found increase in Al, Ca and Na contents while decrease in P, Mg and K contents with increasing Al^{+3} concentration compared to control mycelium of both the fungi.

Egerton –Warber and Griffin (1995) studied that *Pisolithus tinctorius* isolates found in rehabiliated and forest sites showed reduction of mycelial growth with increased concentration of Al and lowered levels of primarily Ca and secondarily Mg. In ectomycorrhizal trees, the impairment of growth caused by Al toxicity was found to be associated with the competitive exclusion of Ca, Mg by Al, but inoculation with *Pisolithus tinctorius* benefited plant through an increased plant Ca and Mg content and reduction in Al content.

Cumming *et al*., (2001) reported the significantly reduced concentrations of Ca, Mg and Fe in mycelia of *Laccaria bicolor* and *Pisolithus tinctorius* by exposure to Al and further, their concentration of Al increased in the mycelia with increasing Al concentration in the medium.

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

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

Gerlitz (1996) detected higher levels of P uptake and mobile polyphosphate concentration in *Suillus bovinus* in the presence of Al 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 hypothesis are not exclusive and it can be concluded that the roles of these enzymes in Al resistance are probably multiple.

2.6 Mycorrhizas and aluminium sensitivity of tree seedlings

The studies on ectomycorrhizal fungi have been demonstrated to alleviate growth depressions of tree seedlings due to toxic effects of aluminium (Cumming and Weinstein, 1990; Hentschel *et al*., 1993; Schier and McQuattie 1995, 1996). Schier and McQuattie (1995) studied the effect of Al on the growth, anatomy and the nutrient content of ectomycorrhizal and nonmycorrhizal seedlings of *Pinus strobus* and found significantly lower K^{+}, Mg^{+2} and foliar concentrations and significantly higher P foliar conc in *Pisolithus tinctorius* inoculated seedlings than noninoculated seedlings of *P. strobus* over all Al levels.

Colpaert *et al*., (1992, 1993) reported that ectomycorrhizal fungi can protect their host trees against heavy metal toxicity. Turner (1994); Wilkinson and Dickinson (1992) and Leyval *et al.,* (1997) indicated that the colonisation of tree roots by ectomycorrhizal fungi can increase tolerance of their hosts to the presence of metals in the soils in toxic concentration. In addition, ectomycorrhizal plants might be used to rehabilitate soils containing large amounts of heavy metals.

Kong *et al*., (2000) studied the biochemical responses of mycorrhizas in *Pinus massoniana* with *Pisolithus tinctorius* to the combined effects of Al, Ca and low pH and found inhibition of enzymes involved in nutritive metabolism particularly acid and alkaline phosphatase in roots, stems and leaves of plant in response to artificial acid rain and Al. 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 Al stress.

Brunner and Frey (2000) reported the localization of Al and other heavy metals such as Cd, Cu, Ni or Zn in *Picea abies* seedlings colonized with *Hebeloma crustuliniforme*.

Haynes (2001) reported that additions of organic residues to acidic soils can reduce Al toxicity and improve phosphorous availability. Complexation of Al by the newly formed organic matter will tend to reduce the concentrations of exchangeable and soluble Al present. As organic residues decompose, phosphorous is released and this can be adsorbed to oxide surfaces. This will, in turn, reduce the extent of adsorption of subsequently added phosphorous thus increasingly 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.

2.7 Mechanisms of tolerance in fungi

Tolerance to heavy metals in plants may be defined as the ability to survive in a soil that is toxic to other plants, and is manifested by an interaction between a genotype and its environment although the term is frequently used more widely in the literature to include changes that may occur experimentally in the sensitive response to heavy metals. In a number of thorough genetic studies, such adaptive metal tolerance has been shown to be governed by a small number of major genes with perhaps contributions from some more minor modifier genes.

Mycorrhizal fungi participate in crucial symbiotic relationships with plants that grow on contaminated sites and alleviate metal toxicity for their host plants (Jentschke and Godbold, 2000; Schutzendubel and Polle, 2002). 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 chelatiing compounds (Denny and Wilkins, 1987a; Cumming and Weinstein, 1990a; Jones and Hutchinson, 1988; Denny and Wilkins, 1987b). It has been reported by Bellion *et al.,* (2006) that metal tolerance of higher plants may be due to a range of potential processes that include (1) extracellular chelation and cell wall binding (2) intracellular complexation by peptides (3) transport across the cell membrane (4) Antioxidative mechanisms. Jentschke and Godbold, (2000); Ernst et al., (1992) reported that the tolerance mechanisms include- a reduction of metal exposure by excretion of chelating substances, extracellular sequestration, modified uptake systems at plasmalemma and 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 directly affecting metal availability and speciation or indirectly modifying plant physiological processes.

(1) Intracellular complexation by peptides:

Large amounts of metal may enter the cells. Using a desorption method with ^{109}Cd, Blaudez et al., (2001) have quantified the proportion of Cd in the cytosol and the vacuole of *Paxillus involutus* and estimated it to be 20% and 30% respectively. Morselt *et al*., (1986) first observed that tolerance to metals in the ectomycorrhizal fungus *Pisolithus tinctorius* was based on the presence of 'metallothionein (MT)- like' peptides. Howe *et al*., (1997) observed Cu binding proteins related to metallothioneins in various isolates of the ectomycorrhizal fungi *Laccaria laccata* and *Paxillus involutus*.

Chelation of metals in the cytosol by high-affinity ligands is potentially a very important mechanism of heavy-metal detoxification and tolerance. Potential ligands include amino acids and organic acids, and two classes of peptides, the phytochelatins and the metallothioneins (Rauser, 1999; Clemens, 2001). The phytochelatins have been the most widely studied in plants, particularly in relation to Cd tolerance (Goldsbrough, 2000).

(2) Transport mechanisms:

Metal transport proteins may be involved in metal tolerance either by extruding toxic metal ions from the cytosol out of the cell or by allowing metal sequestration into intracellular compartments. A crucial step in Cd detoxification in fission yeast involves the accumulation of Cd-conjugated glutathione or Cd-conjugated phytochelatins in the vacuole. This process appears to be mediated by the ATP-binding cassette transporter Hmt1 located at the tonoplast (Ortiz *et al*., 1992). The yeast cadmium factor (Ycf1) gene encodes a Mg ATP-energised glutathione S-conjugate transporter responsible for the vacuolar sequestration of bis (glutathione) cadmium (Li *et al*., 1997) as well as bis (glutathione) mercury (Geldry *et al*., 2003). The presence of this high specific permease in the tonoplast of *Paxillus* could explain the high Cd content in the vacuole (Blaudezb *et al*., 2001). This hypothesis was further supported by X-ray microanalysis, which revealed

that the accumulation of Cd correlated tightly with the accumulation of Sulphur in electron dense bodies in the vacuolar compartment (Ott *et al*., 2002).

(3) Antioxidative mechanisms:

Ott *et al*., 2002, in a study, analysed the antioxidative systems in the ectomycorrhizal fungus *Paxillus* in response to Cadmium, which revealed the induction of superoxide dismutase (SOD) and the accumulation of glutathione, as well as the induction of glutathione related systems at low Cd concentration (glutathione-dependent peroxidase, glutathione reductase). Jacob *et al*., 2004 found a downregulation of hydrophobin genes, a family of small hydrophobic cystein rich proteins implicated in various developmental processes such as the emergence of aerial hyphae.

(4) Metal mobility in the fungal apoplast:

Gadd (1993) reported that fungi could affectively bind metals to cell walls or extracellular polysaccharides. 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).

(5) Fungal hydrophobicity:

Besides metal sorption to fungal cells, metal uptake into host tissues may be affected by the degree of hydrophobicity 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 Cu and Zn 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 Cd and mycorrhizal *Pinus sylvestris* (Colpaert and Van Assche,1993).

2.8 Chelation by Organic acids and other substances released by mycorrhizal fungi

Low molecular weight organic acids are common substances in nature, playing a role in many metabolic reactions. In soils, they are released by plant roots, fungi and bacteria and are produced during microbial decomposition of organic material (Fox and Comerford, 1990). Organic acids can bind elements such as metals, and their role as detoxification agents has been widely discussed (Jones, 1998); they also might play a role

in weathering processes (Lundstrof m, 1994). 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 mobilization of nutrients. Aluminium has been found to increase production of organic acids by roots, especially oxalic acid (Ma *et al*., 1997b), malic acid (e.g. Pellet *et al*., 1997) and citric acid (Pellet *et al*., 1995; Ma *et al*., 1997a). The role of malate in Al tolerance may be to inhibit the blocking of the root cell plasma membrane Ca^{+2}channel (Huang *et al*., 1996). Citric acid has been shown to be an effective Al-chelator and has been proposed to decrease Al toxicity (Jones and Darrah, 1994). The main role of organic acids in Al tolerance in plants may be to prevent Al from crossing the cell wall space in Al sensitive root apices (Kochian, 1995). Higher concentrations of organic acids have been found in heavy metal-tolerant plants than in sensitive plants (Thurman and Rankin, 1982; Godbold *et al*., 1988; Harmens *et al*., 1994; Yang *et al*., 1997).

Production of organic acids, especially oxalate, is a well known phenomenon in (ecto) mycorrhizal fungi. Oxalic acid has been found in soils colonized by ectomycorrhizal fungi (Cromack *et al*., 1982; Griffeths *et al*., 1994) and Ca oxalate crystals have been observed in the mantles of ectomycorrhizas of *Pinus radiata* and *Eucalyptus marginata* (Malajczuk and Cromack, 1982), on mycorrhizas of *Eucalyptus* seedlings (Lapeyrie *et al*., 1987) and in mycorrhizas of *Monotropa uniora* (Snetselaar and Whitney, 1990). Oxalic acid was also found in water droplets added to the surface of ectomycorrhizal mycelium of *Suillus bovinus* (Sun *et al*., 1999). Inside the roots of nonmycorrhizal birch seedlings as well as seedlings colonized by *Paxillus involutus*, citrate and malate were found to be the major organic acids (Blaudez *et al*., 2001). Mycorrhizal roots of *Picea abies* collected from field sites have been found to contain citrate and malate (Nowotny *et al*., 1998) and in a hydroponic system, Ca oxalate crystals have been found in non-mycorrhizal fine roots of *Picea abies* (Fink, 1992).

Oxalate production has been suggested to have an important role in P solubilization (Cromack *et al*., 1982; Knight *et al*., 1992; Grifths *et al*., 1994) and citric acid may play a role in K mobilization (Wallander and Wickman, 1999). Oxalate retained in hyphal mats of mycorrhizal species has been proposed to increase sulphate availability and Ca oxalate crystals may function as a reservoir of Ca in the ecosystem (Dutton and Evans, 1996). In

wheat cultivars grown monoxenically in association with *Azospirillum brasiliense*, transfer of fixed N to plants was found to be higher in an Al tolerant cultivar. This was attributed to higher production of organic acids functioning as substrates for the bacteria (Christiansen-Weniger *et al*., 1992). Jones and Darrah (1994) suggested that organic acids in acid soils may be involved in a general mechanism for micronutrient uptake and potential Al detoxification. Oxalic acid is thought to act in pathogenesis through acidification of host tissue and sequestering of Ca from host cell walls, leading to weakening of cell walls (Dutton and Evans, 1996).

2.9 Organic acid exudation by mycorrhizal Plants

Production of organic acids, especially oxalate, is a well-known phenomenon in (ecto) mycorrhizal fungi. Oxalic acid has been found in soils colonized by ectomycorrhizal fungi (Cromack *et al*., 1979; Griffeths *et al*., 1994) and Ca oxalate crystals have been observed in the mantles of ectomycorrhizas of *Pinus radiata* and *Eucalyptus marginata* (Malajczuk and Cromack, 1982), on mycorrhizas of *Eucalyptus* seedlings (Lapeyrie *et al*., 1990) and in mycorrhizas of *Monotropa unphora* (Snetselaar and Whitney, 1990). The root apex has been shown to be a site for Al toxicity (Delhaize and Ryan, 1995; Kochian, 1995), a well known phenomenon in (ecto) mycorrhizal fungi.

Oxalic acid was also found in water droplets added to the surface of ectomycorrhizal mycelium of *Suillus bovinus* (Sun *et al*., 1999). Ma *et al*. (1998) found that buckwheat secreted oxalic acid in the region 0-10 mm from the root tip but similar data are not available for *Pinus sylvestris* Mycorrhizal roots of *Picea abies* collected from field sites have been found to contain citrate and malate (Nowotny *et al*., 1998) and in a hydroponic system, Ca oxalate crystals have been found in non-mycorrhizal fine roots of *Picea abies* (Fink, 1992).

Studies by Hue *et al*. (1986) classified dicarboxylic acids, oxalic, citric and tartaric acids, as potentially strong detoxifiers of Al compared with malic, malonic and salicylic acids, which were classified as intermediate, while monocarboxylic acids, succinic, lactic, formic and acetic acids, had only a weak detoxifying capacity. In two acid subsoils these authors estimated that Al and organic acid complexes accounted for 76% and 93% of the total solution Al.

Ahonen-jonnarth (2000) developed a cultivation method to enable exposure of ectomycorrhizal plants with intact extramatrical mycelium to solutions containing different concentrations of aluminium or heavy metals. *Pinus sylvestris* seedlings colonized by *Suillus variegatus* (two isolates), *Rhizopogon roseolus* or *Paxillus involutus* (two isolates) were used. Production of organic acids in mycorrhizal and non-mycorrhizal systems was measured by withdrawing samples from the solution and analyzing by HPLC. In most experiments, levels of oxalic acid were significantly higher in mycorrhizal treatments than in non-mycorrhizal controls. The measured levels of organic acids were variable, but the results obtained suggest that production of oxalic acid is stimulated by exposure to elevated Al in mycorrhizal seedlings colonized by *S. variegatus* and *R. roseolus*.

Chapter III

3. Materials and Methods

3.1 Fungal strains

Different ectomycorrhizal fungi viz., *Paxillus involutus* (MAR), *Hebeloma cylindrosporum* (h1), *Pisolithus albus* (Pt-N) and *Pisolithus albus* (Pt- P) were used in this study. *Paxillus involutus* was used to inoculate micropropagated *Populus deltoides* plantlets. All the cultures were maintained in at 25°C on Modified Melin Norkrans media with Heller's micronutrient solution (Appendix 1).

3.2. Plant material

The host plant used in this study was *Populus deltoides* (G 48) and the plantlets were obtained through micropropagation.

3.3. Screening of ectomycorrhizal fungi for their tolerance to Aluminium

Different EM fungi were screened for their tolerance to Aluminium. Fifteen days old mycelial discs (2 discs of 5 mm diameter each) of each fungus, cut from the actively growing mycelia, were inoculated into 250 ml Erlenmeyer flask containing 50 ml of Modified Melin's media with Heller's micronutrients (pH 4.5). To avoid aluminium stress immediately and also to allow fungi to initiate growth in the medium, filter sterilized (0.22 μm) aluminium was added in the form of of $Al_2(SO_4)_3.16H_2O$ (to give the final concentrations of aluminium as 0, 50, 100, 150, 200 and 250 μg/ml into the medium) after three days of fungal inoculation. The flasks were incubated at 25°C in dark and were shaken manually once in a week for 20-30 seconds for uniform distribution of the medium. After 14 and 21 days of growth, the mycelia were harvested and pH of the culture filtrate was recorded. The mycelia were analyzed for growth, pH, phosphatase activity and organic acid production.

3.3.1 Growth

The growth response of different EM fungi to varied concentrations of Aluminium (0, 50, 100 ,150 ,200 and 250 μg/ml) was assessed by growing pure cultures in the presence of Aluminium. After 14 and 21 days of incubation (in dark at 25°C), the mycelia was filtered, washed thoroughly with double distilled water and dried at 70°C for 48 hours in

the oven and weighed. The growth (as dry weight produced in g/L) was recorded for the different cultures.

3.3.2 Phosphatase Activity

Acid phosphatase activity was determined after 14 and 21 days by using the mycelia grown at different concentrations of Aluminium by the method of Tabatabai and Bremner (1969) as mentioned in section 3.5.1.

3.3.3 pH of filtrate

The pH of filtrate was recorded after 14 and 21 days of incubation.

3.4. *In vitro* Experiment (Micropropagation)

3.4.1 Media preparation

The media used for micropropagation was Murashinge and Skoog Media (MS) (Appendix II). For preparing media, a rinsed flask was taken and the required quantities of major salts were put into it. A small amount of double distilled water was added so as to dissolve all the salts. The stock solution of minor salts was added according to the requirement. Vitamins and glycine were added afterwards. Sugar was dissolved in small quantity of water and then this solution was added to the flask containing all the salts. Growth regulators i.e. Cytokinins (eg–Kinetin 0.5mg/ml and BAP 0.5mg/ml) were added. Finally, by adding double distilled water, made the total volume. The pH was adjusted to 5.7 by adding 1N KOH or 1N HCl. Small tissue culture bottles were then taken and agar (0.4 g) was added to each bottle. About 50 ml of media was added to each bottle. The media was then autoclaved at 121 °C for 20 minutes.

3.4.2 Explant collection

The plant selected for the experiment was *Populus deltoides*, which was collected from the Poplar fields in Thapar Institute of Engineering and Technology. Nodal explants were used.

3.4.3 Explant Treatment

The explants were trimmed to approx. 2 cm size and large fleshy leaves were removed. Root portions were also carefully removed; all brown skins were also taken out. These were then washed in detergent soap (0.01% v/v) for 2-3 minutes and put under tap water for rolling water wash in bottles covered with net for 30 min. Then washings with Bavistin solution (0.2%) with a few drops of Tween 20 for 15 min were given. Bavistin solution was then decanted and explants were then taken inside the clean area, inoculation room (laminar air flow). Explants were then washed with sterile double distilled water 2 – 3 times each for 5 min to remove traces of Bavistin. Explants were dried and dipped in 70% Ethyl alcohol for approx 30 sec. Explants were again washed with sterile double distilled water and dipped in Savlon solution 0.1% (v/v) with a few drops of Tween 20 for 7 – 10 minutes. Again washings with sterile double distilled water were given. Finally, the explants were dipped in 0.1% $HgCl_2$ solution for 5-7 minutes and after that washings with sterile double distilled water were given.

3.4.4 Inoculation:

The media was checked for inoculation. The explants were placed on glass plate and trimmed suitably. The cap was removed and flamed and the explants were then placed into the bottles containing media. The bottles were then placed in growth room at 25°C. Subculturing of the plantlets in PD-Shooting media (Appendix III) was carried out after the initiation of cultures.

3.4.5 Rooting:

Small shoot clusters with elongated shoots were taken out from the culture bottles. These are placed on sterilized glass plates, trimmed suitably and were placed in PD Rooting media (Appendix IV). The bottles were then placed in the growth room (at 25°C).

3.4.6 Plant – fungi interaction (Mycorrhiza formation):

For growing the micropropagated plantlets along with fungi, glass beads were used. Glass beads were taken and washed thoroughly with sterile double distilled water followed by concentrated HCl and then again with sterile double distilled water. The beads were then dried. 30 grams of beads were taken in the test tube and 8 ml of MMN media (pH 4.5) was added to it. The tubes were then capped and autoclaved at 121°C for 20 minutes. The

micropropagated plantlets were inoculated into these tubes containing glassbeads with media. The plants were allowed to grow for three days. Then the mycelial discs of *Paxillus involutus* were inoculated near the roots of *Populus deltoides* in case of mycorrhizal plants.

3.4.7 Aluminium- mycorrhiza interaction:

Again after 3 days, when growth of fungi had initiated, 2 ml of MMN (pH 4.5) containing different concentration of Aluminium (0,100,150,200 and 250 µg/ml in the form of $Al_2(SO_4)_3.16H_2O$) was added to all the plantlets. The test tubes were sealed and they were placed at 25°C for 30 days.

3.4.8 Organic acid estimation:

Low molecular weight organic acids produced by fungi (h1, MAR, Pt-N and Pt-P) and mycorrhizal plants in stress were determined using the RP-HPLC method (Reverse Phase High Performance Liquid Chromatography). After required period of incubation, the MMN media was taken out and filtered. Preparatory HPLC was done and samples were prepared as explained in section 3.5.2. Finally, RP-HPLC was done in order to quantify the different organic acids produced. For RP-HPLC, Polypore H column (Perkin Elmer) was used. The mobile phase used was 0.008 N H_2SO_4 (HPLC grade). The flow rate 0.3 ml/min, the pressure 420-450 psi and wavelengh at 210 nm was used for the analysis of organic acids. 10 µl of sample was injected into the column. The chromatograph (Perkin Elmer, series 200) featured a diode array detector. The peaks were identified by comparing retention times of standard acids (Biorad). The standard acid (Biorad) contained oxalic acid, succinic acid, citric acid, formic acid, malic acid and acetic acid. The amount of organic acids produced was calculated from the peak area of the samples and the peak areas of the standard acid.

3.5 Analytical procedures:

3.5.1 Determination of Phosphatase activity (Tabatabai and Bremner,1969):

Reagents :

- Modified Universal Buffer 5X (Skujins *et al*,1962)
  - Tris (hydroxymethyl) aminomethane 12.10 g
  - Maleic Acid 11.16 g
  - Citric Acid 14 g
  - Boric Acid 6.28 g
  - NaOH (1 N) 488 ml
  - Double Distilled Water 1 litre
  - pH 5.5
- **0.115 M p-Nitrophenyl phosphate solution :**
  Dissolved 4.268g of p-nitrophenyl phosphate disodium salt hexahydrate in 100 ml of modified universal buffer.
- **0.5 N NaOH:**
  Dissolved 20 g of NaOH pellets in double distilled water and volume made to 1 litre.
- **1 mg/ml p-Nitrophenol (PNP)** solution in modified universal buffer.

Procedure for Acid Phosphatase (Tabatabai and Bremner, 1969):

- After 14 and 21 days of incubation period, the mycelium grown in different concentration of Aluminium was the filtered, washed with double distilled water, followed by a rinse with filter sterilized universal buffer.
- After washing with modified universal buffer, mycelium was placed in 30-ml screw cap tubes with 4 ml of sterile buffer solution.
- Then 1 ml of filter sterilized 0.115 M Disodium p-Nitrophenyl phosphate solution was added.
- Contents were then incubated at 30°C in water bath for 2 hrs in dark.
- After 2 hrs of incubation, 5 ml of 0.5 N NaOH was added so as to stop the reaction.
- The mixture was swirled and filtered through Whatmann filter No.1 paper.
- The filtrate was transferred to glass cuvettes and the yellow coloured intensity was measured with UV-VIS Spectrophotometer (Hitachi U-2001) at 410 nm.

- The filtered out mycelium from each vial was dried for 48 hrs at 70°C and dry weight was recorded.
- Acid phosphatase activity was indicated as the amount of p-nitrophenol released in the filtrate from p-nitrophenyl phosphate substrate per gram of mycelium. The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 0, 100, 200, 300, 400, 500 μg of p-nitrophenol.
- To perform controls, followed the procedure described for the assay with modified universal buffer but made the addition of 0.5 N NaOH (i.e. immediately before filtration).
- Phosphatase activity was calculated in the unit of μM PNP/g mycelium hr.

Calculation:

Phosphatase activity

$$\mu\text{MPNP/ g mycelium/ hr} = \frac{\text{Conc of PNP } (\mu\text{M})}{2 \times \text{Weight of mycelium}}$$

3.5.2 Organic acid estimation of fungi and mycorrhizal plants:

For HPLC, preparatory HPLC was done and the samples were prepared.

- The filtrate (spent MMN) was filtered through Whatman filter paper No.42. In case of mycorrhizal plants, 10 ml of MQ water was added and the glassbeads along with the media was mixed thoroughly. The solution was filtered and the final volume was noted (so as to calculate the dilution).
- To the filtrate 500 μM of EDTA was added.
- The pH was set to 7 by adding 1M NaOH and they were kept as such for 24 hours so that precipitation of Al-EDTA complex could occur.
- The next day, the samples were centrifuged at 10000 rpm for 10 minutes.
- The supernatant was taken and the pH was adjusted to 4 with 0.008 N H_2SO_4.

- 4 ml of this solution was taken and equal amount of diethyl ether was added to it. The solution was thoroughly mixed well with pasteur pipette and placed as such for 3 minutes so that two layers got formed- the etheral layer and the aqueous layer.
- The etheral layer was transferred to a beaker.
- Again 4 ml of diethyl ether was added and the etheral layer again was transferred to the beaker containing the previous etheral layer.
- Again extraction was done with diethyl ether.
- The beakers were placed at 37 °C in oven for 6 hours for drying.
- After the ether had been dried, 1ml of 0.008 N H_2SO_4 was added to the dried beakers.
- The solution was filtered through 0.22 μm membrane and was then run on polypore H column (Perkin Elmer) in RP-HPLC. The solvent used was 0.008N H_2SO_4.
- The amount of acid was determined by the peak area of standard 10 mM sodium oxalate.

Calculation:

Conc. of organic acid (mM) for 50 ml:

$$= \frac{\text{molarity of standard oxalic acid X peak area of sample solution X 50 X 1000}}{\text{Concentrated factor X Peak area of oxalic acid X Injection volume}}$$

Statistical analysis:

Three replicates were maintained for each treatment in the screening experiment, acid phosphatase activity, pH and organic acid production by different fungi. The data was analyzed by two-way analysis of variance (Two Way ANOVA) for comparison and the means were compared by using Tukey's test.

For *invitro* experiment of organic acid production by mycorrhizal and nonmycorrhizal plantsin at different concentrations of aluminium, six plants for each aluminiuim level were completely randomized in the experimental system. Data was analyzed by two way ANOVA and the means were compared using Tukey's test.

Chapter IV

4. Results

4.1. 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 14 and 21 days of growth. The various parameters such as growth of fungi, phosphatase activity, pH of culture filtrate and organic acid exudation were recorded.

4.1.1 Growth

The fungi tested reacted differently to Al. The growth of ectomycorrhizal fungi was adversely affected with increasing concentration of Al. In general, the growth of fungi continuously decreased with increasing concentration of Al (Tab 3 & 4; Fig 3 & 4). The growth of MAR showed tolerance to Al with not much reduction in the dry weight. MAR was able to grow well at 250 μg/ml of Al even though there was slight reduction in the weight of mycelium. The growth of Pt-N was not much affected (Tab 3, Fig 3). The growth decreased but the difference in growth was not much significant. The growth of Pt-P and h1 decreased to a large extent at 250 μg/ml of Al. Compared to other fungi; Pt-P generally grew poorly on both the control and Al compartment. In case of control, MAR showed the highest dry weight. Nevertheless, the results showed that both h1 and Pt-P fungi were susceptible to Al. A significant decrease in dry weight (compared to control) was observed at 250 μg/ml of Al in both h1 and Pt-P. Statistical analysis (Tab 3 & 4) showed that there was a variation among the fungi and concentration of Al in relation to the growth of fungi. Among the different EM fungi tested, MAR showed the highest tolerance to Al followed by Pt-N, which showed moderate tolerance to Al. Pt-P and h1 were found to be more sensitive to Al than MAR and Pt-N.

Therefore, Al tolerance decreases in the following order:

MAR > Pt-N > h1 > Pt-P

Table 3: Effect of Al on the growth (dry weight in g/L) of ectomycorrhizal fungi after 14 days of growth.

S.No.	Al in the form of $Al_2(SO_4)_3$ (μg/ml)	MAR (g/L)	*Pt-P* (g/L)	H1 (g/L)	Pt-N (g/L)
1.	0	0.85 ± 0.03 a	0.52 ± 0.01 a	0.53 ± 0.04 a	0.58 ± 0.01a
2.	50	0.75 ±0.02 ab	0.29 ± 0.02 b	0.43 ±0.02 b	0.51 ± 0.02 ab
3.	100	0.71 ± 0.01 bc	0.22 ± 0.02 bc	0.32±0.03bc	0.47 ± 0.02 bc
4.	150	0.64 ± 0.02 cd	0.17 ± 0.02 cd	0.23±0.03cd	0.39 ± 0.02 cd
5.	200	0.57 ± 0.018 de	0.12 ± 0.01 cd	0.18 ±0.01 d	0.36 ± 0.02 de
6.	250	0.49 ± 0.02 e	0.07 ± 0.01 d	0.12 ±0.01 d	0.28 ± 0.03 e

Values sharing a common letter within the column are not significant at $P<0.05$ Mean ± S.E.

ANOVA Table:

Source	SS	df	MS	F
Main Effects				
conc	1.34	5	0.2680	192.78**
fungi	1.81	3	0.604	434.74**
conc x fungi	0.157	15	0.0104	7.54**
Error	0.06673	48	0.00139	

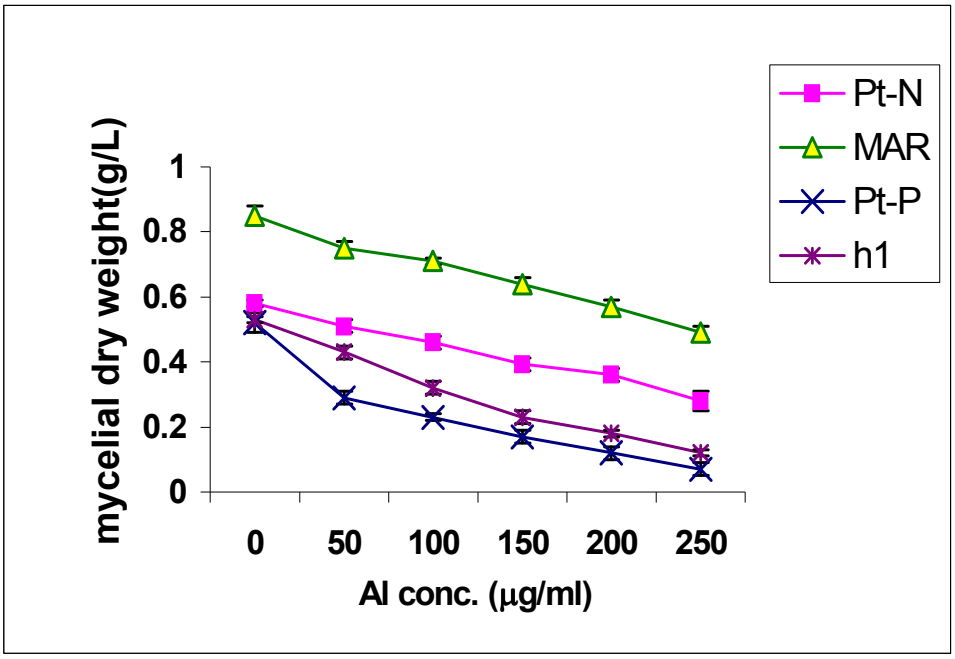


Figure 3: Effect of Al on the growth (dry weight in g/L) of ectomycorrhizal fungi after 14 days of growth. (Mean ± S.E.)

Table 4 : Effect of Al on the growth (dry weight in g/L) of ectomycorrhizal fungi after 21 days of growth.(Mean ± S.E.)

S.No	Al in the form of $Al_2(SO_4)_3$ (µg/ml)	MAR (g/L)	*Pt-P* (g/L)	h1 (g/L)	Pt-N (g/L)
1.	0	1.09 ± 0.02 a	0.6 ± 0.02 a	0.68±0.01a	0.67±0.03a
2.	50	0.89 ± 0.03 b	0.49 ± 0.02 a	0.53±0.02b	0.57±0.02ab
3.	100	0.80 ± 0.01 c	0.3 ± 0.04 b	0.47±0.01b	0.52±0.04b
4.	150	0.73±0.02 cd	0.19 ±0.02 bc	0.32±0.02c	0.49±0.02bc
5.	200	0.67± 0.02de	0.12 ± 0.03 c	0.26±0.01c	0.44±0.01bc
6.	250	0.6 ± 0.01 e	0.08 ± 0.01 c	0.19±0.01d	0.39± 0.02c

Values sharing a common letter within the column are not significant at P<0.05 Mean ± S.E.

ANOVA Table

Source	SS	df	MS	F
Main Effects				
conc	1.59	5	0.3184	213.671***
fungi	2.46	3	0.818	549.118***
conc x fungi	0.1399	15	0.00933	6.262***
Error	0.07153	48	0.0014907	

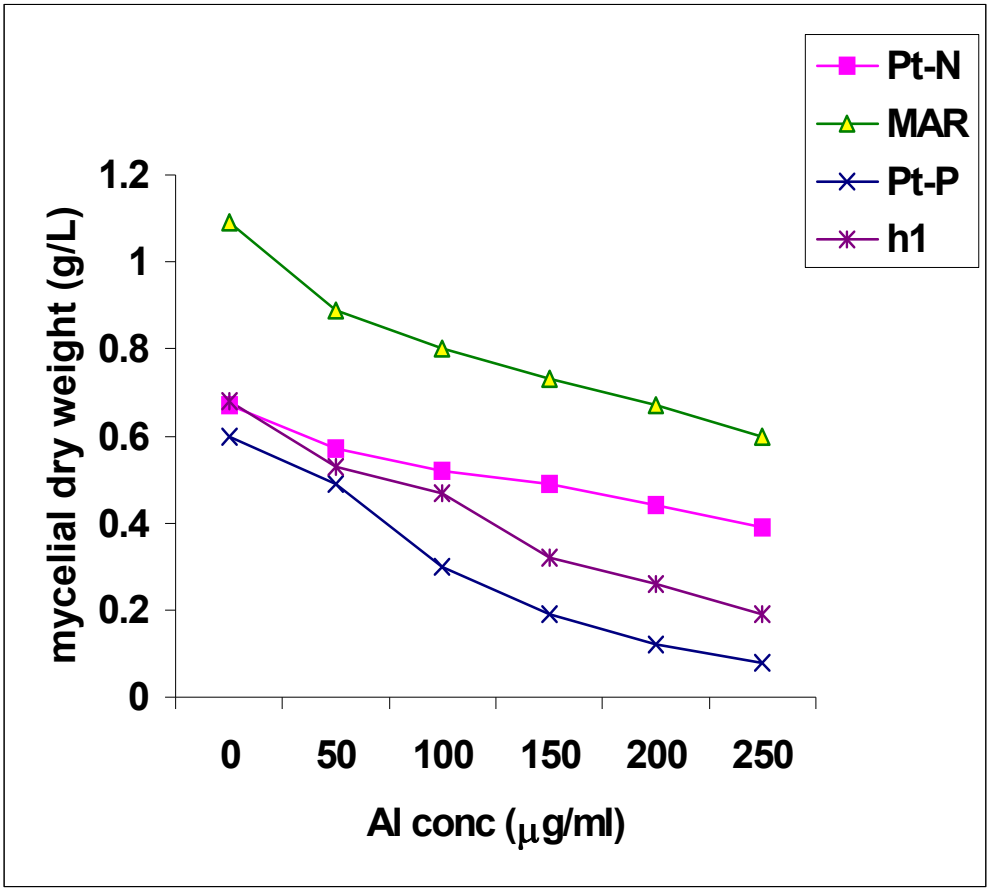


Figure 4: Effect of Al on the growth (dry weight in g/L) of ectomycorrhizal fungi after 21 days of growth. (Mean ± S.E.)

4.1.2 Acid Phosphatase activity

The acid phosphatase activity increased as the concentration of Al increased in the medium. Pt-P showed increased acid phosphatase activity as compared to control at all concentrations of Al. In case of Pt-P, the activity increased significantly compared to control at all the concentration of Al. Similarly, the acid phosphatase activity of h1 increased significantly with an increase in Al concentration in 14 days whereas it decreased to a considerable extent in 21 days. The acid phosphatase activity of MAR decreased but there was not much significant difference in the phosphatase activity (Fig 6 & 7, Tab 6 & 7). In case of Pt-N, the acid phosphatase activity increased with an increase in Al concentration, both in 14 and 21 days. The results showed that the maximum enzyme activity was observed in Pt-P while MAR showed the least enzyme activity when grown in the presence of Al.

Overall the presence of Al in the culture medium increased the fungal acid phosphatase activity.

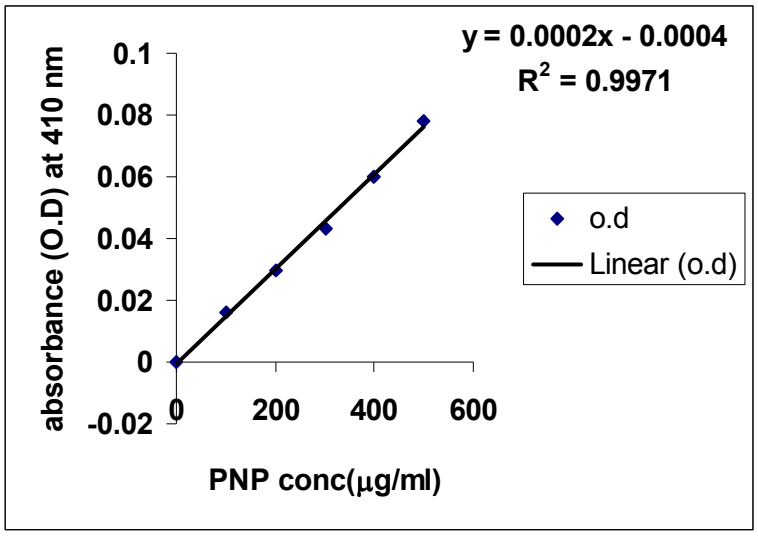


Fig 5: Graph of Standard curve between PNP conc (µg/ml) and absorbance at 410 nm.

Table 6: Effect of Al conc in the nutrient solution on the acid phosphatase activity (μM PNP/hr/g dry wt) after 14 days.

Al conc (μg/ml)	MAR	Pt-P	h1	Pt-N
0	118	131.75	167.6	118.9
50	105.8	325	187.23	139.5
100	97	211.11	274.79	153.3
150	86.18	477	395	158
200	74.2	508.75	543	187.9
250	70.8	740	770	217.6

Anova table:

Source	SS	df	MS	F
Main Effects				
conc	115654.57354	5	23130.914708	39198.01963***
fungi	957360.14292	3	319120.04764	540785.9 ***
conc x fungi	585040.26521	15	39002.684347	66094.57 ***
Error	14.1625	24	0.5901041667	

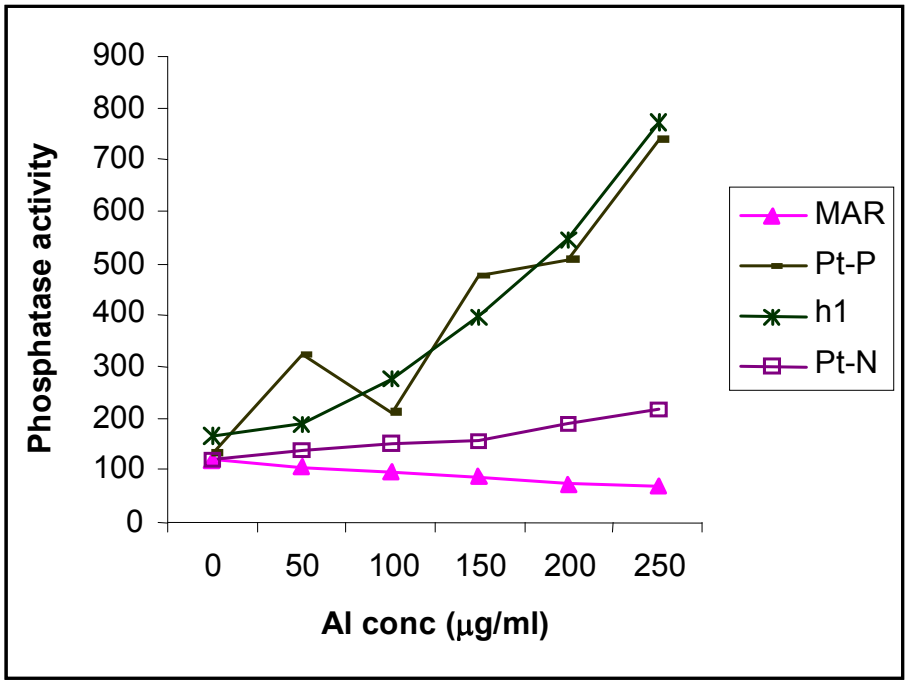


Figure 6: Effect of Al concentration in the nutrient solution on the acid phosphatase activity (µM PNP/hr/g dry wt) after 14 days

Table 7: Effect of Al concentration in the nutrient solution on the acid phosphatase activity (μM PNP/hr/g dry wt) after 21 days.

Al conc (μg/ml)	MAR	Pt-P	h1	Pt-N
0	110	171.7	390.3	28.75
50	90.5	262.5	120.47	29.81
100	82.5	335	143.12	33.65
150	70.4	393.75	59.5	82.6
200	68.2	625	69.4	137.67
250	49.3	750	76.42	196.9

Anova table:

Source	SS	df	MS	F
Main Effects				
conc	544227.98	5	108845.59	56047.053***
fungi	886483.04	3	295494.34	152156.702***
conc x fungi	516125.939	15	34408.395	17717.65***
Error	46.6089	24	1.942	

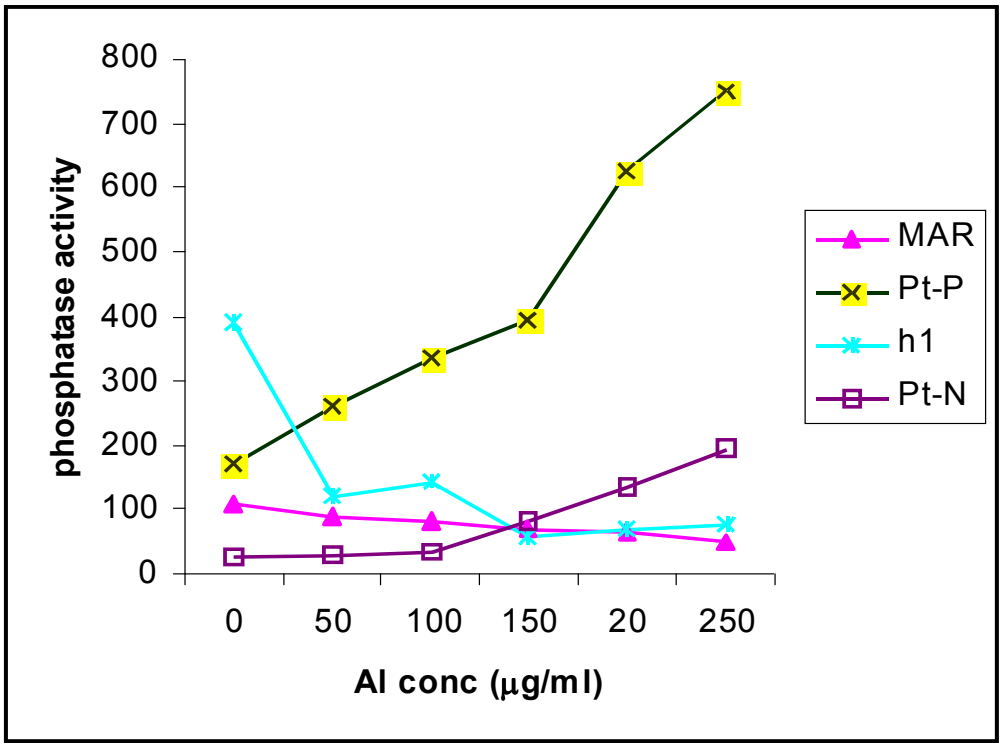


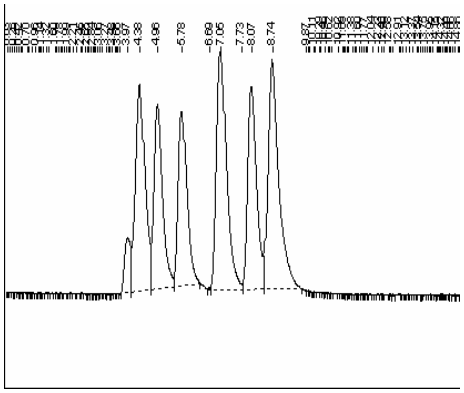
Figure 7: Effect of Al concentration in the nutrient solution on the acid phosphatase activity (µM PNP/hr/g dry wt) of different ectomycorrhizal fungi after 21 days.

4.2 Organic acid exudation by ectomycorrhiza in response to Al

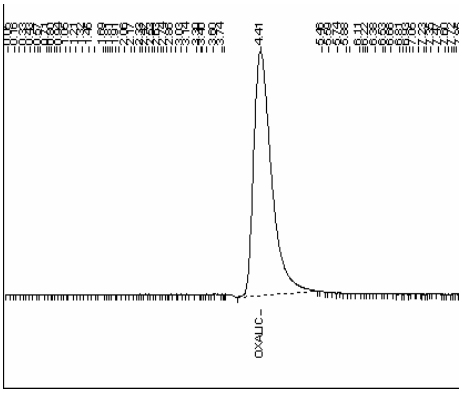
MAR, h1, Pt-P and Pt-N produced similar organic acid profiles, with several exceptions. However, there were quantitative differences in organic acid production among all the fungi. All the fungal cultures produced oxalic acid in large amounts. The standard acid (Biorad) (Figure 8a) contained oxalic acid, succinic acid, citric acid, formic acid, malic acid and acetic acid. The chromatogram (Fig 8 a) of standard acid (Biorad) showed six peaks of different acids at different retention times. The amount of organic acids produced was calculated from the peak area of the samples and the peak areas of the standard acid. The chromatogram of oxalic acid (Fig 8 b) showed a single peak at 4.40 retention time. When the filtrate samples were run on RP-HPLC, a single peak at retention time t = 4 was found, which showed that the peak was of oxalic acid and the cultures produced oxalic acid. The fact that the cultures produced oxalic acid was further verified through the chromatogram (Fig 8 c) produced by the spiked solution of sample (fungal filtrate) and oxalic acid, where only a single peak of oxalic acid was observed. The various chromatograms of fungal cultures at 14 and 21 days are given in figure 8. All the fungal cultures mainly produced oxalic acid in large amounts. Malic acid, citric acid, succinic acid, formic acid and acetic acid were absent or present in such low amounts that they cannot be detected through RP-HPLC (Polypore H Column; Perkin Elmer, Series 200). In controls (0 μg/ml), cultures of Pt-P produced the maximum amount of oxalic acid in 14 days (Fig 9) while Pt-N produced the maximum amounts of oxalic acid in 21 days (Fig 10). In Pt-P, in 14 days (Fig 9), the maximum concentration of oxalic acid was produced at 100 μg/ml, but after that it had decreased. In 21 days, there was not much significant difference in the production of organic acid. It was thus concluded that tolerance mechanism of organic acid exudation was not operative in Pt-P. In case of h1, in controls, the concentration of oxalic acid was very less, but as the conc of Al increased, the conc of oxalic acid, too, increased. At 250 μg/ml of Al, h1 was able to produce significant amounts of oxalic acid, both in 14 and 21 days. ANOVA table (Tab 9 & 10) too, showed that there was a significant variation among the fungi and conc in relation to the exudation of organic acids. It was thus concluded that the tolerance

mechanism of organic acid exudation in response to Al operated in h1. In MAR, in controls, the amount of oxalic acid produced was in considerable amounts. But as the conc of Al increased upto 200 μg/ml, the conc of oxalic acid too decreased but from 250 μg/ml, the concentration of oxalic acid again increased. Thus, in 14 days, the tolerance mechanism of organic acid exudation didn't operate at 200 μg/ml (Fig 9) but only after 250 μg/ml of Al, there was an increase in conc of oxalic acid for the fungi which might be the optimum conc of Al for the fungi to respond to Al. Similarly, the results of 21 days concluded that tolerance mechanism of organic acid exudation didn't operate in MAR. In case of Pt-N, there was not much significant difference in oxalic acid production at different conc of Al both in 14 and 21 days of growth. Thus it was concluded that the tolerance mechanism of oxalic acid production didn't operate in case of Pt-N, there might be other tolerance mechanism operating in it.

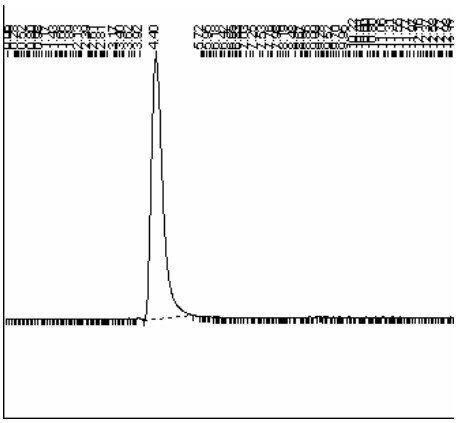
ANOVA table (Tab 9 & 10) showed that there was a significant variation among the fungi and conc in relation to the exudation of organic acids by these fungi. From the results of 14 and 21 days, it was concluded that the tolerance mechanism of organic acid exudation operated in h1, while in Pt-P and Pt-N, the mechanism was not much operative. In case of MAR, the mechanism operated only upto a limited extent.



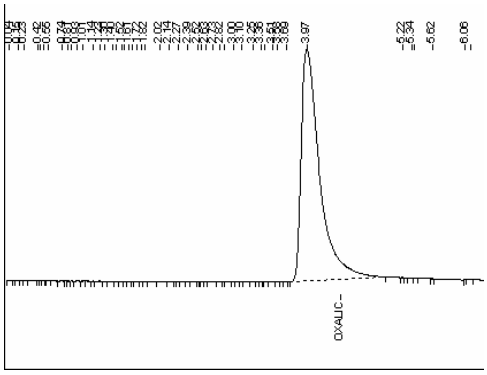
(a) Standard (Biorad) mix



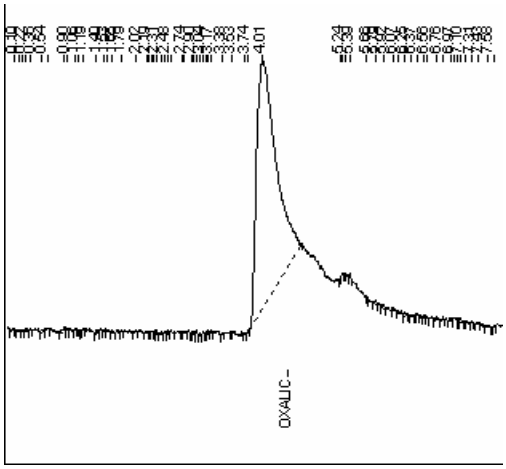
(b) Standard oxalic acid



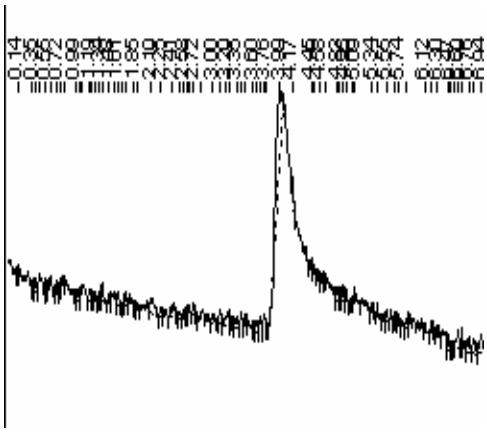
(c) Spiked oxalic acid with 200 µg/ml H1



(d) h1 at 0 µg/ml of Al (14 days)



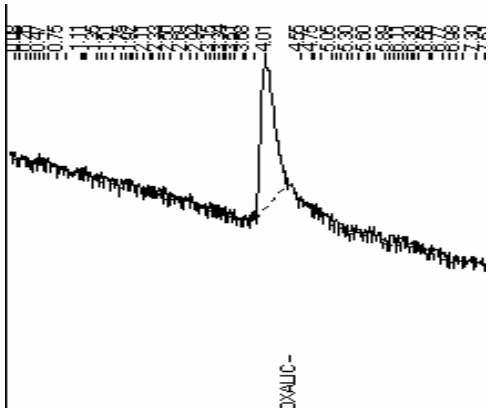
(e) Pt-P at 0 µg/ml (14 days)



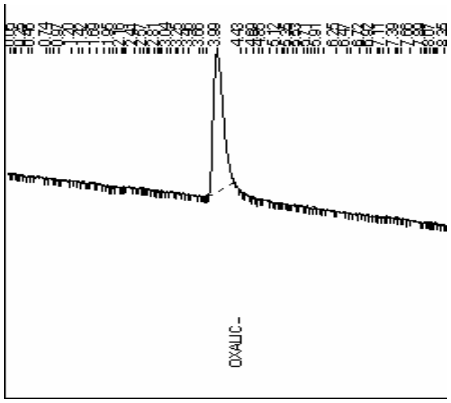
(f) MAR at 0 µg/ml (14 days)

Figure 8: Chromatograms of standards and fungal cultures at different conc of Al.

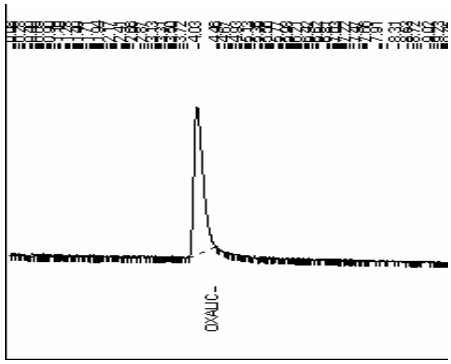
(g) H1 at 250 µg/ml of Al(21 days)



(h) Pt-P at 0 µg/ml of Al(21 days)



(i) Pt-N at 0 µg/ml of Al (21 days)



(j) Pt-N at 50 µg/ml of Al (21 days)

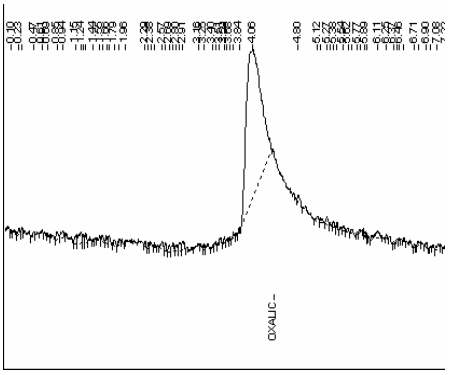


Figure 8: Chromatograms of different fungal cultures at different conc of Al in 21 days.

Table 9: Effect of Al on the oxalic acid exudation (mM) by ectomycorrhizal fungi after 14 days of growth.

S.No	Al in the form of $Al_2(SO_4)_3$ (µg/ml)	MAR	Pt-P	h1	Pt-N
1.	0	1016 ± 8.3a	2661±19.87a	69 ± 0.6 f	110 ± 5.49a
2.	50	64 ± 21.18e	560 ± 15.76c	361 ± 5.68e	37 ± 1.15c
3.	100	122 ± 1.15d	1980 ± 6.2b	416 ± 6.8d	44 ± 0.6bc
4.	150	184 ± 0.66c	196 ± 1.151f	875 ± 12.58c	53 ± 0.88b
5.	200	47 ± 0.66e	499.7±4.7d	1042 ± 6.1b	16 ± 0.6 d
6.	250	950 ± 5.2b	320 ± 4.4e	1545 ± 7.2a	44 ± 0.88bc

Values sharing a common letter within the column are not significant at P<0.05
Mean ± S.E.

ANOVA Table

Source	SS	df	MS	F
Main Effects				
conc	4389391.9	5	877878.	5898.949***
fungi	9677518.44	3	3225839.48	21676.16 ***
conc x fungi	18922730.722	15	1261515.38	8476.81 ***
Error	7143.333	48	148.81	

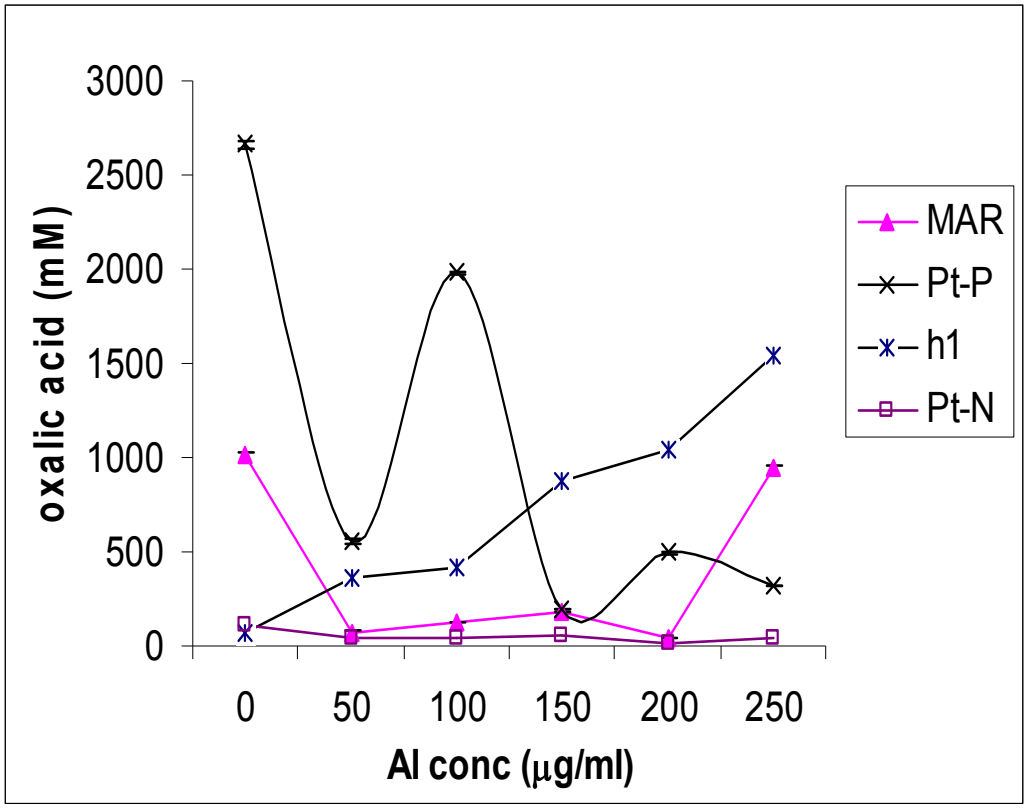


Figure 9: Effect of Al on the oxalic acid exudation (mM) by ectomycorrhizal fungi after 14 days of growth. (Mean ± S.E.)

Table 10: Effect of Al on the oxalic acid exudation (mM) by ectomycorrhizal fungi after 21 days of growth. (Mean ± S.E.)

S.No	Al in the form of $Al_2(SO_4)_3$ (µg/ml)	MAR	Pt-P	h1	Pt-N
1.	0	312±2.33d	1879±5.48c	190±6.4f	6768±34a
2.	50	367±.4.72c	2230±3.3b	479± 9.4e	188± 4.4e
3.	100	1360±6 a	2262± 9.1b	550± 10.7d	242±4.16de
4.	150	269±9.81e	2341± 10.4a	818±10.3c	262± 3.93d
5.	200	170±5.77f	2347±1.7a	1290±5.77b	370± 5.77c
6.	250	538±1.66b	1145±23.4d	2139±6.8a	456±12.25b

Values sharing a common letter within the column are not significant at P<0.05 Mean ± S.E

ANOVA Table

Source	SS	df	MS	F
Main Effects				
conc	17517719.44	5	3503543.88	4969 ***
fungi	23595110.27	3	7865036.759	11155 ***
conc x fungi	100859130.89	15	6723942	9537 ***
Error	33841.333	48	705.027	

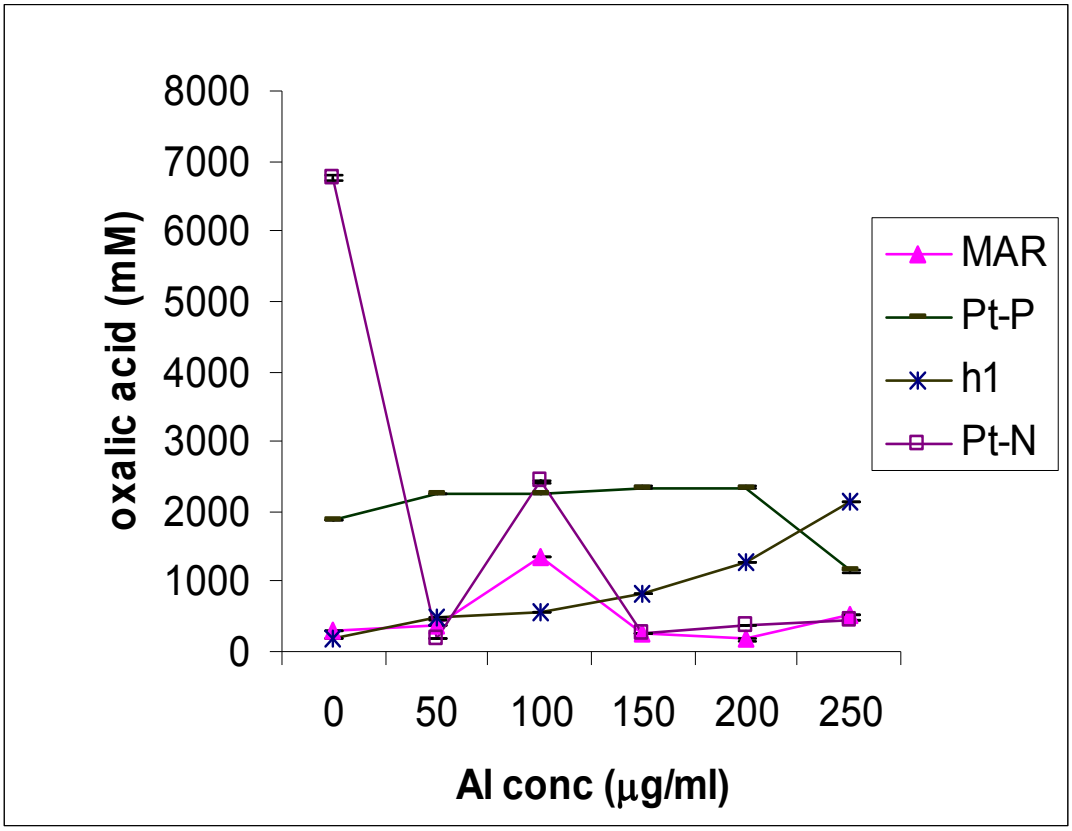
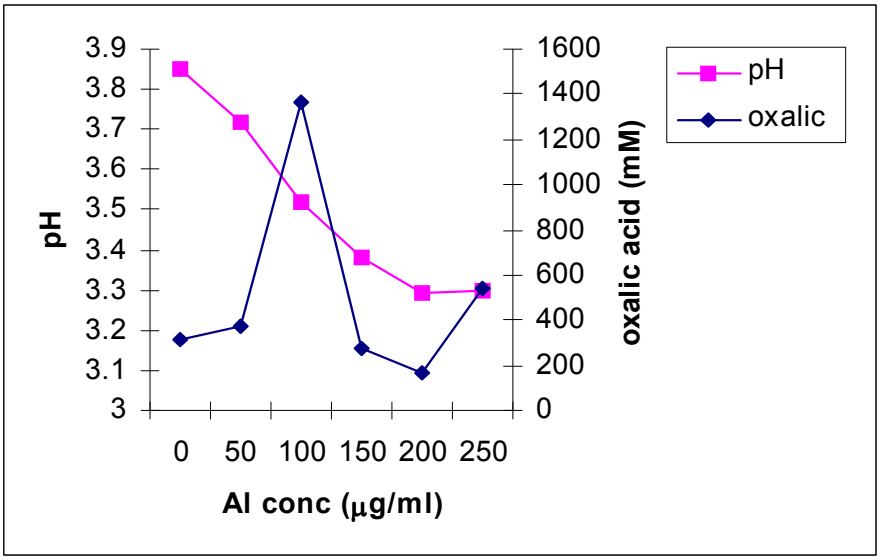


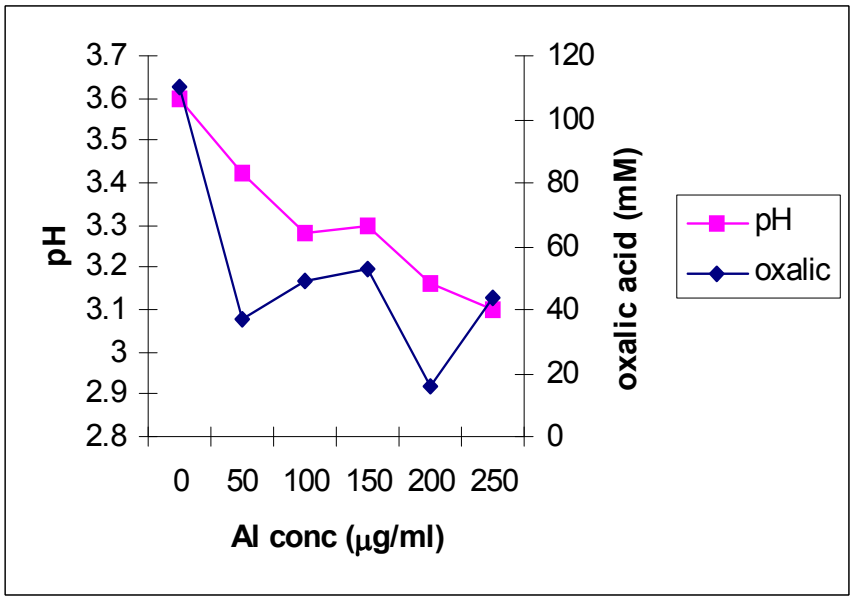
Figure 10 : Effect of Al on the oxalic acid exudation (mM) by ectomycorrhizal fungi after 21 days of growth.

4.3 pH of filtrate in relation to organic acids

The pH of filtrate was measured after 14 and 21 days of growth. pH of filtrate decreased with the increase in the concentration of Al (Fig 11a & 11b; Tab 11a & 11b). Most of the pH of the cultures was lying in the range of 3.1 – 4.2, concluding that acids had been formed during the time period of exposure to Al. The pH of culture filtrate decreased with an increase in Al concentration, thus concluding that there was negative correlation between pH of culture filtrate and Al concentration (Fig 11a & 11b; Tab 12). The trend of decrease in pH with an increase in Al conc was observed in all the cultures. Similarly, the pH of culture filtrate decreased with an increase in oxalic acid, even though there were variations in this trend. The variations could be due to other acids that may be present beyond the detection limit or they could be due to the increased conc of Al in the medium. But, nevertheless, the general trend of decrease in pH with an increase in oxalic acid was observed in all the fungal cultures, particularly, in h1, it was the best visible. A positive correlation (Tab 12) was found between the Al conc and oxalic acid in case of h1, which verified that the fact that with an increase in Al conc, there was more exudation of acids in h1. Thus, tolerance mechanism of organic acid exudation operated well in h1. In the other fungi MAR, Pt-P and Pt-N, no correlation was found, which suggested that these fungi didn't respond well to Al stress (by exuding organic acids). Thus, the tolerance mechanism of organic acid exudation failed in case of Pt-P, Pt-N and MAR.

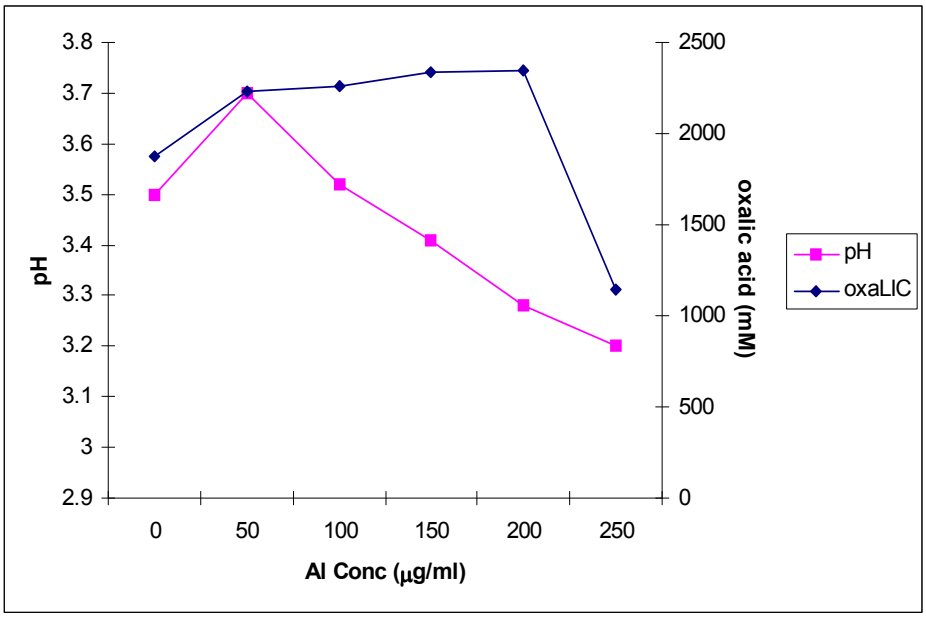


MAR (14 days)

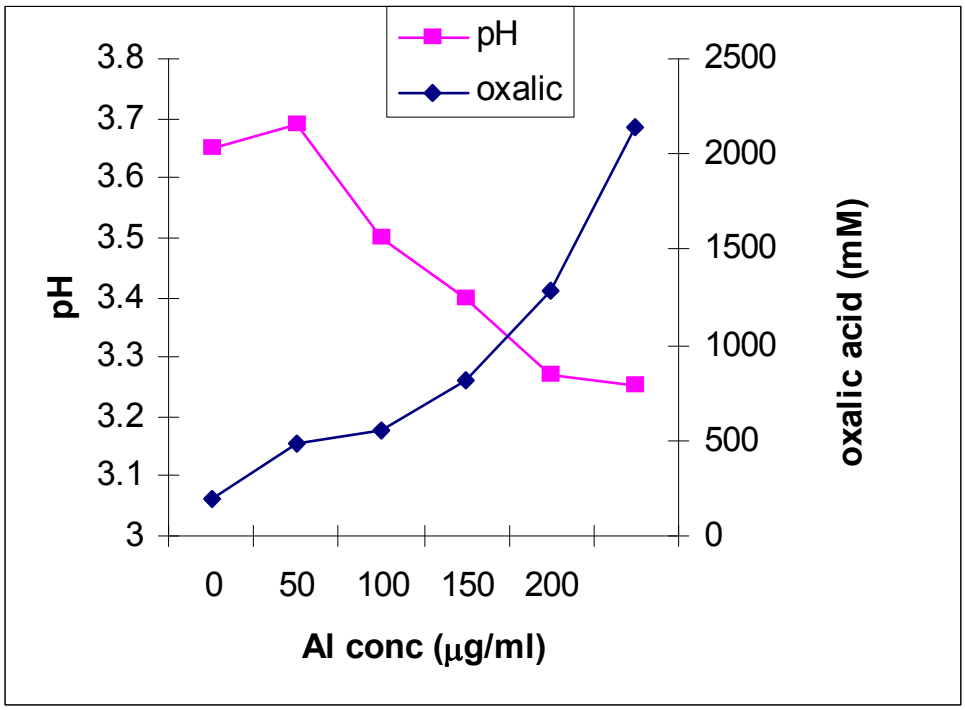


Pt-N (14 days)

Figure 11a: Effect of Al conc on the pH of culture filtrate and oxalic acid production (mM) in Al stress in 14 days.

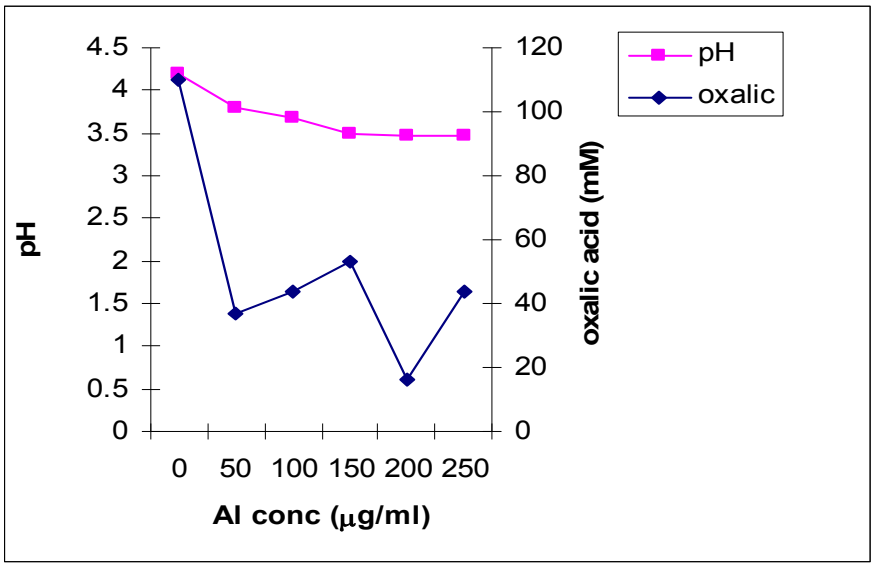


Pt-P

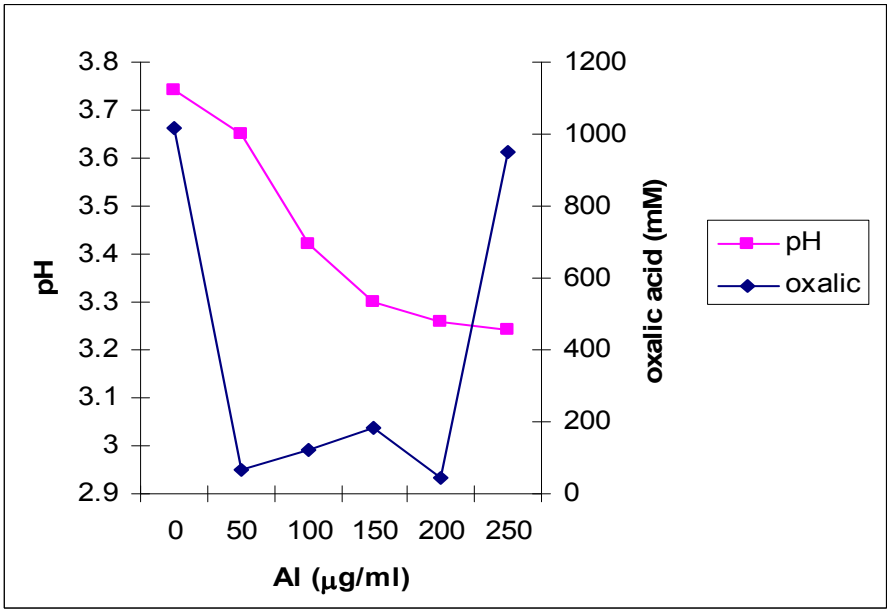


h1

Figure 11a: Effect of Al conc on the pH of culture filtrate and oxalic acid production (mM) in Al stress in 14 days.

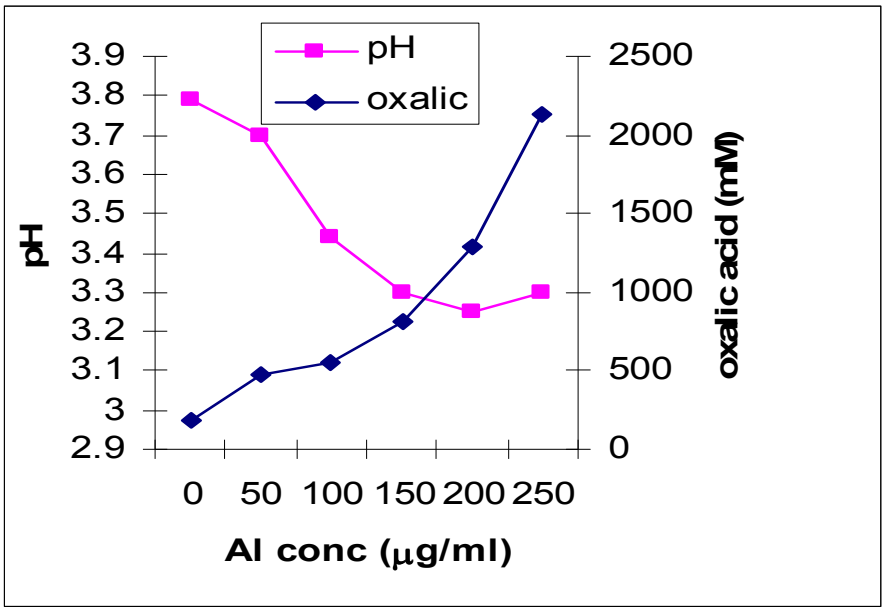


Pt-N (21 days)

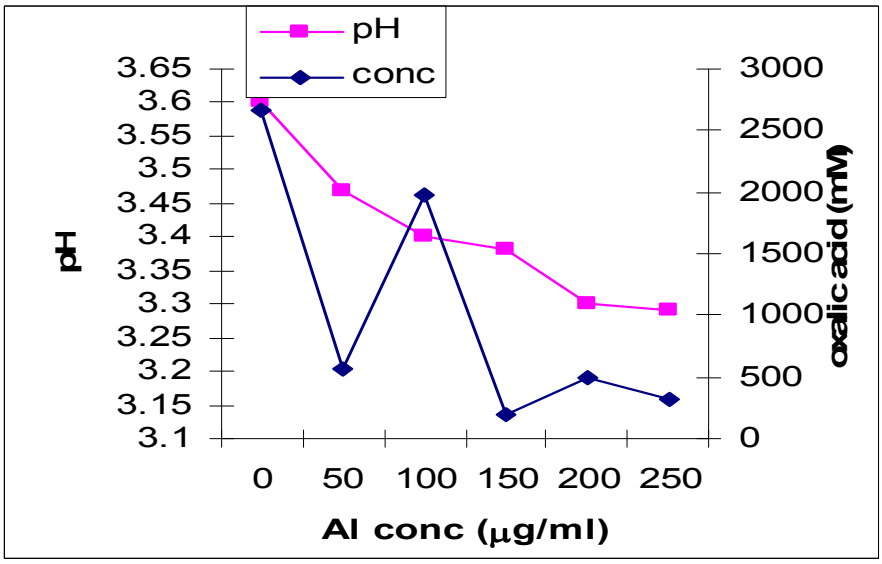


MAR (21 days)

Figure 11b: Effect of Al conc on the pH of culture filtrate and oxalic acid production (mM) in Al stress in 21 days.



h1- (21 days)



Pt-P (21 days)

Figure 11b: Effect of Al conc on the pH of culture filtrate and oxalic acid production (mM) in stress in 21 days.

Table 11: Effect of Al conc on the pH of culture filtrate and oxalic acid production (mM) in stress in 21 days.

S. No.	Fungal cultures	Al conc (µg/ml)	pH (14 days)	Oxalic acid (mM) (21 days)	pH in 21 days	Oxalic acid (mM) in 21 days
	MAR	0				
1.			3.85	312	3.74	1016
		50	3.72	376	3.65	64
		100	3.52	1360	3.42	122
		150	3.38	279	3.3	184
		200	3.29	170	3.26	47
		250	3.3	535	3.24	950
2.	Pt-N	0	3.6	110	4.2	110
		50	3.42	37	3.8	37
		100	3.28	49	3.68	44
		150	3.3	53	3.5	53
		200	3.16	16	3.47	16
		250	3.1	44	3.46	44
3.	h1	0	3.65	190	3.79	190
		50	3.69	479	3.7	479
		100	3.5	550	3.44	550
		150	3.4	818	3.3	818
		200	3.27	1290	3.25	1290
		250	3.25	2139	3.3	2139
4.	Pt-P	0	3.5	1879	3.6	2661
		50	3.7	2230	3.47	560
		100	3.52	2260	3.4	1980
		150	3.41	2340	3.38	196
		200	3.28	2347	3.3	497
		250	3.2	1145	3.29	320

Figure 12: Correlation of Al with pH of culture filtrate and oxalic acid production(mM) in stress in 14 and 21 days.

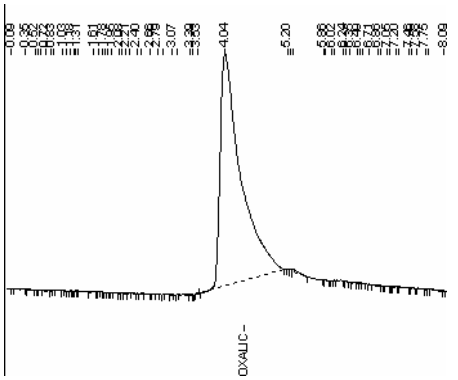
S. No	Fungal cultures	No. of days	Correlation between pH and Al conc	Correlation between pH and oxalic acid	Correlation between Al and oxalic acid
1.	MAR	14	-0.96	0.02	-0.07
2.	MAR	21	-0.95	0.21	-0.04
3.	Pt-N	14	-0.96	0.78	-0.66
4.	Pt-N	21	-0.9	0.83	-0.65
5.	h1	14	-0.96	-0.87	0.94
6.	h1	21	-0.92	-0.74	0.94
7.	Pt-P	14	-0.85	0.05	-0.37
8.	Pt-P	21	-0.96	0.71	-0.71

4.4 Organic acid exudation by mycorrhizal *Populus deltoides*

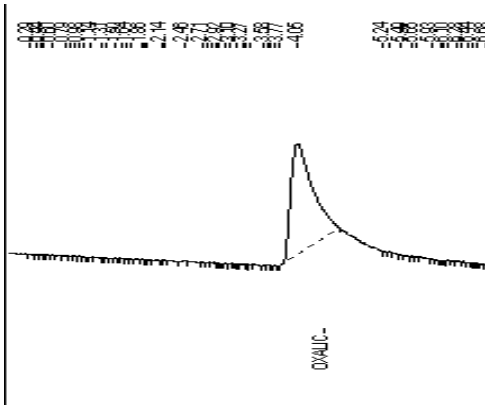
The acid detected in the highest conc was oxalic acid. The conc of oxalic acid was calculated from the peak areas of sample from chromatograms (Fig 13) and the peak area and conc of standard oxalic acid. Concentrations of oxalic acid were significantly higher in mycorrhizal treatments (*Paxillus involutus*-MAR) than in nonmycorhizal treatments (Fig 15 & Tab 15) with mean values between two to ten times greater in mycorrhizal than in nonmycorrhizal treatments. In test tubes containing nonmycorrhizal plants, there was no significant effect of Al on oxalic acid production. In controls, the amount of oxalic acid produced was more in case of mycorrhizal than in nonmycorrhizal plants. In mycorrhizal treatments, the measured mean conc of oxalic acid in the presence of Al was almost double than in test tubes without Al (Fig 15, Tab 15). In nonmycoorrhizal plants, the amount of oxalic acid produced at 250 μg/ml Al was one-hundredth of that produced in case of mycorrhizal plants. Statistical analysis (1 Way Anova) (Tab 15) had shown that there was significant difference within the mycorrhizal plants in response to Al. The amount of oxalic acid produced was maximum at 250 μg/ml of Al in mycorrhizal plants. The amount of oxalic acid produced followed the trend of increased exudation of oxalic acid in response to Al. The toxicity symptoms of Al such as orange-yellow interveinal chlorosis on leaves, poor stunted growth, yellow to white mottling of interveins followed by leaf tip death and leaf margin scorch, necrosis of chlorotic areas were observed in both mycorrhizal as well as in nonmycorrhizal plants (Fig 14). There was an increase in the degree of the appearance of these effects with an increase in the conc of Al (Fig 14).

Overall, it was concluded that organic acid exudation tolerance mechanism operated in case of mycorrhizal *Populus deltoides*.

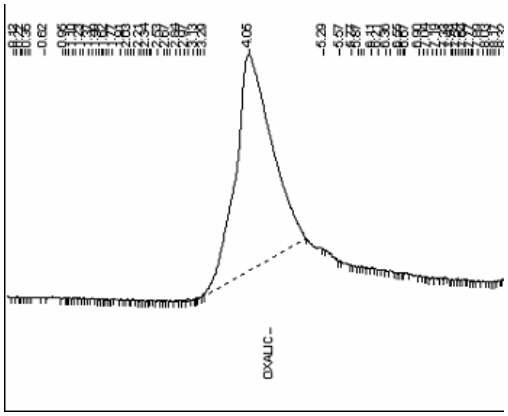
(a) (0 µg/ml Al)- Mycorrhizal



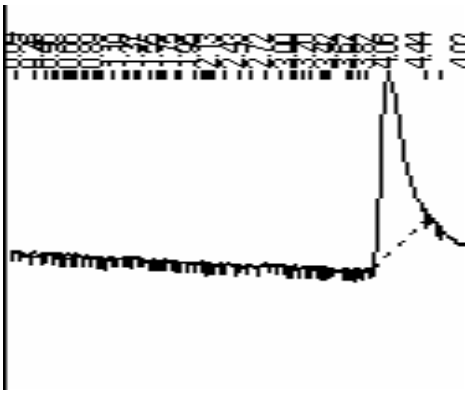
(b) (150 µg/ml of Al) Mycorrhizal



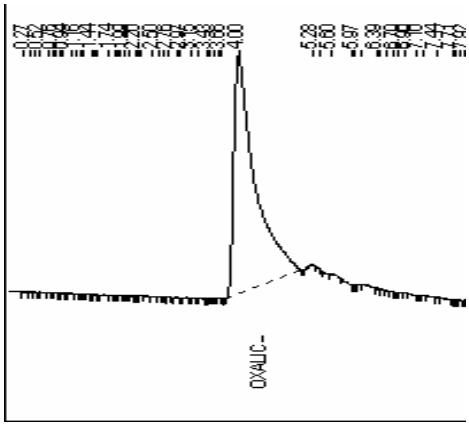
(c) (250 µg/ml of Al) Mycorrhizal



(d) (0 µg/ml of Al) Nonmycorrhizal



(e) (200 µg/ml Al) Nonmycorrhizal



(f) (250 µg/ml Al) NonMycorrhizal

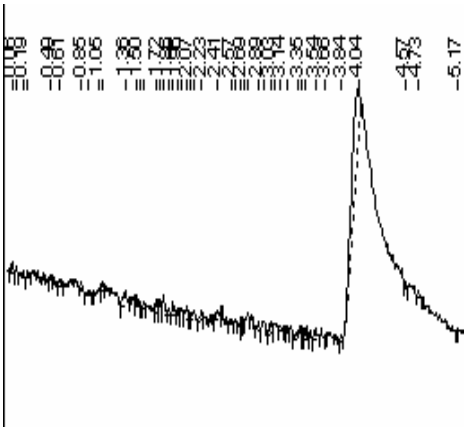


Figure 13: Chromatograms of Mycorrhizal and Nonmycorrhizal plants at different conc of Al.

Table 15: Effect of increase in Al conc on oxalic acid production (mM) by mycorrhizal and nonmycorrhizal *Populus deltoides.*

S.No.	Al (μg/ml)	Oxalic acid production (mM)	
		Nonmycorrhial plants	Mycorrhizal plants
1.	0	3048 ± 30 b	5425 ± 38 c
2.	100	3672 ± 36 a	1031 ± 64 b
3.	150	1770 ± 17 c	3662 ±69.3 e
4.	200	2939 ± 70 b	5105 ± 53 d
5.	250	189 ± 4.5 d	18142 ± 97 a

Values sharing a common letter within the column are not significant at $P<0.05$ Mean ± S.E.

ANOVA Table

Source	SS	df	MS	F
Main Effects				
conc	162900774.13	4	40725193.533	4527.5 ***
plant	288678324.03	1	288678324.03	32093.07***
conc x plant	281698918.13	4	70424729.533	7829.29 ***
Error	179900.666	20	8995	

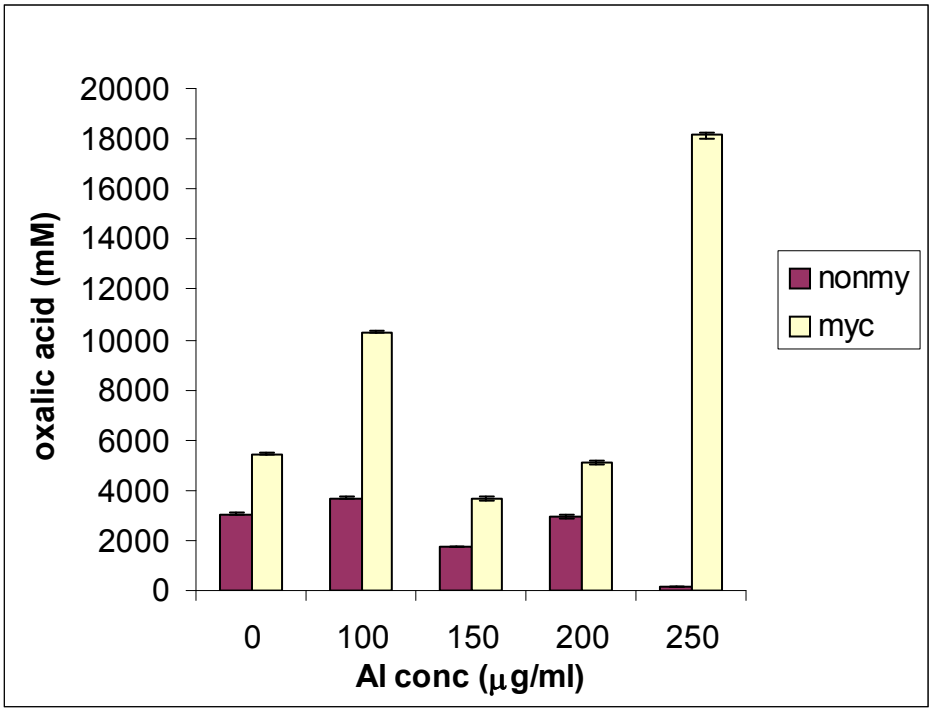


Figure 15: Effect of increase in Al conc on oxalic acid production (mM) by mycorrhizal and nonmycorrhizal *Populus deltoides*.

Chapter V

Discussion

Organic acids perform a diverse range of functions in the soil, and most of these processes appear to be beneficial to resident organisms (Jones, 1998). Depending on the number and dissociation properties of their carboxylic groups, they can complex metal cations to different degrees, and displace anions from the soil matrix. Citrate and malate, in particular, are strong natural chelators of trivalent cations such as Al^{+3} and Fe^{+3} and are involved in the scavenging of metal ions from insoluble compounds in the soil. These organic acids are released in the soil by bacteria, fungi and plant roots (Jones, 1998). Their production in fungi has been reported to increase at growth limiting concentrations of soluble metal ions such as Mn^{+2}, Fe^{+2} and Zn^{+2} (Gadd, 1993). The recent findings that metal tolerant ectomycorrhizal fungi grew and solubilized metal-containing minerals better than nontolerant species (Foramina *et al*., 2005) confirm a possible relationship between tolerance to metals and extracellular chelation by extruded ligands. However, organic acid exudation should not be regarded as a general tolerance mechanism as it is both metal and species dependent (Meharg, 2003). This can be explained by the fact that EM fungi do not adapt to metal toxicity through a downward adjustment of their organic acid capacity but use other mechanisms for tolerance such as binding of metals to cell wall, transport mechanisms, intracellular complexation by peptides and antioxidative mechanisms.

EM 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 Al toxicity may be important factors for the decline in the vitality of many forest trees and associated ectomycorrhizal fungal flora. 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 the significant intra and interspecific variations exists in metal sensitivity of ectomycorrhizal fungi (Jones 1988; 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 Al on plants are well known, data on the toxicity of Al to mycorrhizal fungi are scarce. EM fungi exhibit differential responses to Al (Zel and Bevc, 1993; Jones, 1994; Reddy *et al*., 2002). In the present study, different EM fungi were tested for their ability to tolerate various concentrations of Al. Gadd (1983) reported the disadvantages of using agar media to assess the sensitivity of EM fungi to metals, as it forms complexation with metals and alters the metal availability to fungi. Hartley *et al*., (1997) suggested that fungal biomass production determined in liquid media provide a more accurate assesment of metal sensitivity, as it is independent of growth form.

In the present study, different isolates of EM fungi, Pt-P, Pt-N, H1 and MAR were grown in MMN (pH 4.5) in the presence of different concentration of Al (0, 50, 100, 150, 200 & 250 μg/ml) and the growth was assessd after 14 and 21 days. The growth of EM fungi decreased as the concentration of Al incresed in the growth media (Fig 3 & 4). MAR was found to be the most tolerant to Al among the other fungi, followed by Pt-N. Pt-N was found to be moderately tolerant to Al. h1 and Pt-P were found to be sensitive to Al. The growth of Pt-P and h1 decreased to a large extent at 250 μg/ml of Al. The growth of fungi Pt-N, too, decreased. Reports are available on different ectomycorrhizal fungi such as *Paxillus involutus MAI, P. involutus 533, P.involutus NAU* and *Pisolithus tinctorius* in response to Al and Pb. The growth of *P. involutus MAI* decreased to 50 % at 2000 μM of Al whereas in case of *P. involutus NAU*, *P. involutus 533* and *Pisolithus tinctorius 956,* there was 50 % decrease in growth at 200 μM of Al. In the present study, MAR was able

to grow well at 250 μg/ml of Al without much reduction in growth. These results indicate that MAR showed high tolerance followed by Pt-N. The other ectomycorrhizal fungi Pt-P and h1 seemed to be very sensitive to Al.

In the present study, fungal acid phosphatase activity increased in Al treated mycelia of ectomycorrhizal fungi. Reddy *et a*l., (2002) reported increased acid phosphatase activity of *Pisolithus sp*. and *Cantharellus cibarius* in presence of Al at lower conc (100 μg/ml). Since one of the main effects of Al on phosphate in 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 Al. It may also correspond to a more general stress resistance process, since acid phosphatase of ectomycorrhizal fungi can also be induced by Cd (Turnau and Dexheimer, 1995) and can play a role in heavy metal detoxification (Turnau *et al.,* 1994). The acid phosphatase activity increased as the concentration of Al increased in the medium. Pt-P showed increased acid phosphatase activity as compared to control at all concentrations of Al. In case of Pt-P, the activity increased significantly compared to control at all the concentration of Al. Similarly, the acid phosphatase activity of h1 increased significantly with an increase in Al concentration in 14 days whereas it decreased to a considerable extent in 21 days. The acid phosphatase activity of MAR, too, decreased but there was not much significant difference in the phosphatase activity (Fig 6 & 7, Tab 6 & 7). In case of Pt-N, the acid phosphatase activity increased with an increase in Al concentration, both in 14 and 21 days. The results showed that the maximum enzyme zctivity was observed in Pt-P and MAR showed the least enzyme activity when grown in the presence of Al.

Overall the presence of Al in the culture medium increased the fungal acid phosphatase activity.

Increasing evidence suggests that exudation of organic acids (Jones, 1998) plays a major role in Al tolerance of higher plants. In fungi, metal tolerance in some cases has been linked to extracellular chelation by organic compounds (Gadd, 1993). In the present study, the production of organic acids have been linked to Al exposure, as these systems have been linked to Al tolerance in higher plants (Delhaize and Ryan 1995; Kochian 1995). Oxalate exudation is widespread among fungi. Compared with other organic acids, oxalate is highly effective at complexing multivalent cations (Lapeyrie *et al.,* 1987). The

induction of oxalic acid efflux correlated closely with Cu tolerance in brown rot fungi (Green and Clausen, 2003) and overexcreation of oxalic acid probably contribuited to the metal tolerance exhibited by *Beauveria caledoonica* (Foramina *et al*., 2005a). However, organic acid exudation should not be regarded as a general tolerance mechanism as it is both metal and species dependent (Mehrag, 2003).

In the present study, the production of organic acids had been linked to Al exposure. MAR, h1, Pt-P and Pt-N produced similar organic acid profiles, with several exceptions. However, there were quantitative differences in organic acid production among all the fungi. All the fungal cultures produced oxalic acid in large amounts. The standard biorad acid contained oxalic acid, succinic acid, citric acid, formic acid, malic acid and acetic acid. The fact that the cultures produced oxalic acid was verified through the chromatogram (Fig 8 c) produced by the spiked solution of sample (fungal filtrate) and oxalic acid, where only a single peak of oxalic acid was observed. The chromatogram of standard biorad acid showed six peaks of different acids. The chromatogram of oxalic acid showed a single peak at 4.40 retention time. All the fungal cultures mainly produced oxalic acid in large amounts. In controls (0 μg/ml), cultures of Pt-P produced the maximum amount of oxalic acid in 14 days (Fig 9) while Pt-N produced the maximum amounts of oxalic acid in 21 days (Fig 10). In Pt-P, in 14 days, the maximum concentration of oxalic acid was produced at 100 μg/ml, but after that it had decreased. In 21 days, there was not much significant difference in the production of organic acid. In controls, there was less organic acid in 21 days than in 14 days, the organism might have used the acid. Possibly, another tolerance mechanism, other than organic acid exudation might be operating in Pt-P. Morselt *et al*. (1986) observed that tolerance to metals in the ectomycorrhizal fungus *Pisolithus sp.* was based on the presence of metallothionein like peptides. It was thus concluded that tolerance mechanism of organic acid exudation was not operative in Pt-P. In case of h1, in controls, the concentration of oxalic acid was very less, but as the conc of Al increased, the conc of oxalic acid, too, increased. At 250 μg/ml of Al, h1 was able to produce significant amounts of oxalic acid, both in 14 and 21 days. ANOVA table, too, showed that there was a significant variation among the fungi h1 and conc in relation to the exudation of organic acids by h1. It was thus concluded that the tolerance mechanism of organic acid exudation in response to Al operated in h1. In case

of Pt-N, there was not much significant difference in oxalic acid production at different conc of Al both in 14 and 21 days of growth. Thus it was concluded that the tolerance mechanism of oxalic acid production didn't operate in case of Pt-N, there might be other tolerance mechanism operating in it. In MAR, in controls, the amount of oxalic acid produced was in considerable amounts. But as the conc of Al increased upto 200 μg/ml, the conc of oxalic acid too decreased but from 250 μg/ml, the concentration of oxalic acid again increased. Thus, in 14 days, only after 250 μg/ml of Al, there was an increase in conc of oxalic acid for the fungi which might be the optimum conc of Al for the fungi to respond to Al. Similarly, the results of 21 days concluded that tolerance mechanism of organic acid exudation didn't operate in MAR. In case of MAR, the other tolerance mechanisms might be operating. Binding of Cd to cell walls was shown to represent a substantial fraction of the metal accumulated by MAR and may also be part of the mechanisms by which mycorrhizal fungi tolerate high amounts of metals (Blaudez *et al*., 2000; Brunner *et al*., 2000). Binding proteins such as Cu binding proteins related to metallothioneins in various isolates of the ectomyxcorrhizal fungi *Laccaria laccata* and *Paxillus involutus* have also been reported by Howe (1997). More recently, glutathione was found to be increased under Cd exposure in MAR (Courbot *et al*., 2004). Putative gene sequences encoding enzymes involved in the synthesis of glutathione have been identified in expression sequence tag (EST) databases obtained from ectomycorrhizal fungi h1 and MAR. Cd detoxification mechanism in vacoules has also been reported by Blaudez *et al*., (2000) in MAR. Thus from all these findings it appears that extracellular chelation mechanism occurs only to a limited extent in case of MAR and other mechanisms such as cell wall binding, intracellular complexation by peptides and detoxification mechanisms might be operating in MAR.

From the results of 14 and 21 days, it was concluded that the tolerance mechanism of organic acid exudation operated in H1, while in Pt-P and Pt-N, the mechanism was not much operative.

pH of filtrate in relation to organic acids

In order to check whether there was decrease in pH of the filtrate i.e. whether acid was produced or not, pH of filtrate was noted. The pH of filtrate was measured after 14 and 21 days of growth. pH of filtrate decreased with the increase in the concentration of Al (Fig 11). Most of the pH of the cultures was lying in the range of 3.1 – 4.2, concluding that acids had been formed during the time period of exposure to Al. The pH of culture filtrate decreased with an increase in Al concentration, thus concluding that there was negative correlation between pH of culture filtrate and Al concentration (fig 11a, 11b, Tab 11 & 12). The trend of decrease in pH with an increase in Al conc was observed in all the cultures. Similarly, the pH of culture filtrate decreased with an increase in oxalic acid, even though there were variations in this trend. The variations could be due to other acids that may be present beyond the detection limit or they could be due to the increased conc of Al in the medium or due to the tolerance mechanisms. But, nevertheless, the general trend of decrease in pH with an increase in oxalic acid was observed in all the fungal cultures, particularly, in h1, it was the best visible. A positive correlation (Tab 12) was found between the Al conc and oxalic acid in case of h1, which verified that the fact that with an increase in Al conc, there was more exudation of acids in h1. Thus, tolerance mechanism of organic acid exudation operated well in h1. In the other fungi- MAR, Pt-P and Pt-N, no correlation was found, which suggested that these fungi didn't respond well to Al stress (by exuding organic acids). Thus, the tolerance mechanism of organic acid exudation failed in case of Pt-P, Pt-N and MAR.

Organic acid production by mycorrhizal and nonmycorrhizal *Populus deltoides:*

Infection with mycorrhizal fungi can increase the ability of plants to resist environmental stress (Wilkinson, 1989). The plants infected with these fungi may be suitable for contamination sites. Ectomycorrhizal fungi have in some cases been found to increase tolerance of their host plants to Al and heavy metals (Jentschke and Godbold, 2000). The main role of organic acids in Al tolerance in plants may be to prevent Al from crossing the cell wall space in Al sensitive root apices (Kochian, 1995). Higher concentrations of organic acids have been found in heavy metal-tolerant plants than in sensitive plants (Godbold *et al*., 1984; Harmens *et al*., 1994; Yang *et al*., 1997). Several different organic

acids, principally oxalic (Ma *et al*., 1997b), citric (Pellet *et al*., 1995; Ma *et al*., 1997a) and malic (e.g. Pellet *et al*., 1997) acids, have been found to be produced in increased amounts by non-mycorrhizal roots exposed to Al. In buckwheat (*Fagopyrum esculentum*), oxalic acid was shown to be a specific detoxification substance against Al (Ma *et al*., 1997b).

In the present study, the acid detected in the highest conc was oxalic acid. Concentrations of oxalic acid were significantly higher in mycorrhizal treatments (*Paxillus involutus*-MAR) than in nonmycorhizal treatments with mean values between two to ten times greater in mycorrhizal than in nonmycorrhizal treatments (Tab 15, Fig 15). Oxalic acid is thought to act in pathogenesis through acidification of host tissue and sequestering of Ca from host cell walls, leading to weakening of cell walls (Dutton and Evans, 1996). In pure culture tests of inhibition of *Pythium ultimum* by ectomycorrhizal fungi (Rasanayagam and Jeåries, 1992) acidification of the media was thought to be responsible for antibiosis, and *Paxillus involutus* produced large amounts of organic acids but only small amounts of oxalic acid. In the present study, in controls, the amount of oxalic acid produced was more in case of mycorrhizal than in nonmycorrhizal plants (Fig 15; Tab 15). In nonmycoorrhizal plants, the amount of oxalic acid produced at 250 μg/ml Al was one-tenth of that produced in case of mycorrhizal plant. The toxicity symptoms of Al such as orange-yellow interveinal chlorosis on leaves, poor stunted growth, yellow to white mottling of interveins followed by leaf tip death and leaf margin scorch, necrosis of chlorotic areas were observed in bothmycorrhizal as well as non mycorrhizal plants (Fig 14). There was an increase in the degree of the appearance of these effects with an increase in the conc of Al (Fig 14). The amount of oxalic acid produced followed the trend of increased exudation in response to Al. Thus, it was concluded that organic acid exudation operated in case of mycorrhizal *Populus deltoides*.

Chapter VI

Summary

Mycorrhizas are symbiotic associations that form between the roots of most plant species and fungi. Bi-directional movement of nutrients characterizes this symbiosis 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 various tolerance mechanisms operating extracellularly and intracellularly in response to heavy metals. In contrast to plants, some fungi exhibit heavy metal tolerance to high concentrations of metals, which normally cause severe toxicity symptoms. Tolerance of vascular plants to Al is now a major area of research worldwide because Al is abundant and occasionaqlly toxic component of acidic soils. Low pH and nutrient availability enhance the competitive adsorption of Al over Ca and Mg by their displacement from apoplast exchange sites and also forms complexes with inorganic phosphorous and inhibit the uptake of phosphorous and potassium in the apoplast. Ectomycorrhizal fungi exhibit varying degrees of Al tolerance and improve metal tolerance of their host plants by extracellular chelation of organic acids, binding of metals to cell walls, through intracellular complexation by peptides and by antioxidative mechanisms. Thus the passage of metals to shoot is restricted.

In the recent decades, great attention has been paid to the extracellular chelation of organic compounds by the fungi in response to Al. There have been many reports, which suggest that some organic componds are released by fungi in response to metal stress.

In the present study, different ectomycorrhizal fungi were screened for their tolerance to Al. The growth pattern, pH of filtrate, acid phosphatase activity and organic acid production were studied in these fungi. A study was conducted to see whether mycorrhial plantlets produce larger amounts of organic acids in response to Al than nonmycorrhizal plants.

Screening of different ectomycorrhizal fungi for their tolerance to Al

The different ectomycorhizal fungi were screened for their tolerance to Al in MMN (pH 4.5). Al was added (as $Al_2(SO_4)_3$.16H2O) in the medium after 3 days of inoculation to have a final concentration of 0, 50, 100, 150, 200 and 250 μg/ml. After 14 and 21 days of fungal growth, the biomass was harvested by filtration and the parameters such as pH of filtrate, dry weight of mycelia, phosphatase activity and organic acid production were determined.

The results showed that the growth of fungi decreased with increasing concentration of Al. MAR was found to be the most tolerant to Al among the other three, followed by Pt-N. Pt-N was found to be moderately tolerant to Al. h1 and Pt-P were found to be sensitive to Al. The growth of Pt-P and h1 was inhibited at 250 μg/ml of Al. The growth of fungi Pt-N, too, decreased. In the present study, MAR was able to grow well at 250 ug/ml of Al without much reduction in growth. These results indicate that MAR showed high tolerance followed by Pt-N. The other ectomycorrhizal fungi Pt-P and h1 seemed to be very sensitive to Al. The phosphatase activity of many fungi also increased as the concentration of Al increased in the culture medium. The results recorded in the present study showed that Al tolerance of ectomycorrhizal fungi in pure culture involves complex nutritional interactions, which are probably varying depending on the fungus.

Organic acid production by fungi in response to Al:

After 14 and 21 days of incubation, the filtrate was collected. pH of culture filtrates was noted so as to have an indication whether acid is produced or not. The pH of almost all the samples was found in the range of 3.1-4.2, which suggests that acid might be present in the medium filtrate as it is lying in the acidic range. Samples were prepared and they were run on RP-HPLC (Polypore H, Perkin Elmer, Series 200). Altogether six standards were used. The peaks were identified by comparing the retention timkes of known (Biorad) standards. The results showed that oxalic acid was produced in large amounts in all the fungi. In case of Pt-P, the maximum concentration of oxalic acid was produced at 100 μg/ml, but after that it had decreased. Possibly, other tolerance mechanism might be operating in Pt-P. Thus, extracellular chelating mechanism is absent in Pt-P. In case of h1, at 250 μg/ml of Al, h1 was able to produce significant amounts of oxalic acid. This suggested that the tolerance mechanism of organic acid exudation in response to Al

operated in h1. In case of Pt-N, there was not much significant difference in oxalic acid production at different conc of Al. Possibly; other tolerance mechanism might be operating in Pt-N as in case of Pt-P. In MAR, the maximum amount of oxalic acid produced was at 100 μg/ml in 21 days. But after that there had been a decrease in oxalic acid production. Other tolerance mechanisms might be operating in MAR. Further studies are required to verify this fact.

In order to check whether there was decrease in pH of the filtrate i.e. whether acid was produced or not, pH of filtrate was noted. pH of filtrate decreased with the increase in the concentration of Al. Most of the pH of the cultures was lying in the range of 3.1 – 4.2, concluding that acids had been formed during the time period of exposure to Al. There was negative correlation between pH of culture filtrate and Al concentration (Fig 11a, 11b & Tab 11, 12). The trend of decrease in pH with an increase in Al conc was observed in all the cultures. Similarly, the pH of culture filtrate decreased with an increase in oxalic acid, even though there were variations in this trend. A positive correlation was found between the Al conc and oxalic acid in case of h1, which verified that the fact that with an increase in Al conc, there was more exudation of acids in h1. Thus, tolerance mechanism of organic acid exudation operated well in h1. In the other fungi- MAR, Pt-P and Pt-N, no correlation was found, which suggested that these fungi didn't respond well to Al stress (by exuding organic acids). Thus, the tolerance mechanism of organic acid exudation failed in case of Pt-P, Pt-N and MAR.

Organic acid production by mycorrhizal Populus deltoides in response to Al

Micropropagated *Populus deltoides (G 48)* was used in this case. The plantlets were placed in the glasstubes containing beads. They were inoculated with *Paxillus involutus (MAR*). Al in the form of $Al_2(SO_4)_3$.16H2O was added so as to give the final conc of 0,100,150,200 and 250 μg/ml of Al. After 30 days, organic acid estimation by RP-HPLC was done so as to quantitate the acids. The results showed that there was an increase in oxalic acid in mycorrhizal plants as compared to nonmycorrhizal plants.

Concentrations of oxalic acid were significantly higher in mycorrhizal treatments (*Paxillus involutus* - MAR) than in nonmycorhizal treatments with mean values between two to ten times greater in mycorrhizal than in nonmycorrhizal treatments. In controls, the amount of oxalic acid produced was more in case of mycorrhizal than in

nonmycorrhizal plants. In nonmycoorrhizal plants, the amount of oxalic acid produced at 250 μg/ml Al was one-hundredth of that produced in case of mycorrhizal plant. The amount of oxalic acid produced followed the trend of increased exudation of oxalic acid in response to Al. From these results, it was thus concluded that the tolerance mechanism of organic acid exudation operated in case of mycorrhizal *Populus deltoides*.

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APPENDIX I

Modified Melin's medium (Melin 1921)

Salts	concentration
$CaCl_2$. $2H_2O$	50 mg/L
NaCl	25 mg/L
KH_2PO_4	500 mg/L
$(NH_4)_2HPO_4$	250 mg/L
$MgSO_4.7H_2O$	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 Micronutriennts (100 X)	10.0 ml/L
pH	5.5

Heller's Micronutrients (100 X) (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 °C

APPENDIX II

Murashinge and Skoog media (Basal MS media) :

Major Elements	**Concentration (mg/L)**
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor Elements	
H_3BO_3	6.2
$MnSO_4.H_2O$	16.9
KI	0.83
$ZnSO_4.7H_2O$	8.6
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.5
$FeSO_4.7H_2O$	27.8
Vitamins and organics:	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Glycine	2.0
Sugar	30000
pH	5.8
Solidifying agent	
Agar	**0.8%**

MS (Shooting media with phytohormones):

MS Basal + Phytohormones:

Phytohormones:

BAP	1mg/ml
Kinetin	1mg/ml

APPENDIX III

Poplar media shooting (PDI media):

Major Elements	**Concentration (mg/L)**
$(NH_4)_2SO_4$	250
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	450
KH_2PO_4	170
Minor Elements	
H_3BO_3	6.0
$MnSO_4.H_2O$	20
KI	0.80
$ZnSO_4.7H_2O$	8.0
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.5
$FeSO_4.7H_2O$	27.8
Vitamins and organics:	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Glycine	2.0
Ascorbic acid	10
Adenine Sulphate	15
Sugar	30000
pH	5.8
Solidifying agent	
Agar	0.8%

PDI (Shooting media)

PD Media + Phytohormones

Phytohormones:

BAP	0.5mg/ml
Kinetin	0.5mg/ml

APPENDIX IV

PDR (Rooting Media):

Major Elements	**Concentration (mg/L)**
KNO_3	1900
NH_4NO_3	1650
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	450
KH_2PO_4	170
Minor Elements	
H_3BO_3	6.0
$MnSO_4.H_2O$	20
KI	0.80
$ZnSO_4.7H_2O$	8.0
$Na_2MoO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Na_2EDTA	37.5
$FeSO_4.7H_2O$	27.8
Vitamins and organics:	
Myoinositol	100
Nicotinic acid	0.5
Pyridoxine-HCl	0.5
Thiamine-HCl	0.1
Glycine	2.0
Ascorbic acid	10
Adenine Sulphate	15
Sugar	30000
pH	5.8
Solidifying agent	
Agar	0.8%

PDI (Rooting media)

PD Media + Phytohormones (IAA-1mg/ml)

APPENDIX V

ABBREVATIONS

Al : Aluminium

ANOVA : Analysis of Variance

BAP : Benzyl amino purine

Ca : Calcium

Df : Degrees of Freedom

EM : Ectomycorrhizal fungi

F : F-value

HCl : Hydrochloric acid

Kn: Kinetin

KOH : Potassium Hydroxide

LMW : Low Molecular Weight

Mg : Magnesium

MMN : Modified Melin Norkran's media

MQ :

MS : Mean Sum of Squares

Ni : Nickel

O.D. : Optical Density

P : Probability

Pb : Lead

PNP : para- Nitro Phenol

RP-HPLC : Reverse Phase High Performance Liquid Chromatography

SS : Sum of Squares

VAM : Vesicular Arbuscular Mycorrhiza