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Salinity Tolerance of Selected Ectomycorrhizal Fungi (Pisolithus tinctorius Pers.) and Ectomycorrhizal Eucalypts

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Salinity tolerance of selected ectomycorrhizal fungi (*Pisolithus tinctorius* Pers.) and ectomycorrhizal eucalypts.

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Abstract

Increasing soil salinity has become a major problem worldwide. It has led to a reduction in the amount of arable land, has put at risk the supply of freshwater and threatens the existence of many natural habitats. The major increase in salinity has been attributed to human activities such as clearing of natural vegetation and large-scale irrigation programmes. The alleviation of this problem has focussed on changed management strategies, the most significant of which is the re-establishment of deep rooted plants in salt affected areas. This, however, is difficult because of the variation in salt tolerance of such plants and the problems created through nutrient deficiencies characteristic of such sites. This study investigated the role of ectomycorrhizal (ECM) associations in assisting eucalypts tolerate soil salt.

The response of specific isolates of *P. tinctorius* Pers. To salinity *in vitro* was used to determine which may be the most effective when transferred to saline soils. All isolates tested appeared to be at least tolerant or semi-tolerant to 150 mM NaCl. However, the different isolates produced different patterns of colony growth, making assessment of growth rates, and therefore salt tolerance, difficult.

Inoculation of *E. camaldulensis* Dehnh. and *E. diversicolor* F. Muell. with spores of field collected *Scleroderma* species and *Pisolithus* tinctorius improved salt tolerance of *E. camaldulensis* but not *E. diversicolor*. Inoculation of *E. diversicolor* and *E. camaldulensis* seedlings and clones with *P. tinctorius* isolates used in *in vitro* studies, showed no significant growth response to salinity. This may be attributed to poor development of ECM structures within the root zone of these plants.

Root and shoot proline content showed significant responses to both inoculation with ECM fungi and salt treatment. These results did vary between experiments. Further research into the use of ECM to alleviate the problem of soil salinity is justified by this study. The development of new, and improvement of current techniques is discussed in light of these findings.

DECLARATION

I certify that this thesis does not, to the best of my knowledge and belief:

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Ben Bradshaw

26th June 2000

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CHAPTER 1: INTRODUCTION

Salinity is one of the most significant problems threatening the productive capacity of arable land and freshwater resources necessary to sustain an increasing global population. It is human induced salinisation (secondary salinity) that has received greatest attention in recent years, particularly in Australia. Large scale changes in the natural environment since European settlement and subsequent land management practices are believed to have been the cause of secondary salinisation of both dryland and irrigated farmland (Oliver *et al.*, 1996). Removal of deep rooted native plants and their replacement with shallow rooted crop and pasture species results in increased groundwater recharge and associated mobilisation of natural salts that are stored in the soil profile (Salinity Committee, 1984). The level of salt in groundwater that has reached the surface significantly reduces agricultural productivity and generally renders the land unsuitable for any form of agriculture. In addition, it has impacted upon natural systems where natural ecological processes are affected, reducing biodiversity and degrading the aesthetic character of these natural areas (Saunders and Hussey, 1996).

The rehabilitation of salt affected land in many situations requires the management of groundwater levels. Typically this involves increasing water use by employing a number of strategies including the planting of perennial species, particularly woody shrubs and trees (Government of Western Australia, 1996; Macar *et al.*, 1991). Selection of trees and shrubs for rehabilitation of saline areas has attracted greatest attention in recent years with the increasing need to reduce the effects of salinity on agricultural land, infrastructure and potable water supplies. Tree species selected for salinity control must have the ability to

survive and grow in salt affected soils and as far as possible provide other products and benefits (Morris and Thompson, 1983).

The mechanisms of salt tolerance in plants have been reviewed extensively by a number of authors including, Munns (1993),Yeo (1983), Greenway and Munns (1980) and Flowers *et al.* (1977). Many of the mechanisms identified, in both halophytes and non-halophytes, have been recognised as important attributes of plants used in the reclamation of salt affected areas as well as the long term control of rising water tables.

The mechanisms by which plants alleviate salt stress vary between and among major plant groups. Halophytes grow at soil concentrations of between 200 and 500 mM NaCl. Some halophytic species do this by accumulating and compartmenting ions (specifically Na^+ and Cl^-) to maintain turgor (Larcher, 1975). High levels of these ions in the vacuole ensure osmotic balance is maintained, and that excessive ion concentrations do not affect the metabolic processes of the cytoplasm. In addition to ion regulation, the accumulation of neutral organic solutes or osmoprotectants (such as betaines and proline) contributes to the maintenance of the osmotic potential of the cytoplasm (Hare and Cress, 1997; Greenway and Munns, 1980). Some plants, termed salt regulators, may actively exclude Na^+ and Cl^- from the roots such as the case with many mangrove species. Other salt regulators may take up salt readily but will often employ salt glands as a means of removing excess salt from the leaf tissue (Flowers *et al.*, 1977).

The degree of sensitivity of non-halophytes to external NaCl also varies within and among plant species. While excessive ion uptake is uncommon, compartmentation of ions into

vacuoles and the accumulation of neutral organic solutes are adaptations for coping with high external ion concentrations. The relative efficiency of such processes, however, is much reduced and has greater variability compared to halophytes (Greenway and Munns, 1980).

Avoidance of excessive ion concentrations within non-halophytes can occur through control of ion uptake and subsequent transport to the shoot where ions can be removed (Greenway and Munns, 1980). Ion exclusion is also employed by non-halophytes which rely on turgor maintenance as a means of alleviating the internal water deficit. Synthesis of organic solutes is responsible for turgor maintenance as well as an increased leaf thickness which optimises water use efficiency. Greenway and Munns (1980) also suggest turgor is maintained by increasing root permeability to water. One means by which this may occur is via the infection of the plant root by mycorrhizal fungi that can increase water absorption and potentially alleviate water stress associated with salinity (Juniper and Abbott, 1993). Mycorrhizal fungi have been identified as significant components of many terrestrial ecosystems and have a number of functional roles within plant and soil communities including increased water and nutrient absorption (Pankow *et al.*, 1991). They are extremely diverse in terms of their physiology and morphology in association with roots of host plants.

Fundamental differences in morphological and physiological characteristics differentiate the two most common forms of mycorrhizas. Ectomycorrhizas (ECM) are characterised by the profuse hyphal growth forming a sheath around plant root tips that is described as the mantle. Hyphae from the mantle penetrate the intercellular spaces of the root cortex but do not penetrate the cells of roots. In comparison, vesicular-arbuscular mycorrhizas do not

form a sheath enclosing the root tip. Hyphae of these fungal species penetrate root cortical cells where arbuscles are formed. Vesicles for product storage will also form within cortical cells of most but not all plant species (Brundrett *et al.*, 1996).

The ability of mycorrhizal fungi to mobilise important key nutrients such as phosphorus (P) and nitrogen (N) influences plant fitness and survival. Increasingly, they are being recognised as important for general health and nutrition of agricultural crops and plantation forests, and have also been applied in land reclamation. For example, the alleviation of heavy metal toxicity in plants as a result of mycorrhizal infection has been recognised in a number of studies (e.g. Wilkins, 1991; Aggangan *et al.*, 1998). Other studies have indicated mycorrhizal plants may benefit from the association under other environmental stresses such as high soil temperatures, acidic soils, water deficits, and low levels of soil phosphorus (Brundrett *et al.*, 1996). An important aspect of mycorrhizal associations not often considered is the mitigation of nutritional imbalances in plants grown in soils where nutrients may be in excess or in short supply (Bermudez and Azcon, 1996). In all of the above, little is understood of the mechanisms by which mycorrhizal fungi benefit the host plant.

The effects of saline soil conditions on the life cycle of mycorrhizal fungi and indeed the formation of the symbiosis with potential host plants is not well understood. Juniper and Abbott (1993) have reviewed the response of VAM to salinity, however, no studies have focused on the response of ectomycorrhizas to salinity and the subsequent effect on the host plant. An understanding of the physiological response of both the host plant and the symbiont during salt stress has practical application in the reclamation of salt affected farmland and irrigated agricultural systems affected by salt.

1.1 Salinity - a Global Problem

The occurrence of salinity across the major continents has increased with the increasing intensity of human activity. Agriculture has perhaps been the most influential of all such activities and has drastically altered the landscape. Large scale removal of native vegetation to make way for intensified field cropping has resulted in rising water tables in many arid and semi-arid regions of the world. Irrigation, in conjunction with poor drainage, has also resulted in the accumulation of many different salts (predominantly NaCl) within the soil. It is important to recognise there are a number of different approaches to estimating the severity of salinity and this largely depends on the criteria used for its classification. However, all estimates indicate a disturbing increase in the land area affected by salt.

Salinisation of rivers, streams and dams has serious consequences not only for agriculture but also for human populations dependent on a constant supply of potable water. Indeed, salinisation is associated with many of the great river systems of the world including the Tigris and Euphrates flowing through Syria and Iraq, the Ganges north-west of India, the Mekong in Thailand's north-east, the Colarado River of the U.S.A., the Nile of Egypt and the Murray-Darling catchment of south-east Australia (McWilliam, 1986).

Irrigated land is significantly affected by salinisation. Of the approximately 270 million ha of irrigated land worldwide, between 20 and 30 million ha are severely affected by salinity and another 60–80 million ha are affected to some extent. In Pakistan, as much as 11 million ha of the 15 million ha of irrigated land are affected by salinisation and waterlogging (Qureshi *et al.*, 1996; Scheumann, 1997). Afghanistan, Egypt, Iran, Iraq,

Sudan, Syria and the former USSR also have similar problems. In the Xinjiang, of north-west China, more than half of the 3.4 million ha of arable land has been abandoned due to salinisation (Scheumann, 1997).

Dryland salinity (seepage salinity) is common in areas that have water tables relatively close to the surface, high rates of evaporation, and sporadic, variable levels of precipitation. Removal of the deep-rooted perennial vegetation of these areas results in a reduced interception of rainfall and a reduced rate of evapotranspiration, this increases the rate of groundwater recharge, resulting in a general rise in the water table. A rising watertable brings with it dissolved salts that accumulate at the soil surface as a result of evaporation of the surface water. Sporadic and variable rainfall does not allow sufficient leaching of the exposed salts through the soil profile further compounding soil salinity (McWilliam, 1986; Ralph, 1993).

The salts commonly associated with soil salinity have a number of origins. The principle source is weathered rock material but wind-borne oceanic salts and salt that has remained following the recession of marine waters during past geological periods are also recognised as important sources (Salinity Committee, 1984). The principle causes of the salinity problems observed throughout the world today are those human activities that contribute to the mobilisation, accumulation or exposure of salt that is already in the landscape. Accumulated salts are transported and redistributed throughout the landscape in close association with water. Hence, an understanding of the process of salinisation requires knowledge of the physicochemical interactions of the salts in the environment and also the hydrogeological characteristics of the landscape. Considerable research effort has focused

in these areas in an attempt to reduce the effects of salinity. Research has also focused on the salt tolerance of crop, fodder and tree species for use in saline soils, indicating the inevitability of dealing with salinity in agricultural systems in the future.

1.2 Salinity in Australia

Prior to European settlement the Australian continent had an estimated 28.2 million ha of naturally occurring saline land. This included salt marshes and large areas of dry saline land. Since European settlement a further 4.2 million ha (as at 1982) of saline land has been induced by changes in the landscape mainly through clearing for agriculture (Robertson, 1996). Robertson (1996) estimated this figure would increase at a rate of 5% per annum. Australia has the highest ratio of salt affected soils in relation to the total surface area of the continent (Szabolcs, 1989). For this reason, salinity (particularly secondary salinity) has become one of the most important environmental issues in Australia.

In Western Australia salinity has become the most immediate problem facing the agriculture industry. Robertson (1996) suggests there has been a more than 6-fold increase in the amount of land affected by dryland salinity in Western Australia since 1982. Ferdowsian *et al.* (1996) evaluated the extent of dryland salinity in Western Australia using a number of different data sources and suggested as much as 9.4% (or 1.8 million ha) of cleared land is already affected to some extent. More disturbing is that this area could double in the next 15 to 25 years (depending on rainfall) and then double again before reaching a stable hydrological equilibrium. It is this potential outcome that has research efforts focused on the alleviation of secondary salinisation.

Lost agricultural production in Australia is estimated to cost $130 million annually and the lost capital value of salt affected land has been estimated to be $700 million annually (Prime Minister's Science, Engineering and Innovation Council, 1998). The loss of productive agricultural land to salinity impacts on farming systems and therefore society. Rising saline watertables has a significant impact on infrastructure such as roads, buildings, parks, sewerage, water supply, telephone and electricity supply (Oliver *et al.*, 1996). Saline rivers and streams greatly affect urban water supply systems, necessitating expensive treatment of saline water, particularly that used by industry. These are all significant costs to government agencies and communities.

The environmental impact of salinity is not confined to loss of soil fertility and productivity. Rising water tables and saline watercourses also threaten native remnant vegetation. In the wheatbelt of Western Australia nature conservation areas have been adversely affected by salinisation. In addition, a further 80% of remnant vegetation on private land and 50% in public reserves is potentially at risk from increased salinity (Saunders and Hussey, 1996). Degradation of remnant vegetation and the associated fauna will influence the aesthetic character of, and the ecological processes operating within the landscape. Biological diversity and the integrity of the remaining natural ecosystems is a cost not often taken into consideration when evaluating the effects of salinisation.

1.3 Plant Response to Salinity

The most common plant response to salinity is a reduction in growth and yield, however, the threshold level and rate of growth reduction varies both within and among species (Maas, 1996). The tolerance of the plant to saline soil will depend on the mechanisms available for alleviating the osmotic and specific ion effects. Excessive Na^+ and Cl^- accumulation in the protoplasm results in osmotic imbalance and ion-specific effects on enzyme proteins and the membrane. This results in low levels of photophosphorylation with a subsequent reduction in nitrogen assimilation and protein metabolism, and an increase in the accumulation of polyamines (Larcher, 1975).

1.3.1 Salinity and Halophytes

The degree of tolerance varies among plant groups. Obligatory halophytes, found in saline habitats, have been shown to improve growth rates with a moderate uptake of salt. However, excessive salt uptake will impair growth (Flowers *et al.*, 1977). The principle means by which halophytes alleviate salt stress is maintenance of high internal Na^+ and Cl^- concentrations. These ions are sequestered in the vacuole rather than the cytoplasm thus minimising specific ion affects on cytoplasmic processes.

Maintenance of low water potentials in the cytoplasm may also be achieved through production of organic solutes such as glycinebetaine, proline and sucrose, however, evidence for the protective benefit of these solutes is lacking (Greenway and Munns, 1980; Munns, 1993). With low ion uptake in such plants, the contribution of organic solutes for

osmoregulation would require mechanisms for the accumulation of these solutes in the vacuole. This would also require significant amounts of photosynthate because the vacuole occupies up to 95% of the cell volume.

Tolerance of excess salt involves a number of mechanisms and may be grouped into three categories: avoidance, osmoregulation and adaptation. All these mechanisms involve the co-ordinated interaction of cells and tissues to facilitate salt tolerance in the plant. For example, transport and ion exchange mechanisms such as ion pumps of individual cells and tissues operate to remove excess Na^+ and Cl^- ions. Similarly, other mechanisms of salt tolerance require anatomical organisation that exists only in specific plant groups. Examples of such mechanisms can include salt exclusion, salt elimination, succulence and redistribution of salt (Larcher, 1975).

1.3.1.1 Salt exclusion

The greatest barrier to the passive movement of Na^+ and Cl^- into plant roots is the suberised endodermis. This causes all water to pass through the cell membrane which has low permeability to both Na^+ and Cl^-. Such transport barriers in the roots prevent excessive salinity in the water of the conducting system. Whilst predominantly a halophytic adaptation, most non-halophytes are generally considered to be salt excluders. In non-halophytes, salt ions, particularly Na^+, are also held back in the roots, lower stems, petioles and pedicels. This reduces the amount of salt reaching the meristems, developing leaves and fruits. (Larcher, 1975).

1.3.1.2 Salt elimination

Excess salts can be exuded by glands throughout the plant or excreted at the surface of the shoot, and lost by shedding older parts of the plant that are heavily loaded with salt. While this is occurring, the concentration of salts in the younger leaves remains within tolerable limits due to their expansion. Many halophytic species have this adaptation and include *Plantago*, *Aster* and *Atriplex* species.

1.3.1.3 Succulence

The development of succulence is a common morphological feature of many halophytes. An increase in leaf volume to enable greater water intake as a result of increased salt uptake ensures salt concentration remains low in the cells (Salisbury and Ross, 1992). Halophytes found in wet saline environments commonly exhibit this form of salt regulation. *Mesembryanthemum crystallinum* is known to utilise succulence as a means of salt regulation (Flowers *et al.*, 1977).

1.3.1.4 Redistribution of salt

Na^+ and Cl^- are readily redistributed throughout the plant in the phloem, reducing concentrations in actively growing regions. Sodium and chloride ions are likely to be mobilised via source to sink mechanisms in the plant. For this reason excessive concentrations of these ions in actively growing regions may be prevented through the same mechanisms that mobilise photoassimilate within the plant (Larcher, 1975).

1.3.2 Salinity and non-halophytes

Salinity limits vegetative and reproductive growth of non-halophytes by inducing severe physiological dysfunctions that may have many direct and indirect effects on physiological processes at the cellular and whole plant level (Kozlowski, 1993). The accumulation and compartmentalisation of ions and organic solutes as a means of dealing with NaCl stress has received considerable attention in studies of salt resistance. Non-halophytes may accumulate organic acids and neutral organic solutes to lower internal osmotic potential (Hare and Cress, 1997). The compartmentalisation of specific ions is another means of reducing specific ion effects on cellular processes. This is done by means of actively transporting specific ions primarily into the vacuole where their accumulation creates an osmotic gradient and alleviates specific ion effects that occur within the cytoplasm.

Greenway and Munns (1980) suggest non-halophytes are affected by either ion excess in the expanded tissues or by water deficits in expanding tissues. Concentrations of 100 mM NaCl are optimal for halophytes, but significantly reduce the growth of non-halophytes (Greenway and Munns, 1980). High ion concentrations or "ion excess" may affect membrane permeability or enzyme activity of non-halophytes. Expanding tissues are likely to handle ion excess by sequestering Na^+ and Cl^- in the growing vacuole whereas fully expanded tissues have reduced storage capacity in the vacuole reducing net uptake. Excessive concentrations of salt ions will accumulate rapidly in the cell wall prior to their build up in the cytoplasm due to the efflux of salt from the cytoplasm (Munns, 1993). The build up of salt in the cell wall results in the rapid dehydration of the cell.

1.4 Mineral Nutrition in Saline Soils

Non-halophytes in saline soils have low nutrient-ion activity and extreme ratios of Na^+/Ca^{2+}, Na^+/K^+, Ca^{2+}/Mg^{2+} and Cl^-/NO_3^-, causing ionic imbalance and competitive substitution in plant cells and tissues (Gratten and Greive, 1992; Maas, 1996). A number of studies have shown that salinity reduces N uptake. This is most likely the result of an increase in Cl^- uptake at the expense of NO_3^- (Helal *et al.*, 1975; Aslam *et al.*, 1984). Munns and Termaat (1986) suggest that although N deficiency occurs in NaCl treated plants there is limited evidence to suggest that this limits growth significantly. Gratten and Greive (1992) reported that despite the drastic reductions in NO_3^- levels in the leaves as a result of salinity, other N-containing fractions such as proline and glycine-betaine may either increase or decrease in concentration. It has, therefore, been suggested that N deficiency does not result in salt injury of non-halophytes in saline soils.

Phosphorus (P) nutrition in salt stressed plants varies depending on the level of the salt and the availability of P in the growing medium. The addition of P improves plant growth in saline soils but it may simply be the result of increased availability of P and it is not likely to improve the tolerance of the plant to high levels of salinity (Gratten and Greive, 1992). Nonetheless, salinity may increase the P requirement of certain species and cultivars. Gratten and Greive (1992) suggest that P accumulation in plant tissues is the result of enhanced uptake by plant roots in response to salinity. This raises the question of what is happening at the membrane level that causes excessive P uptake despite the reduced P activity in the presence of salinity. It is unlikely that Cl^- is competitive with $H_2PO_4^-$ in terms of uptake by the plant (Champagnol, 1979).

Potassium (K) is also required in adequate quantities by plants but is often found in very low concentrations in the soil. Typically, it is unavailable as it is readily adsorbed onto the surface of soil particles. The plasma membranes of root cortical cells have a high affinity for K^+, but Na^+ may compete for K^+ binding sites (Grattan and Greive, 1992). This is of importance in saline and sodic environments where Na^+ concentrations are high, particularly given evidence for the substitution of K^+ by Na^+ in some non-halophytes (Marschner, 1986). A number of studies cited by Grattan and Greive (1992) suggest that salt-sensitive plants have a lesser affinity for K^+ than do salt-tolerant varieties and uptake mechanisms more readily substitute Na^+ for K^+. Little evidence exists that growth of these plants is increased as a result of Na^+ substitution. The addition of K^+ in both pot culture and field experiments has shown little tendency to fully alleviate the K^+ deficiency induced by competitive Na^+ substitution (Grattan and Greive, 1992). This implies that plants growing in saline soils will encounter K^+ deficiency depending on the concentration of Na^+ in the soil medium and the degree of selectivity for K^+ over Na^+ in such plants.

Calcium plays a critical role in plant nutrition and the physiology of metabolic processes. It is involved as a second messenger of cellular proteins (Bressan *et al.*, 1998), the maintenance of structural and functional integrity of cell membranes and particularly in enzyme activities of the cell wall (Grattan and Greive, 1992). Interference with normal function of the enzymes of the cell wall is significant due to the occurrence of competitive substitution. Substitution of Ca^{2+} with other competitive cations can occur and significantly reduce the activity of functional proteins that are Ca^{2+} dependant.

As an example, Cramer *et al.* (1987) studied the effects of NaCl in cotton roots and found that Na^+ displaced Ca^{2+} increasing the permeability of the membrane such that a loss of K^+/Na^+ selectivity resulted (K^+ leakage). Subsequent exogenous application of Ca^{2+} restored membrane integrity and reduced K^+ leakage. This form of nutrient disorder was also observed in corn plants (Maas and Greive, 1987). Therefore, the high K^+/Na^+ selectivity within plants is dependent on the calcium status of the root. Calcium is the dominant cation of most soils, and is therefore not considered to limit growth by means of deficiency. However, under saline conditions the activity of Ca^{2+} is typically reduced and subject to ionic interactions, particularly with Na^+ and Mg^{2+}.

The concentration of micronutrients in the soil is generally low depending on the characteristics of individual soils. The solubility of many micronutrients is increased in saline soils (Gratten and Greive, 1992). However, the availability of micronutrients is likely to depend on macronutrient ratios (Mass *et al.*, 1972). The complexity of plant nutrition in saline environments is highlighted in studies attempting to evaluate the status of micronutrients in the plant and within the soil. Many studies show conflicting results and inconsistent conclusions as to the status of specific micronutrients. Despite their importance in plants growing in non-saline soils, their relative importance in saline conditions may be insignificant when compared to macronutrients.

1.5 Mycorrhizas as Ecosystem Components

Mycorrhizal associations are those formed between mutualistic host plants and fungal symbionts. Mycorrhizal symbioses are characteristic of most terrestrial plants including many crop plants (Pankow *et al.*,1991). They are highly evolved associations between a plant root and a fungus and are known to aid plant growth by means of increased nutrient acquisition (Marschner and Dell, 1994; Read, 1991). The primary means by which this occurs is the increase in the absorbing surface area of the fungal hyphae.

Basidiomycetes (*Basidiomycota*), ascomycetes (*Ascomycota*) and zygomycetes (*Zygomycota*) all have species that form mycorrhizal associations across a broad range of vascular plants (Newman and Reddell, 1987). The morphological patterns and physiological characteristics of different species in association with the host plant allows the recognition of a number of different association types. The two most common are vesicular-arbuscular mycorrhizas (VAM) and ectomycorrhizas (ECM) that are formed primarily by zygomycetes and basidiomycetes, respectively (Brundrett *et al.*,1996; Harley, 1986).

The morphological characteristics of VAM and ECM associations are strikingly different. In VAM associations the fungus forms a network of intercellular hyphae within the root cortex which branch and penetrate the cell wall and form arbuscules (Dexheimer and Pargney, 1991). Vesicles then develop for the storage of accumulated products. Fungal hyphae of ECM associations contact and adhere to root epidermal cells close to the apex of an actively growing lateral root. Mycelia develop and envelop the root apex covering the epidermal cells forming a mantle. Hyphae then penetrate between epidermal cells to form

the characteristic Hartig net that becomes the principle exchange site in the association (Harley and Smith 1983 and Brundrett *et al.*, 1996).

Pankow *et al.* (1991) described the mycorrhizal association as an extension of a plants potential for exploring the soil. The ability of mycorrhizas to mobilise key nutrients from within the soil and deliver them to the plant has a profound influence on the health and vigour of the host. However, the increase in available nutrients comes at a cost to the host which provides carbon compounds to the growing fungal hyphae.

Another suggested functional role of mycorrhizas is increased water absorption (Harley and Smith, 1983; Brundrett *et al.*, 1996). The presence and maintenance of extramatrical hyphae has been suggested as a functional extension of the root system in mycorrhizal plants where water absorption is of paramount importance (Read, 1986). As an example, Goicoechea *et al.* (1998) found that symbiotic alfalfa were better able to cope with water stress conditions imposed under controlled conditions. Such rapid recovery from induced water stress in these mycorrhizal plants indicates that specific mechanisms devoted to efficient absorption of water are in place. Little is known of these specific mechanisms, however speculation centers on greater water absorptive capacity of the extramatrical hyphae.

The alleviation of heavy metal toxicity in mycorrhizal plants has received considerable attention recently (Brundrett *et al.*, 1996). The sequestration and storage of heavy metals in hyphal structures has been proposed as a means of alleviating toxicity in mycorrhizal plants (Galli *et al.*, 1994; Leyval *et al.*, 1997). The physiological mechanisms of heavy metal tolerance are poorly understood and are complicated by genetic interactions between the host plant and fungal partner, and soil characteristics, particularly pH.

Soil pH has a significant impact on the availability of specific nutrients, particularly phosphorus. Gupta and Krishnamurthy (1996) have demonstrated that mycorrhizal plants have greater protection from extremes of pH. Their study examined the effects of acid (HCl) stress on peanut (*Arachis hypogea*). Infection by VAM in these plants alleviated acid stress, however, the mechanisms of HCl tolerance were not explored. Aggangan *et al.* (1996) examined the effects of pH extremes on ectomycorrhizal formation and subsequent growth of *Eucalyptus urophylla.* This study showed that not all fungal isolates of *Laccaria*, *Pisolithus* and *Scleroderma* were capable of alleviating extremes in soil pH. Nevertheless all inoculated plants showed greater biomass production at pH 6.6 than uninoculated control plants. It is not known if mycorrhizas offer any form of protection to the host plant or whether tolerance to acid soils is a product of increased nutrient availability in mycorrhizal plants (Sylvia and Williams, 1992). Attempts to differentiate between direct tolerance of pH extremes and increased tolerance due to increased nutrient uptake have not been documented.

Disease resistance in mycorrhizal plants has seen limited exploration despite the potential applications of such knowledge. Read (1986) suggests mycorrhizal plants could be resistant to disease simply by improved host vigor and by the physical presence of the fungus competing for scarce resources in soil. This is particularly true of ECM that form profuse hyphal structures around the short roots providing a physical barrier to potential pathogens.

Marx (1973) reported the potential role of mycorrhizas, particularly ectomycorrhizas, as a biological deterrent to pathogenic fungi. Mechanical and chemical barriers to pathogenic infection were suggested as possible mechanisms for combating infection of short roots.

Malajczuk and Hingston (1981) reported unpublished data indicating that ectomycorrhizal infection of *Eucaluptus marginata* may reduce the pathogenicity of *Phytophthora cinnamomi*. More recently, Branzanti *et al.* (1999) studied the effect of ECM colonisation of *Castanea sativa* (chestnut) seedlings on chestnut ink disease caused by *Phytophthora cambivora* and *P. cinnamomi*. Results of this study suggested inoculation with a number of ECM species increased plant biomass, reduced the presence of zoospores and prevented root penetration by the pathogenic fungi. Electron microscopy demonstrated that no hyphae of the pathogenic fungi penetrated the Hartig net. Hence the presence of a fungal mantle was suggested as a physical barrier to both spores and hyphae of both *P. cambivora* and *P. cinnamomi*. Chemical suppression (e.g. antifungal compound production) and competition between mycorrhizal fungi and pathogenic fungi were also indicated as possible mechanisms for protection against chestnut ink disease. It was further suggested that the presence of ECM fungi on short roots inhibited zoospore germination and reduced hyphal growth of *P. cambivora* and *P. cinnamomi*.

The alleviation of salt stress in mycorrhizal plants has been examined in a number of studies including: Azcon and El-Atrash (1997), Ruiz-Lozano *et al.* (1996), Juniper and Abbott (1993), Rosendahl and Rosendahl (1991), Pfeiffer and Bloss (1988) and Ojala *et al.* (1983). Initial observations of experimental data suggested P nutrition was critical in alleviating salt stress. More recently, however, studies have indicated nutrition is one of many possible factors contributing to salinity tolerance of mycorrhizal plants and is discussed in a later section of this review.

A review by Pankow *et al.* (1991) concluded that the main ecological significance of mycorrhizas was their protective rather than their productive capacity within the ecosystem that is of greatest importance. Researchers have tended to focus on the productive capacity of mycorrhizas in individual plant growth and ecosystems rather than the protective role during the early stages of development and at maturity. Hence an understanding of the protective role of mycorrhizas in specific environments is required to develop a greater understanding of their ecological significance. Read (1991) suggests the major means of protection to plants by mycorrhizal infection is associated with the provision of key growth-limiting nutrients at crucial stages of the plants development. However, this will depend on the nature of the environment in which the host plant and the fungal symbiont is found. The survival and functionality of the symbiosis is therefore of paramount importance in assessing the protective ability of mycorrhizas in specific environments.

1.6 Nutrition of Mycorrhizal Plants

Nutrition of mycorrhizal plants has been the focus of many studies since the recognition of the relationship. Most studies have examined the benefit to host plants of increased nutrients made available by mycorrhizal fungi. Examination of plant material in earlier studies demonstrated that concentrations of nutrients in mycorrhizal plants were higher in comparison comparison with non-mycorrhizal plants (Harley and Smith, 1983). The range of nutrients more readily available to mycorrhizal plants via fungal hyphae include P, N, K, Ca, S, Cu, Zn and Fe (Marschner and Dell, 1994).

The uptake of P and N of mycorrhizal plants has been studied extensively. Increased uptake of P is primarily the result of increased exploration by the external hyphae of mycorrhizas (Grove *et al.*, 1996). The extension of external hyphae to the surrounding soil greatly increases the absorptive capacity of soluble P sources. In addition, the storage of P within the hyphae in the form of polyphosphates maintains low internal phosphate concentrations thereby facilitating greater P uptake (Marschner and Dell, 1994).

The availability of N to plant roots is dependant on the source of N present in the soil. Nitrate (NO_3^-) is the most mobile source of N in soils and is therefore the primary source of N uptake in plant roots. Another, less mobile, source of N in soils is NH_4^+ and this also contributes to N nutrition. Marschner and Dell (1994) suggest external VAM hyphae are more likely to utilise NH_4^+ as the principle source of N as opposed to NO_3^-. Interestingly, France and Read (1983) found that ECM are more likely to utilise, and may even prefer, NH_4^+ as the principle source of N in culture studies. This would suggest a greater availability of N to mycorrhizal plants via the external hyphae that are better placed in the soil matrix than plant roots, given the immobility of NH_4^+ in the soil.

The secretion of ectoenzymes by the external hyphae of ECM fungi is another means by which limited nutrients become available to mycorrhizal plants. These enzymes greatly influence the availability of nutrients that are present in organic forms and also those nutrients that are adsorbed on Fe or Al oxides in the soil. For example, oxalic acid produced by mycorrhizas and plant roots in calcareous soils can release phosphates from Fe and Al oxides (Marschner and Dell, 1994) and Ca phosphates (Lapeyrie *et al.*, 1990). In conjunction with the release of P from Ca phosphates, oxalic acid binds Ca to prevent

excessive uptake into fungal hyphae (Lapeyrie *et al.*, 1990).

Similarly, the secretion of extracellular acid proteinases by ECM releases organically bound N that would normally be unavailable to non-mycorrhizal plants. It is stressed by Marschner and Dell (1994) that the degree of total N assimilated by this means has not yet been quantified and may vary greatly between fungal species and even individual isolates. The presence of such secretary substances in mycorrhizal fungi may therefore be of particular benefit in soils in which specific nutrients are limited. Similar studies have failed to determine the presence of similar enzymes in VAM.

Studies of the uptake of other macro and micronutrients by mycorrhizal plants are not yet as thorough. Generally it has been noted that those nutrients that have low mobility in the soil are more likely to be increased in the roots of mycorrhizal plants. Similarly, highly mobile nutrients in the soil may have increased uptake in mycorrhizal plants given the increased absorptive surface area of mycorrhizal roots and associated external hyphae. Uptake of highly mobile nutrients such as K, Ca and Mg, is limited by the uptake capacity of roots (Vogt *et al.*, 1991). The availability and mobility of such ions is dependent on soil type and studies have shown variable levels of uptake of these ions by mycorrhizal plants (Read, 1991; Bermudez and Azcon, 1996).

Studies of micronutrient uptake in mycorrhizal plants have focused on heavy metals both those important for growth and those that have no known biological function such as Cd, Pb and Hg. A number of reviews discuss heavy metal tolerance in mycorrhizas and their possible use in bioremediation of contaminated sites (Galli *et al.*, 1994; Hartley *et al.*, 1997; Leyval *et al.*, 1997).

Some experimentation under controlled conditions has allowed researchers to propose possible mechanisms by which mycorrhizas deal with excess heavy metals (Galli *et al.*, 1994). For example, heavy metals may be bound in the extramatrical mycelium of mycorrhizas with Zn and Cd being sequestered, not in the cytoplasm of hyphal cells, but in the cell walls of hyphae and the interhyphal spaces. Zinc has also been found in the extra-hyphal polysaccharide slime. Components such as chitin, cellulose and also melanins may be the primary means of cell wall binding of heavy metals in fungal hyphae (Leyval *et al.*, 1997). It has been suggested the abundant production of extramatrical mycelium provides increased protection to the host plant, particularly of ECM plants and that a high turnover of mycelium is required to maintain this binding capacity (Hartley *et al.*, 1997).

The intracellular compartmentation of heavy metals by polyphosphate granules has been suggested for sequestration in ECM. Initial observations showed a relationship between increased presence of heavy metals in mycelium and increasing levels of P suggesting polyphosphate granules were involved in heavy metal sequestration. However, debate continues as to whether polyphosphate granules exist in fungal cells or whether they are artifacts of specimen preparation (Galli *et al.*, 1994; Hartley *et al.*, 1997).

Other proposed mechanisms of protection from heavy metals include; intracellular chelation and compartmentation in the fungal vacuole (Hartley *et al.*, 1997; Galli *et al.*, 1994), and the fungal sheath acting as a physical barrier preventing the apoplastic movement of heavy metals (Hartley *et al.*, 1997).

The mechanisms proposed for heavy metal tolerance, particularly of ECM, suggests strongly the protective capacity of mycorrhizas in disturbed areas such as disused minesites undergoing plant recolonisation. Hartley *et al.* (1997) suggest that if the mycorrhizas are protecting the host plant from heavy metal toxicity then mechanisms might also be in place to protect the fungus from these same, if not higher, levels of heavy metals. The health and functional status of the symbiont is, therefore, of critical importance when considering the protective role of mycorrhizas in any stress environment.

1.7 Mycorrhizas and Salinity

Studies of mycorrhizas in saline environments have focused on plant growth and fitness with relatively little attention directed at the protective role of mycorrhizas in saline soils. Few studies have related specifically to the biology and ecology of mycorrhizal fungi in saline soils, and none have involved ectomycorrhizas (ECM).

1.7.1 Vesicular-arbuscular mycorrhizas (VAM) and salinity

Juniper and Abbott (1994) focused on the affects of salinity on VAM in both field and controlled conditions. They examined the effect of salinity on the life cycle of the fungus, hyphal growth and formation of the symbiosis. They suggest experimental design and methodologies have not allowed the effects of salinity on fungal growth to be separated from plant mediated effects. The difficulty, to date, in initiating or maintaining VA fungi in culture has no doubt contributed to the absence of data.

The occurrence of mycorrhizas in naturally saline soils has been evaluated in a number of studies (Kim and Weber, 1985; Bhaskaran and Selvaraj, 1997). Kim and Weber (1985) found fungal spores in soil core samples taken from salt playas. A negative correlation between spore numbers and mycorrhizal roots with increasing Na concentration was apparent and no spores were observed where Na concentrations exceeded 20 000 ppm (~340 mM). The spores were identified as those of VA mycorrhizas and there was no report of the presence of ECM spores.

The incidence of mycorrhizal halophytes is generally quite low (Brundrett, 1991) although an earlier study conducted by Allen and Cunningham (1983) found that *Distichlis spicata* inoculated with VA mycorrhizas and exposed to three different salinity levels was unaffected by mycorrhizal infection. They concluded that mycorrhizal infection had no affect on the level of salt tolerance in *D. spicata.* Another study involving leguminous *Strophostyles helvola* (a non-halophytic occupant of beaches and foredunes) showed increased chlorophyll content and increased shoot dry weight in VAM plants exposed to high NaCl concentrations (Tsang and Maun, 1999). Growth of *S. helvola* was affected by increased salt concentrations but the effects were partially mitigated by VAM colonisation.

Many studies have shown positive responses of VAM inoculated non-halophytic plants in controlled saline soil conditions. Hirrell and Gerdemann (1980) showed that P concentrations increased in onion and pepper plants infected with VAM and overall yield was improved, as was salinity tolerance (as measured by plant survival). Similarly, Ojala *et al.* (1983) found that growth and yield in onion infected with VAM was increased and the nutritional status of mycorrhizal plants was similar to, if not greater than, treatment

plants supplemented with additional phosphorus. Hence, improved P nutrition as a result of mycorrhizal infection has been seen as the mechanism by which salt stress can be alleviated in mycorrhizal plants.

A more recent study of lettuce (*Lactuca sativa* L.) inoculated with three species of *Glomus* indicates that the alleviation of salt stress by VAM infection is based on physiological processes other than nutrition (Ruiz-Lozano *et al.*, 1996). No differences in P content of plants were observed between mycorrhizal treatments at three levels of salinity (2.5, 3.2 and 4.0 dS m^{-1}). The rates of CO^2 exchange, transpiration, stomatal conductance and water use efficiency were improved with increasing salinity, but only in mycorrhizal plants. They concluded that *Glomus* spp. alleviated salt stress by means other than P nutrition. Azcon and Eltrash (1997), working with alfalfa (*Medicago sativa* L.) and *Glomus mosseae* (VAM fungi) concluded that mycorrhizal plants had alternative means of satisfying nutritive requirements in saline conditions. They suggested this may be due to the prevention of excessive cation uptake affecting the cation/anion balance in the plant.

Whilst improved growth and yield of mycorrhizal plants in saline environments have been shown in a number of studies, the mechanisms operating to alleviate salt stress are relatively unknown. The ability of non-mycorrhizal fungi to grow in saline conditions may be dependent on their ability to maintain low internal osmotic potentials either by uptake of ions or by the synthesis of polyols (Juniper & Abbott, 1993). Whether similar mechanisms of osmoregulation occur in mycorrhizal fungi is speculative and requires further investigation.

1.7.2 Ectomycorrhizas (ECM) and salinity

There are limited published investigations of ECM plants in saline conditions. Tresner and Hayes (1971) found basidiomycetous fungi, as a group, to be relatively intolerant of NaCl compared to other fungal groups. In contrast, Dixon *et al.* (1993) suggested that selected ectomycorrhizal fungi can tolerate soil salt stress and consequently improve host nutrition. An investigation of the *in vitro* salt tolerance of *Pisolithus tinctorius* (an ECM species) suggests that at optimum Na salt concentrations, mycelial production may be promoted (Nagarajan and Natarajan 1999). The increased levels of the three Na salts used in this experiment (NaCl, Na_2SO_4 and $Na_3C_6H_5O_7$) is not necessarily comparable to increased levels of NaCl typical of saline soil. However, it does provide an indication of potential salt tolerance in ECM fungi. The lack of published material on salinity tolerance of ECM plants and fungus is unusual given the relative ease of *in vitro* maintenance of cultures and the initiation and maintenance of the symbiosis in culture conditions (Brundrett *et al.*, 1996; Peterson and Chakravarty, 1991). The salt tolerance of the ECM association is neglected and requires attention given the potential application of such technology in forestry and agriculture.

1.8 Basis for Research

The protective role of mycorrhizas in ecosystems has been largely neglected and focus has been given to increased productivity by mycorrhizas. The response of mycorrhizal plants to salinity has been the subject of few studies. Plants in association with VAM have shown increases in growth and yield indicating mycorrhizal fungi can protect the host plant from salt stress. The mechanisms involved in salt tolerance of ECM fungi have not been studied in any practical terms. The response of ECM plants to salinity has not been studied. The *in vitro* response of ectomycorrhizal fungi to NaCl suggests certain species and certain isolates may tolerate such stress conditions (Nagarajan and Natarajan, 1999).

The mechanisms of salt tolerance by mycorrhizal plants, whilst largely unknown, may be a result of increased P and N supply to the host plant alleviating the effects of nutrient disorders associated with saline soils. Alternatively, mycorrhizal infection may reduce the toxicity or deficiency of specific ions by the regulation of ion uptake into the plant root. It follows that the maintenance of membrane integrity in the root cells of host plants will better enable the plant to maintain cation/anion balance in stress conditions.

The ability of ECM to sequester and mediate specific ions in the soil of sites polluted with heavy metals warrants the application of ECM technology to the problem of salinity. Despite there being little evidence for the sequestration of either Na^+ or Cl^- in hyphae of mycorrhizal fungi, alternative mechanisms of dealing with excessive concentrations of these ions is possible. If it can be shown that ECM plants have an increased tolerance of saline soil conditions then studies of potential mechanisms assume particular importance.

The examination of ECM infection of plants in saline soils is particularly important in eucalypt species given their role in the rehabilitation of salt affected land (Morris and Thomson, 1983; Pepper and Craig, 1986; Marcar *et al.*, 1993). Since ECM are associated with the majority of eucalypts, there may be considerable benefit to these species in adverse soil conditions. A large number of species and sub-species of ectomycorrhizal fungi can be screened to identify particular isolates that improve plant growth and yield in saline soils. This can be done without specific knowledge of tolerance mechanisms operating within and between the host and the symbiont.

The purpose of this study was to evaluate the response to salinity of selected ECM isolates and the subsequent mycorrhizal response of inoculated seedlings of *E. diversicolor* F. Muell. (salt sensitive) and *E. camaldulensis* Dehnh. (salt tolerant) and clonal lines of *E. camaldulensis* Dehnh.

The *in vitro* tolerance of a number of isolates of *Pisolithus tinctorius* Pers. were studied to examine their relative degree of tolerance of elevated levels of NaCl. Considerable genetic variation in isolates of *P. tinctorius* suggests functional variation may also occur in mycorrhizal associations of these isolates (Bougher, 1995; Cairney, 1999). Experimentation with these isolates allowed for the selection of fast growing and potentially salt tolerant isolates of *P. tinctorius* for inoculation of seedlings and clonal material for glasshouse experiments.

Spore inoculum of field-collected isolates of *Pisolithus* and *Scleroderma* species was applied to evaluate mycorrhizal response to salinity. It was expected the high level of infection of spore based inoculum would provide a sufficient level of infection to evaluate the affect of soil salinity on the presence of mycorrhizal structures in *E. diversicolor* and *E. camaldulensis* seedlings. Similarly the response of *E. diversicolor* and *E. camaldulensis* seedlings to inoculation with axenic culture material was evaluated. Growth and the levels of proline accumulation in the plants, was the primary means of evaluating the significance of inoculation with ECM fungi in saline conditions.

Consideration of the response of the mycorrhizal host plant to salinity was evaluated using salt sensitive and salt tolerant clonal lines of *E. camaldulensis*. Of importance was the response to salinity following inoculation with ECM isolates. The significance of the physiological well-being of the host plant to the symbiosis under saline conditions was of particular interest in this case. Levels of the organic solute proline were also determined to evaluate the stress response of inoculated and uninoculated plants to NaCl.

CHAPTER 2: IN VITRO SALT TOLERANCE OF SELECTED *P. tinctorius* ISOLATES.

2.1 Introduction

The salt tolerance of mycorrhizal fungi in axenic culture has seen limited investigation. Tresner and Hayes (1971) examined the tolerance of a range of genera within the ascomycetes and basidiomycetes and found all to be relatively intolerant of sodium salts. Later studies examining the degree of tolerance of ECM species to salt, suggested that at optimum concentrations of NaCl, hyphal growth may be promoted (Dixon *et al.,* 1993; Nagarajan and Natarajan, 1999).

Dixon *et al.* (1993) used survival and hyphal growth and protein content as a measure of salt tolerance of 6 species of ectomycorrhizas. The study found there were differences among species and isolates in salt tolerance, with species of *Pisolithus*, *Laccaria* and *Suillus* showing greater tolerance. Some treatments of low concentrations of NaCl stimulated growth in some test fungi, particularly two isolates of *P. tinctorius*. Biomass figures indicated *P. tinctorius* had greater biomass production at 30 and 120 mM NaCl in comparison to 0 mM. Similar figures were shown for the isolate of *Suillus luteus*. Protein content of *Suillus luteus* increased significantly with increasing salt concentration. The experimental isolate of *P. tinctorius* showed a significant decrease in protein content at 120 mM NaCl. By comparison, isolates of the genera *Thelephora* and *Cenococcum* were identified in the study as relatively intolerant with comparatively low rates of colony growth

with increasing NaCl concentrations. The isolate of *Thelephora terrestris* showed no significant change in protein content with increasing salt concentration. This study identified considerable variation in the relative tolerance of isolates within species.

Nagarajan and Natarajan (1999) worked with only a single *P. tinctorius* isolate that was found to be relatively tolerant of NaCl and $NaSO_4$ where medium concentrations did not exceed 60 mM. Similar to the findings of Dixon *et al.* (1993) it was found that the sodium salt, trisodium citrate ($Na_3C_6H_5O_7$), was particularly toxic at all increased concentrations. Again, similar to the findings of Dixon *et al.* (1993), the authors found protein content decreased with elevated salt concentrations greater than 60 mM, suggesting salinity has a significant influence on cytoplasmic metabolic activity. Mexal and Reid (1973) suggested that tolerance to salt and low water potential of fungus may involve cytoplasmic osmoregulation. One means by which this may occur is the accumulation of intracellular proteins (Mexal and Reid, 1973; Nagarajan and Natarajan 1999). The results of Dixon *et al.* (1993) and Nagarajan and Natarajan (1999) suggest protein content may be a means of evaluating the relative tolerance of fungal species and isolates within species.

In all of the above mentioned studies, no definition of tolerance was provided to describe the basis of salt tolerance in these fungal groups. Most studies examining the growth response of fungal cultures *in vitro* to environmental factors (e.g. fungitoxic compounds), utilise linear growth measurements as a means for comparison between control and treatment groups. Hutchison (1990a) presents a means of evaluating the relative tolerance of particular fungal isolates to a range of fungitoxic compounds in axenic culture conditions. Linear growth of colonies is determined and categorised into one of three

growth response categories; (i) tolerant, where growth is >50% compared with the control treatment; (ii) semitolerant, where growth is 20-50% of control; and (iii) sensitive, where growth is <20% of control. Results of this experiment are evaluated briefly in accordance with this tolerance model.

This experiment was carried out to determine the salinity tolerance of selected *P. tinctorius* isolates in axenic culture. The considerable functional variation within species of ectomycorrhizal fungi, particularly *P. tinctorius*, may provide for unique characteristics that allow for specific mechanisms of salt tolerance and therefore may be of use in association with host plants (Cairney 1999; Marx, 1977). Exposing individual isolates to saline conditions *in vitro* allows for rapid detection of tolerant isolates for their potential application in a stress environment. Growth and biomass production by the fungal component of the ECM relationship may provide an indication of the potential benefit of ECM associations of tree species used for the reclamation of salt affected soils.

The experiment also attempted to identify isolates showing variation in relative salt tolerance such that they could be used in subsequent glasshouse trials. A number of isolates were used in the experiment in an attempt to identify isolates that showed variation in their response to a saline growth substrate. In doing so inoculation of seedlings in subsequent glasshouse trials with fungal isolates that vary in their response to salinity, can be carried out to study the response of ectomycorrhizal seedlings to salinity. Of particular interest is the examination of any correlation between the *in vitro* salt tolerance of a particular isolate and the response to salinity of a plant inoculated with this same isolate.

2.2 Materials and Methods

Five isolates of *P. tinctorius* (MH39, MH2, LuH78, LuH25 and LuH43) were obtained from Associate Professor B. Dell, Murdoch University. Cultures were maintained on modified Melin Norkans (MMN) solid agar medium (Marx, 1969) according to Brundrett *et al.* (1996), with D-maltose substituted for glucose (Dell, Pers. Comm.) Cultures were maintained under cool white light in a 16h light/8h dark photoperiod at 25 °C.

For experimental purposes, 1cm^2 block of agar was removed from the edge of the growing parent colony. This block was placed on sterilised 70mm glass fibre membranes that overlaid the appropriate agar medium. The membrane prevented fungal hyphae penetrating the agar and enabled transfer of the growing colony between plates of different salt concentration. The membrane also allowed the complete removal of fungal hyphae at the completion of the experiment for biomass determination.

The different isolates were grown on MMN medium containing different concentrations of NaCl (0, 50, 100 and 150 mM). These concentrations were in addition to the 0.43 mM NaCl already present in the medium. To avoid osmotic shock associated with NaCl treated media, salt concentrations were introduced incrementally at a rate of 50 mM every 48 hours until the final NaCl concentrations were reached. All treatments were replicated 6 times.

Culture plates were maintained in growth cabinets at 25 °C in darkness for the duration of the experiment. Colony diameter was recorded regularly to determine the rate of growth. The growth rate of different isolates is known to vary substantially (Cairney, 1999). The

duration of the experiment varied between isolates; faster growing isolates were harvested when hyphae reached the membrane edge, slower growing isolates were harvested after 4 weeks (28 days). Direct comparisons between the growth of isolates was not possible due to the variable time periods for which cultures were grown, therefore, relative growth rates were used to determine which isolates were most affected by salt.

Colony diameter measurements were used to calculate the relative growth rate (RGR) of each of the colonies within each treatment. Hyphae was carefully scraped from the membrane and fresh and dry weights determined. Statistical analysis was carried out using one-way analysis of variance (ANOVA). Where significant differences were found, appropriate multiple range tests were carried out (e.g. Tukey's HSD).

2.3 Results

Considerable variation in the growth of the isolates was observed. Isolates MH39 and LuH78 grew most rapidly and reached the membrane edge at day 7 and 9 respectively. Isolate MH2 also showed rapid growth and was harvested at day 18. The remaining two isolates (LuH25 and LuH43) were harvested at day 28, although neither had hyphae that had approached the membrane edge. The growth form of the chosen isolates varied from consistent profuse growth, observed in MH39, LuH78 and MH2, to the sparse, scattered branching patterns in LuH43 and LuH25. These patterns of morphology are described by Hutchison (1990a).

Significant variation of dry weights of fungal isolates and fungal colonies was observed among salt treatments (Figure 2.1). Dry weights of colonies within the isolates of MH39, MH2 and LuH78 did not show statistically significant variation between salt treatments ($P = 0.133$; $P = 0.177$; $P = 0.612$, respectively). LuH43 colonies showed significant variation ($P = 0.000$) with those at higher salt concentrations producing more hyphae. Dry weights of LuH25 colonies varied, though only at 100 mM salt which was significantly higher than all other treatments.

Relative growth rates (RGR) provided additional comparisons between control and treatment groups with growth rates of salt treatment groups observed as a percentage of the control (Figure 2.2). Isolate MH39 showed no significant difference ($P = 0.227$) between NaCl treatments. Similar variation in relative growth rates of both LuH25 and LuH43 are observed. RGR of colonies of these two isolates, grown at different salt levels, did not vary ($P = 0.518$; $P = 0.245$ respectively). By comparison MH2 shows a significant reduction in RGR at all increased salt treatments ($P = 0.000$). A less than 50% RGR is observed of colonies exposed to 100 and 150 mM NaCl. Isolate LuH78 showed a significant reduction in relative growth rate at the higher salinity treatments of 100 and 150mM ($P = 0.000$). A less-than 60% RGR is observed of both these treatment levels.

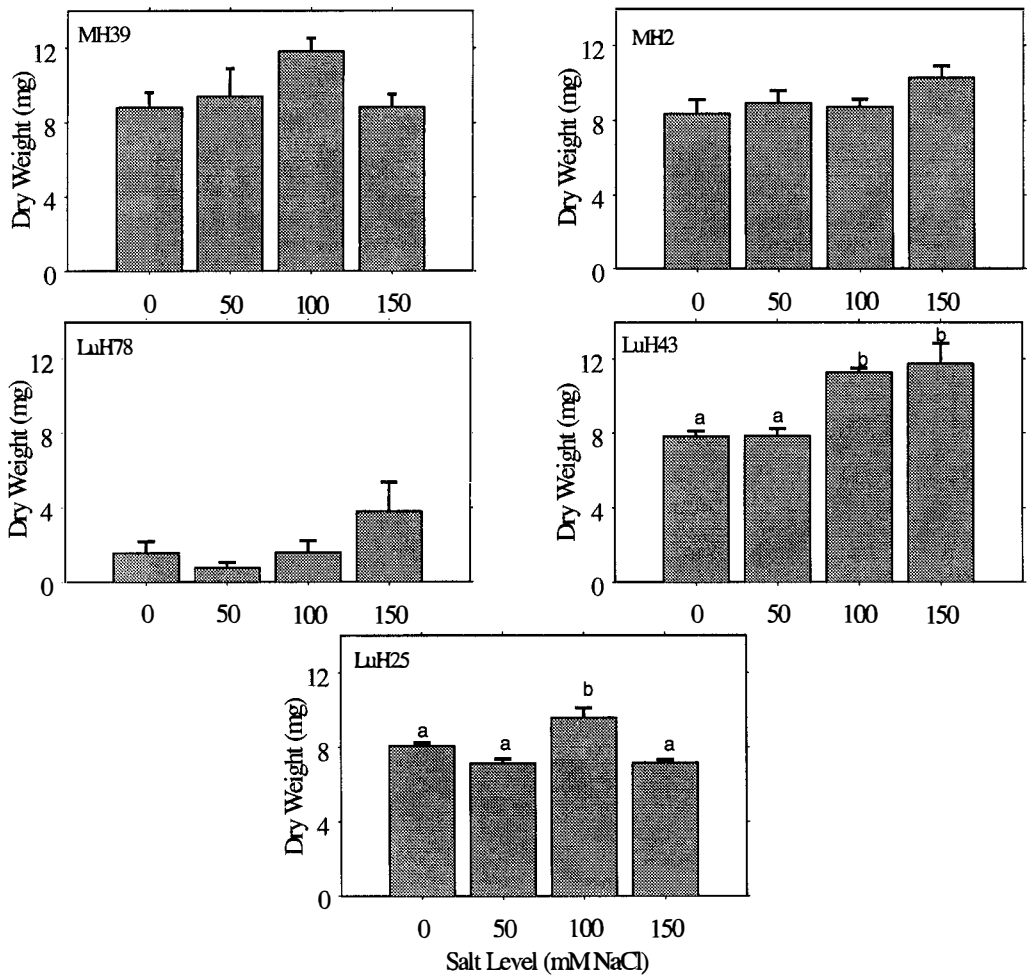

Figure 2.1: Mean dry weight of *P. tinctorius* colonies exposed to NaCl. Error bars indicate standard error. Values with the same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

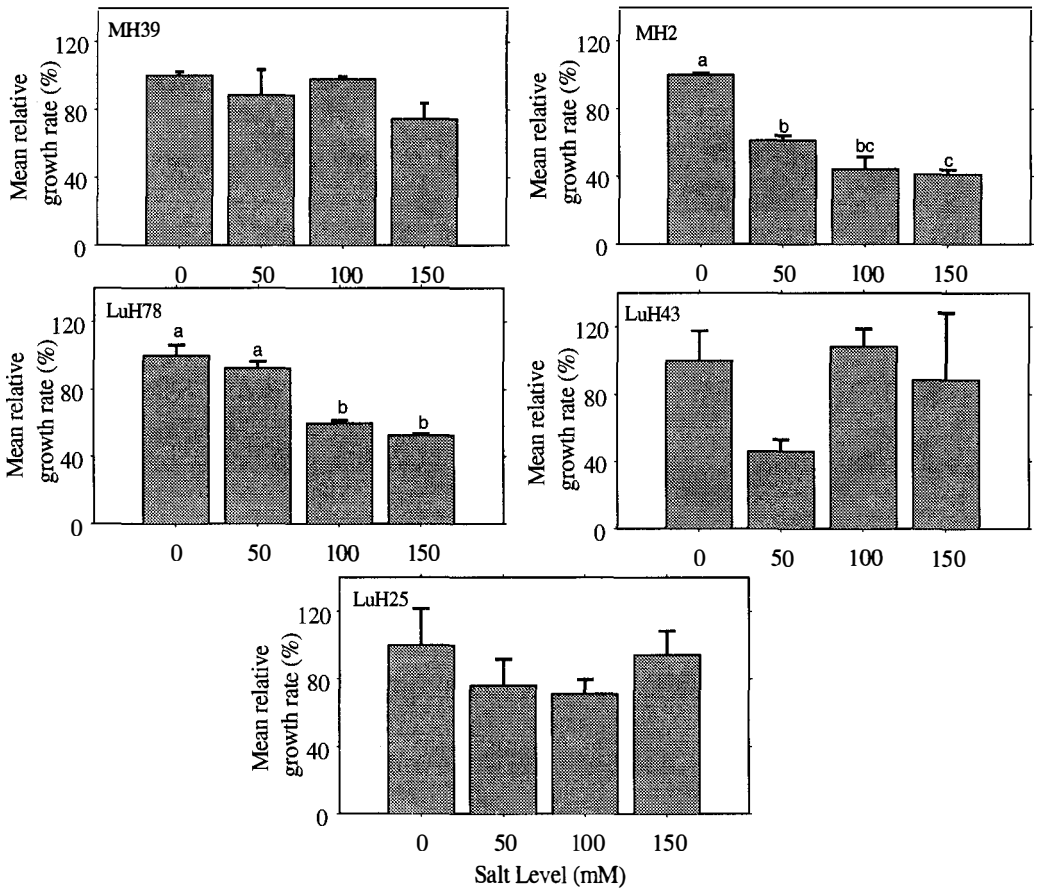

Figure 2.2: Mean relative growth rate of *P. tinctorius* colonies exposed to NaCl. Error bars indicate standard error. Values with the same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

2.4 Discussion

The rapid growth of individual isolates such as MH39 and LuH78 was in marked contrast to those growth rates observed of LuH43 and LuH25. This limited direct comparisons between isolates and highlights the need to use isolates possessing similar rates of growth for future experiments. Variation in growth rates of ECM fungi grown in axenic culture may be attributed to a number of factors such as; temperature, pH and nitrogen composition of the substrate (Lianqing and Zhida, 1994). Hutchison (1990b) studied the temperature dependance of a range of ectomycorrhizal species in axenic culture using linear growth patterns as a taxonomic character. Similar studies reported by Cairney (1999) have found temperature to be important for optimal and maximal mycelial growth *in vitro*. Variation in growth rates in axenic culture may be attributed to variation in genotypes within ectomycorrhizal species, just as there is variation in provenance varieties in plants (Cairney, 1999).

Tresner and Hayes (1971) first recorded the relative intolerance of basidiomycetous fungi to NaCl. Mycelial growth response of isolates in this experiment varied in their response to elevated NaCl in the growth medium. Three of the 5 isolates showed no significant growth response to increased salt concentration. Colonies of both MH2 and LuH78 showed a significant reduction in relative rate of growth at higher levels of NaCl. This pattern of decline is not reflected in biomass measurements of the same isolates. Variation in growth form may be attributed to this discrepancy. Both MH39 and MH2 isolates demonstrated dense mycelial growth of colonies by comparison with colonies of LuH78, LuH43 and LuH25. Therefore, while radial growth of colonies may be affected by elevated NaCl it may

not necessarily limit biomass production. This appears particularly true of MH2 which shows a clear, and significant reduction in relative growth rate with increased salt but no apparent reduction in dry weight. A similar result is observed of LuH78 colonies, whilst these colonies grew rapidly, biomass values appear much lower than any other isolate.

The sparse yet consistent growth form of these colonies appears to have influenced dry weight values. Colonies of LuH43 and LuH25 showed variable growth rates throughout replicate samples at all salt levels and there was a significant increase in biomass at higher levels of NaCl. Colonies of LuH43 and LuH25 exhibit an increase in biomass production at 100 and 150 mM, respectively. Hence biomass results suggest all isolates examined are tolerant of elevated NaCl. Dixon *et al.* (1993) found biomass measurements were consistent in two *P. tinctorius* isolates across a range (0 - 120 mM) of salt treatments and similarly found a reduction in linear growth rates with increasing NaCl.

Relative growth rate of colonies of isolates MH2 and LuH78 may suggest intolerance of elevated salt in the growing medium. However, application of the growth response categories described by Hutchison (1990a, b), suggests all isolates tested are tolerant (RGR > 50%) of elevated NaCl, except colonies of LuH43 exposed to 50 mM which exhibit semi-tolerance (RGR 20-50%). Colonies of MH2 exposed to 150 mM are borderline tolerant of that concentration. This is consistent with the findings of Hutchison (1990a) in which two isolates of *P. tinctorius* were found to be semi-tolerant and tolerant to 10 mg mL^{-1} NaCl (~150 mM).

Dixon *et al.* (1993) attributed the decline in growth rates to a response to increased osmotic potential of the treated medium thereby reducing hyphal extension. However, both Mexal and Read (1973) and Coleman *et al.* (1989) suggest it is the toxicity of NaCl that adversely influences growth rate as opposed to osmotic stress. The authors suggest that organic compounds such as proteins may provide the additional osmotic potential to counter the increase in external osmotic potential. However, it was found that protein content of isolates of 3 different ECM species generally decreased with increasing salt. Similarly, Nagarajan and Natarajan (1999) found protein levels decreased in a *P. tinctorius* isolate where sodium salts exceeded 60 mM.

Determination of protein content of fungal isolates saw preliminary investigation in relation to this experiment, however, a substantial amount of fungal material is required to carry out the analysis and precluded the addition of such analysis to support this experiment. Such analysis in future experiments would provide greater indication of the possible role of proteins in osmoregulation and metabolic activity of ECM fungi under stress conditions. It is possible that protein determination may be used as a means of determining variation in salt tolerance between isolates. The use of tolerance categories is also a means of defining the relative tolerance of fungal isolates to elevated NaCl and is likely to be particularly useful in future studies.

From the results of this experiment it is possible to determine those isolates that potentially may benefit a host plant via inoculation in subsequent glasshouse trials. MH39 and LuH78 were both considered to show rapid growth, however, they show variation in their relative tolerance to increased NaCl. Rapid and relatively profuse growth of fungal colonies is necessary for glasshouse experiments and may be well suited to MH39 and LuH78.

CHAPTER 3: RESPONSE OF ECM *Eucalyptus* SEEDLINGS TO INCREASED NaCl IN SOIL MEDIUM

3.1 Introduction

In Australia there is an increasing emphasis on developing the eucalypt plantation estate to reduce pressure on native forests, and to provide an alternative source of income for landowners. Australia has large tracts of land available for the establishment of eucalypt plantations, much of which may be degraded farmland affected by salt or erosion (Bell, 1999; Barson and Barrett-Lennard, 1995). The establishment of plantations on sites partially affected by salt provides the additional benefit of lowering the watertable locally, thereby reducing the likelihood of a buildup of salt in the upper soil horizons. This form of forestry in Australia is rapidly growing and there is a need to develop new techniques to improve growth and survival on degraded and moderately saline sites.

The use of mycorrhizas in forestry has seen its greatest exploitation in north America where important tree species are planted with their associated ectomycorrhizas. In Australia, an example of the significance of providing appropriate mycorrhizal fungi is provided by the introduction of *Pinus* species for the production of timber. Initial plantings of *Pinus* and other conifers were not successful because of the nature of Australian soils (low nutrient availability) and the low level of compatibility between native mycorrhizal fungi and the introduced plant species (Dunstan *et al.*, 1998a). With the introduction of compatible non-native fungi to these plantings, conifer plantations were established across a wide range of sites. Indeed, it is now standard practice to inoculate nursery stock with appropriate mycorrhizas for the successful establishment of seedlings.

Research on eucalypt mycorrhizas has not received as much attention as that on gymnospermous mycorrhizas. Yet plantation growers of eucalypts in Australia have a great deal to gain from improved management of mycorrhizas in such systems (Miller *et al.*, 1994; Grove and Le Tacon, 1993). In general, while the benefits of mycorrhizas in terms of increased growth and yield are well recognised, less is known of the protective capacity of mycorrhizas associated with eucalypts, particularly with respect to salinity.

The physiological response of mycorrhizal fungi to high levels of soil salt is an important aspect of plant salt tolerance studies. A number of studies of VAM fungi and VAM plants in saline soils suggests mycorrhizal fungi may be able to tolerate high levels of NaCl and high external osmotic potential. Evaluation of the response of ECM and ECM plants will contribute significantly to current knowledge of the physiology of ECM eucalypts in stress environments. Also of considerable potential benefit is the use of ECM and ECM plants in the reclamation of salt affected land, particularly where moderate levels of salt persist. This would have significant consequences for the development of plantations on moderately saline sites where growth rate and yield of eucalypts may be improved by inoculation with ECM fungi.

The purpose of the three experiments reported here was to evaluate the significance of ECM in the response of *Eucalyptus* seedlings to salinity. This was done by measuring growth and proline production in *E. diversicolor* and *E. camaldulensis* that were inoculated with ECM fungi and grown in saline soil. Inoculation of plants was with field collected spores and specific isolates of *Pisolithus tinctorius* grown in axenic culture.

3.2 Material and Methods

3.2.1 Plant material

Seed of *Eucalyptus camaldulensis* and *Eucalyptus diversicolor* for experiments 1 and 2 were obtained from a commercial seed supplier. Clonal plants were produced from cuttings of glasshouse growing stock available at Edith Cowan University. Seeds were germinated in a glasshouse under normal light conditions with temperatures ranging between 20 and 30°C. Cuttings from stock material were treated with root induction hormones (Richgro® striking hormone - medium wood) and were established in high humidity misting cabinets with 25 °C bottom heat. Following establishment (between 3 and 4 weeks) cuttings were maintained under normal glasshouse conditions prior to inoculation. Seedlings and cuttings were maintained in steam pasteurised 1:1 coarse sand/peat soil mix appropriate for the establishment of mycorrhizal symbioses (Brundrett *et al.*, 1996).

3.2.2 Fungal material

Spore material for inoculation of seedlings in experiment 2 was obtained from field collected fruiting bodies during the winter and spring months. Fruiting bodies of *Pisolithus tinctorius* and a *Scleroderma* species were used for spore suspension slurries for seedling inoculation (Dell, pers. comm.). Fruiting bodies of *P. tinctorius* were collected from the Darling scarp region near Perth, Western Australia. *Scleroderma* sp. fruiting bodies were collected from Manjimup, south-western Western Australia. The fruiting bodies were allowed to dry and then the spores were scraped from sporocarps and suspended in Distilled

Deionised H_2O (DDH_2O) and placed on an orbital shaker overnight. Following saturation, spores were stored at 4 °C. Serial dilution of these initial spore suspensions was carried out to determine the number of spores in 1 μL.

Fungal isolates of *P. tinctorius* in axenic culture were obtained from Associate Professor B. Dell, Murdoch University. These axenic cultures were maintained on modified Melin-Norkans (MMN) solid agar medium (D-maltose substituted for glucose. Dell, pers. comm.) (Brundrett *et al.* 1996) at 25 °C beneath fluorescent tubes with a 16h light/8h dark photoperiod.

3.2.3 Inoculation and maintenance

Inoculation procedures for all glasshouse experiments were carried out on seedlings growing in 50mm plastic pots. This was aimed at facilitating maximum infection prior to out-planting in larger pots. All experiments were carried out in 5L pots (3 seedlings/clones per pot) with 4.5kg of steam pasteurised 50:50 mix of course and fine sand. Before and after inoculation, plants received 1/4 strength Thrive® fertiliser supplemented with Fe (Librel® FeLo) at a rate of 0.25 g $10L^{-1}$.

3.2.3.1 Experiment 1

Spore inoculation of *E. camaldulensis* and *E. diversicolor* was carried out 8 weeks prior to out planting into sand culture. Inoculum of each of the two fungal species used were applied as a spore slurry containing approximately 2.55 x 10^6 spores ml^{-1}. Spore suspensions were applied at a rate of 10 mL weekly for 4 weeks. Uninoculated control plants were treated with 10 ml DDH_2O at each application. Eight weeks after the initial inoculation, seedlings were planted into 5L sand culture pots, 3 seedlings per pot. The root system was minimally disturbed at planting. Plants were grown for a further 6 weeks before being exposed to the salt treatments.

3.2.3.2 Experiment 2

Seedlings of *E. camaldulensis* and *E. diversicolor* were inoculated 21 days following germination with two fungal isolates that were previously maintained in culture. The two isolates chosen (MH39 and LuH78) showed rapid growth and were considered appropriate for rapid establishment of the symbiosis. The seedlings were inoculated in 50mm pots prior to establishment in sand culture. A 15mm diameter corer was used to remove actively growing hyphae from established culture colonies. These discs were placed near growing roots of seedlings and were grown for a further 6 weeks before transferring to 5L pots. When transferred to these pots another fungal plug was placed near the growing roots. Plants were grown for a further 5 weeks prior to application of the salt treatments.

3.2.3.3 Experiment 3

Four clonal lines of *E. camaldulensis* were inoculated with the same two isolates used for experiment 2 (MH39 and LuH78). Clones used are identified as salt sensitive C903 and C919 and salt resistant C066 and C502. Inoculation of clonal material was performed using the same procedures as described for experiment 2. Again, a 6 week period was allowed for the development of symbiosis in 50mm pots prior to transplanting into sand culture. A further 4 weeks elapsed prior to the application of salt solutions.

3.2.4 Application of salt

Salt treatments (and similarly control treatments) were applied as a saline nutrient solution that allowed for the incremental increase in salt concentrations (50 mM every 48 hours) to avoid osmotic shock in experimental plants. A basal nutrient solution consisting of 1/4 strength (4.00 g $10L^{-1}$) Thrive® and chelated iron (Librel® FeLo) (0.45 g $10L^{-1}$) was considered appropriate for maintenance of mycorrhizal plants in sand culture. Salt was applied in the form of technical grade NaCl at molar concentrations of 0, 50, 100 and 200 mM with the exception of experiment 3 where only 0 and 200 mM salt concentrations were applied. Sodicity in these solutions was counteracted with the addition of CaCl and $MgSO_4.7H_2O$ at a rate in accordance with the molar concentration (Table 3.1).

Table 3.1: Application rate of CaCl and $MgSO_4.7H_2O$ to counteract sodicity in saline nutrient solutions.

Molar Concentration (mM)	NaCl (g L^{-1})	$MgSO_4.7H_2O$ (g L^{-1})	$CaCl_2$ (g L^{-1})
0	0	2.46	0.55
50	2.92	2.46	0.55
100	5.84	4.93	1.11
150	8.77	7.39	1.66
200	11.69	9.86	2.22

Salt solutions were applied by flushing each pot with the appropriate solution until the conductivity of the solution applied equalled the conductivity of the solution exiting through a single drainage hole. Following application of salt solutions, pots were allowed to drain for 24 hours and then the drain holes were plugged. Pots were maintained at field capacity for the duration of the experiments. At the midpoint of each of the experiments (14 days) the application of the appropriate salt/nutrient solution was repeated to avoid nutrient deficiency of the plants.

3.2.5 Plant measurements and harvest

Height of plants was determined at 7-day intervals and growth characteristics of individuals and treatment groups were recorded. Plants in all experiments were harvested at 28 days after the application of the final desired salt concentration, and shoot and root fresh weights determined. Leaf samples for proline analysis were removed prior to plant harvest. Root

material was stored in sealed plastic bags at 4 °C for assessment of mycorrhizal infection. Shoots were oven dried at 70 °C and dry weights determined. Following assessment for mycorrhizal structures and removal of material for proline analysis, root dry weights were similarly determined.

3.2.6 Mycorrhizal assessment

Random samples of root material were taken from individual plants and examined beneath a dissecting microscope. The presence of mycorrhizal structures, such as the characteristic short root tips enveloped by fungal hyphae (mantle), were recorded using the methods of Brundrett *et al.* (1996) and Grand and Harvey (1982). The method, known as the line intercept method, allowed for the determination of the percentage of short root tips infected by mycorrhizal fungi.

3.2.6 Proline determination

Analysis of free proline in leaf and root material was determined using the acid ninhydrin reaction method described by Bates *et al.* (1973). The analysis required 0.5 g of fresh leaf and root material per plant.

3.2.7 Statistical analysis

The factorial design of all glasshouse experiments allowed for the application of ANOVA for making statistical comparisons. The type of ANOVA applied was dependant on the design of the individual experiments (2 and 3 factorial designs). Where significant

differences were observed a one-way ANOVA was carried out in conjunction with Tukey's multiple range comparison. Statistical analysis of growth of clonal material (experiment 3) to compare effects of fungal treatments within each salt treatment was performed using one-way ANOVA. Analysis of effects of salt treatments on growth of clonal material was conducted using Student's t-test.

All manipulation and statistical analysis of experimental data used Excel 97® and SPSS® for Windows version 10.0. Graphical presentation of data was carried out using Axum® for Windows version 6.0.

3.3 Results

Harvested plants showed little mycorrhizal infection and mycorrhizal short root development. Closer inspection of root material showed no indication of complete development of short roots and associated ECM structures (i.e. fungal mantle). This did not allow for quantitative assessment of the level of mycorrhizal infection and was a consistent feature in all three experiments. A qualitative assessment of root material was considered appropriate in which the presence of mycorrhizal infection was recorded and tabulated for presentation. Hyphal presence in inoculated plants appeared to be greater than that of uninoculated plants. However this is a broad generalisation, and it must also be noted that hyphae were present among roots of uninoculated plants.

3.3.1 Experiment 1

The results of root assessment are presented in tables 3.2 and 3.3. Differences in plant growth response to fungal treatment were significant between species (P = 0.004). Within both *E. camaldulensis* and *E. diversicolor* plant growth was affected by both salt and fungal treatments. *E. diversicolor* inoculated with *Scleroderma* showed a significant (P = 0.000) decline in mean whole plant weights with increasing soil salt (Figure 3.1). This decline was not observed in uninoculated or *Pisolithus* inoculated plants.

Mean whole plant weights of uninoculated *E. camaldulensis* declined significantly with increasing salinity (P = 0.004, figure 3.2) A similar result was seen in *Scleroderma* and *Pisolithus* inoculated plants, although differences were not significant (P = 0.133; P = 0.187, respectively).

Table 3.2: Assessment for the presence of hyphae on roots of inoculated and uninoculated *E. diversicolor*. Value given is the proportion of total plants sampled within each treatment. A total of 105 plants were assessed.

	Salt Level	Hyphae		
	(mM)	**Absent**	**Present**	**Abundant**
Uninoculated	**0**	0.66	0.34	0
	50	0.77	0.23	0
	100	0.77	0.23	0
	200	0.71	0.29	0
Scleroderma	**0**	0.22	0.44	0.33
	50	0.11	0.44	0.44
	100	0.125	0.75	0.125
	200	0.23	0.77	0
Pisolithus	**0**	0.45	0.55	0
	50	0.11	0.66	0.23
	100	0.45	0.55	0
	200	0.34	0.66	0

Table 3.3: Assessment for the presence of hyphae on roots of inoculated and uninoculated *E. camaldulensis*. Value given is the proportion of total plants sampled within each treatment. A total of 102 plants were assessed.

	Salt Level	Hyphae		
	(mM)	Absent	Present	Abundant
Uninoculated	**0**	0.625	0.125	0
	50	0.875	0.125	0
	100	0.88	0.12	0
	200	1	0	0
Scleroderma	**0**	0.125	0.625	0.25
	50	0.11	0.66	0.23
	100	0.22	0.66	0.12
	200	0.45	0.55	0
Pisolithus	**0**	0.33	0.33	0.34
	50	0.10	0.55	0.35
	100	0.34	0.44	0.22
	200	0.33	0.55	0.11

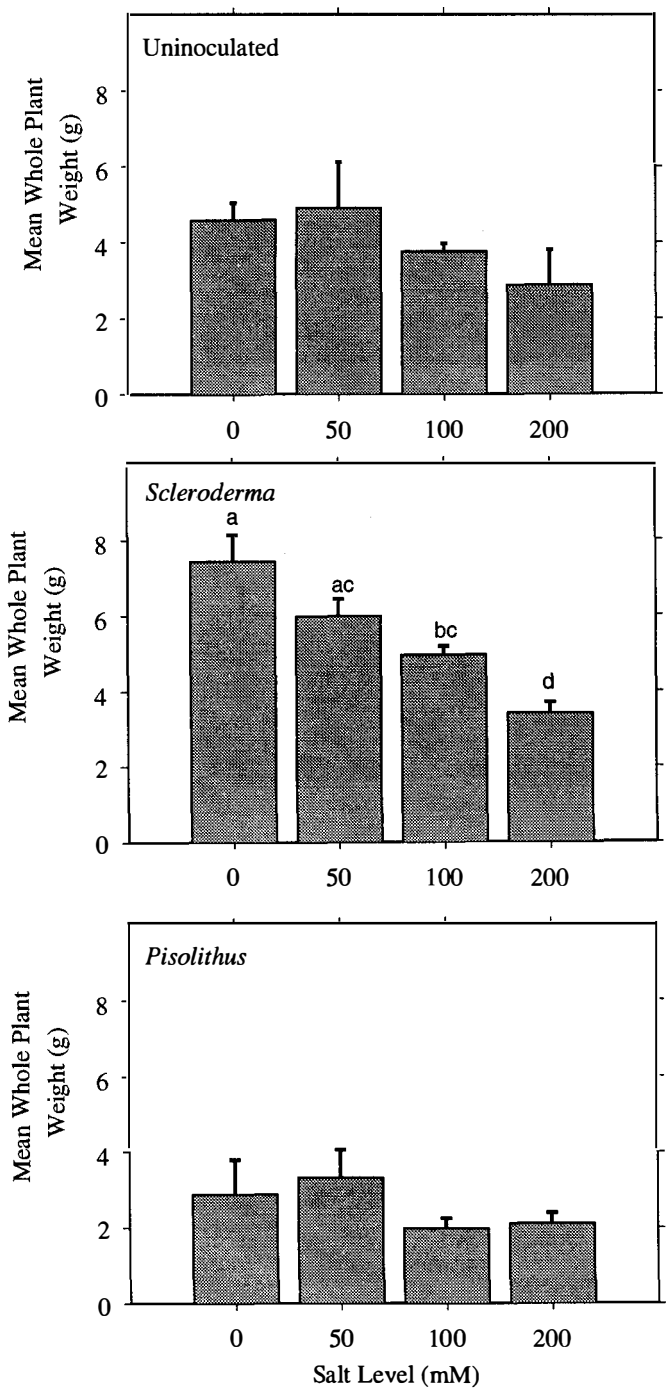

Figure 3.1: Mean whole plant weight of *E. diversicolor* in response to fungal and salt treatment. Error bars indicate standard errors. Values with the same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

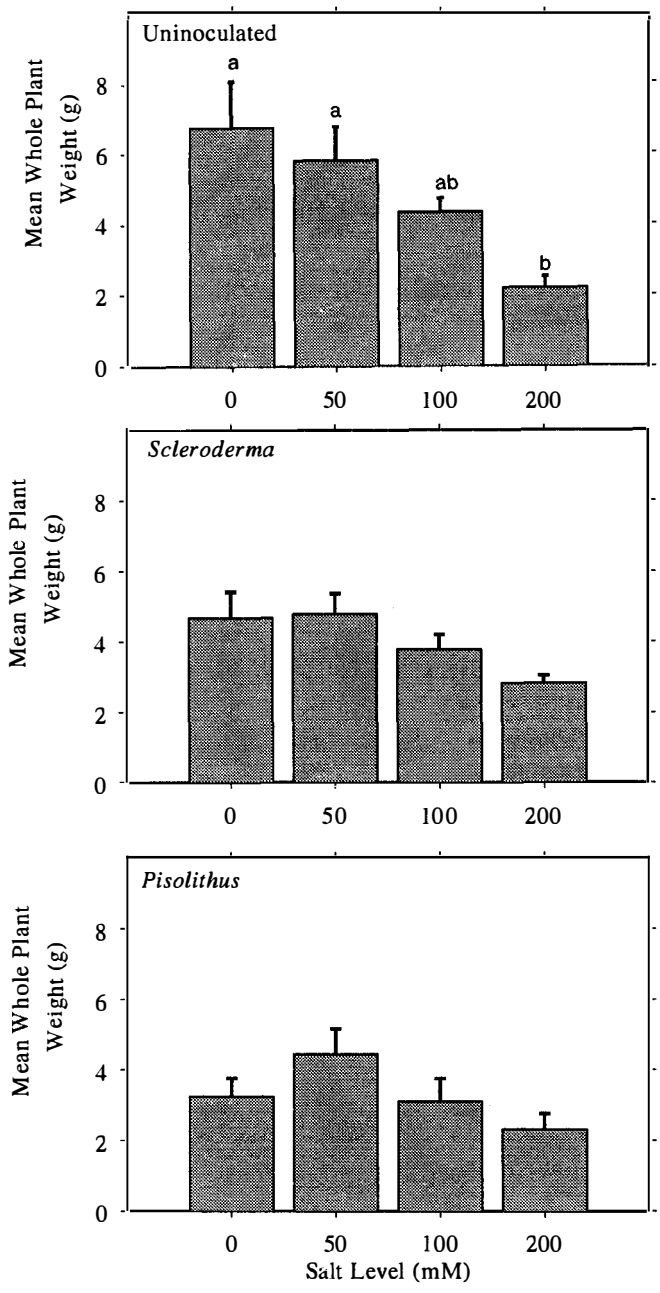

Figure 3.2: Mean whole plant weight of *E. camaldulensis* in response to fungal and salt treatment. Error bars indicate standard errors. Values with the same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

Proline levels varied between species, between shoots and roots within species, among salt treatments and among fungal inoculation treatments. Proline levels in *E. diversicolor* roots only varied due to salt treatments in those plants inoculated with *Scleroderma* (Figure 3.3). For these plants, the proline increased at 200 mM salt above the levels of the 50 and 100 mM treatments but was the same as the control. Shoot proline content for *E. diversicolor* was more variable than the roots (Figure 3.3). In the uninoculated plants there was significantly more proline in the 200 mM (1.03 μmol g^{-1} fresh weight) salt treatment than either the controls (0.53 μmol g^{-1} fresh weight), or the 50 and 100 mM treatments. For *Scleroderma* inoculated plants a similar trend to that observed of the roots was obtained, with the exception that the control had significantly higher proline content than the 50 and 100 mM treatments. In plants inoculated with *Pisolithus* there was no effect of the salt treatments.

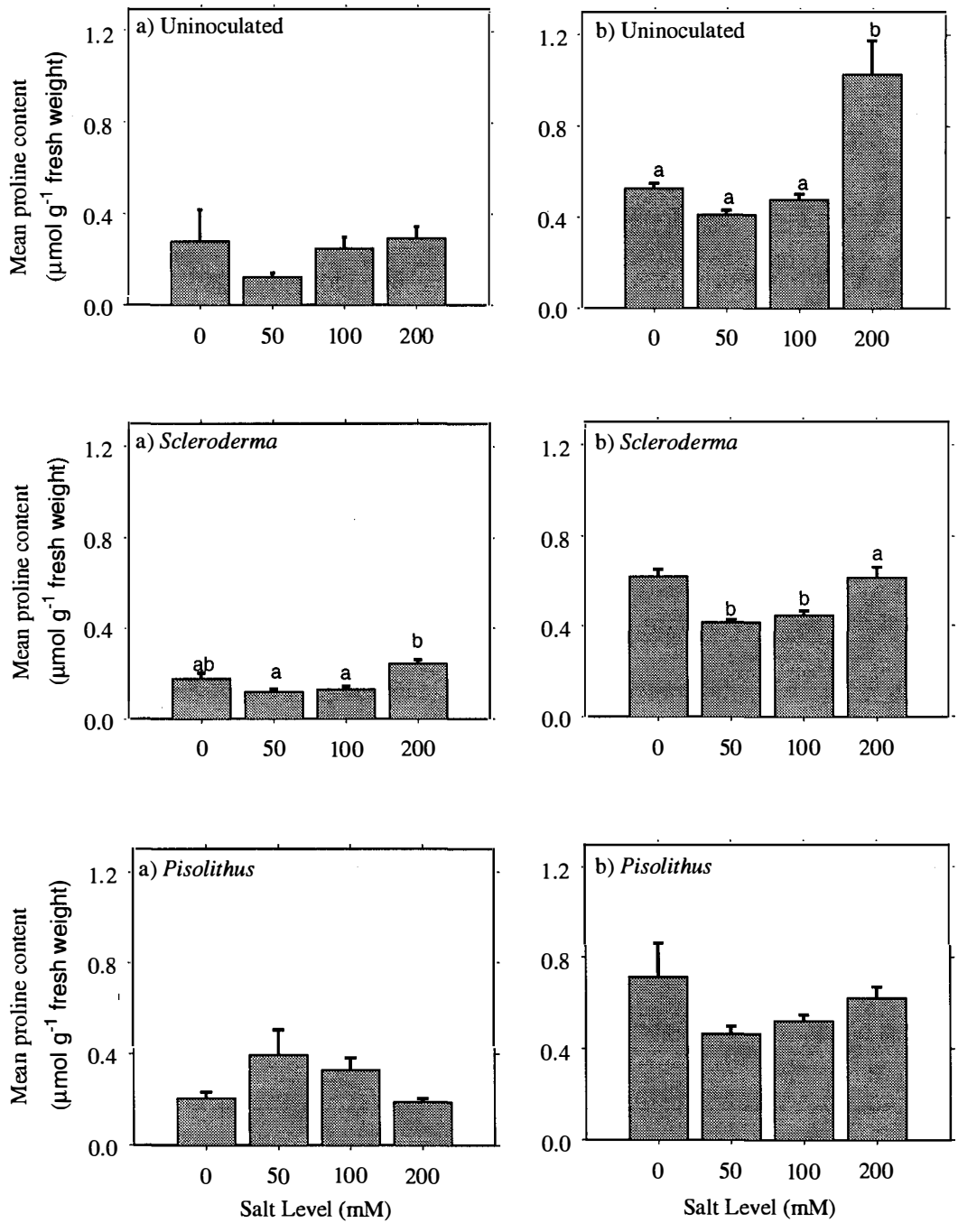

Figure 3.3: Mean root (a) and shoot (b) proline content of *E. diversicolor* in response to fungal and salt treatment. Error bars indicate standard errors. Values with same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

For *E. camaldulensis* proline also varied significantly due to salt treatment as well as fungal inoculation (Figure 3.4). As with *E. diversicolor*, there was a difference in the proline content between roots and shoots. However, root proline of *E. camadulensis* content was higher than shoot proline content. In uninoculated plants, root proline content increased with increasing salt and was significantly higher at 100 and 200 mM from the controls and 50 mM salt treatments (Figure 3.4).

For plants inoculated with *Scleroderma* there were higher proline levels than the uninoculated plants but a similar trend was produced with the highest level of proline being produced at the 200 mM salt treatment. *Pisolithus* inoculated plants had the highest levels of proline in the controls which decreased with increasing salt concentration; 50, 100 and 200 mM salt concentrations all had lower proline content. Shoot proline content for *E. camaldulensis* showed similar trends (Figure 3.4). An apparent increase in proline content with increasing salt was observed of the uninoculated plants ($P = 0.004$) and those plants inoculated with *Scleroderma* ($P = 0.020$). Plants inoculated with *Pisolithus* showed a reduction in proline content compared to the control ($P = 0.000$).

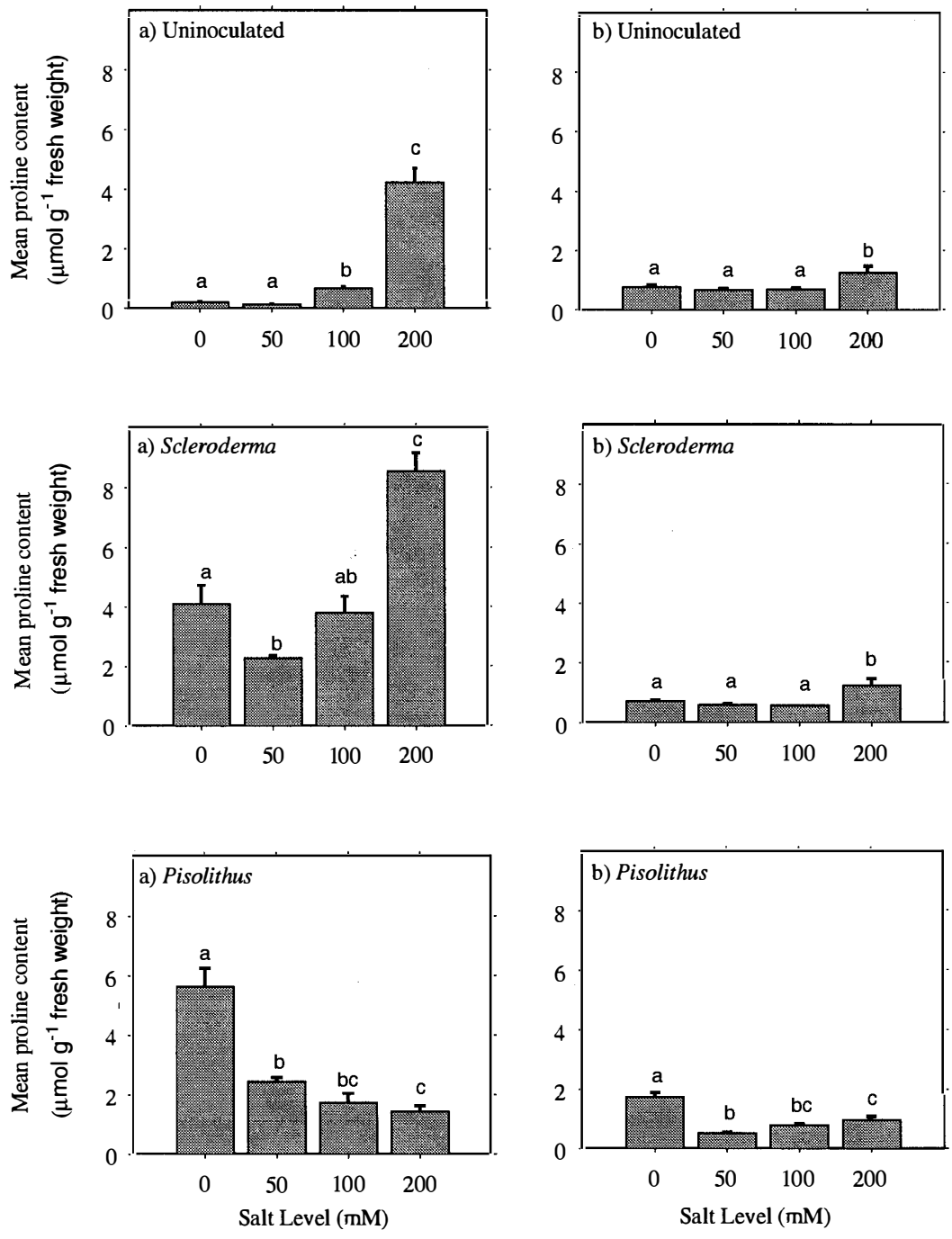

Figure 3.4: Mean root (a) and shoot (b) proline content of *E. camaldulensis* in response to fungal and salt treatment. Error bars indicate standard errors. Values with same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

3.3.2 Experiment 2

A qualitative assessment of hyphal presence was recorded (Tables 3.4 and 3.5). Hyphal presence appeared more abundant in inoculated plants. However, hyphae were also present in uninoculated plants.

Table 3.4: Assessment for the presence of hyphae on roots of inoculated and uninoculated *E. diversicolor*. Value given is the proportion of total plants sampled within each treatment. A total of 108 plants were assessed.

	Salt Level	Hyphae		
	(mM)	Absent	Present	Abundant
Uninoculated	**0**	0.77	0.23	0
	50	0.55	0.45	0
	100	0.77	0.115	0.115
	200	0.66	0.34	0
MH 39	**0**	0.22	0.33	0.55
	50	0.33	0.67	0
	100	0.33	0.67	0
	200	0.22	0.55	0.23
LuH 78	**0**	0	0.66	0.34
	50	0.11	0.77	0.12
	100	0.22	0.66	0.12
	200	0.33	0.67	0

Table 3.5: Assessment for the presence of hyphae on roots of inoculated and uninoculated *E. camaldulensis*. Value given is the proportion of total plants sampled within each treatment. A total of 108 plants were assessed.

	Salt Level	Hyphae		
	(mM)	Absent	Present	Abundant
Uninoculated	**0**	0.66	0.34	0
	50	0.33	0.55	0.12
	100	0.55	0.33	0.12
	200	0.88	0.12	0
MH 39	**0**	0	0.44	0.56
	50	0	0.66	0.34
	100	0.22	0.44	0.34
	200	0.33	0.55	0.12
LuH 78	**0**	0.12	0.55	0.33
	50	0.12	0.44	0.44
	100	0.33	0.44	0.23
	200	0.22	0.44	0.24

Plant growth responses differed significantly to salt and fungal treatments (P = 0.030). Salt and fungal treatments affected growth responses in both eucalypt species, although no significant interactions were apparent (*E. diversicolor*: P = 0.073; *E. camaldulensis*: P = 0.469). Comparisons made within fungal treatments of both plant species show significant differences between salt treatments. The response of *E. diversicolor* to all three fungal treatments was to decline in growth with increasing salinity (Figure 3.5). Growth of inoculated plants was significantly reduced at 200 mM, as was the case with those inoculated with MH39. Plants inoculated with LuH78 show a significant reduction (in comparison to the control) in whole plant weights at all salt levels with the exception of 100 mM.

Growth of *E. camaldulensis* showed similar trends across all three fungal treatments (Figure 3.6). Uninoculated *E. camaldulensis* plants at 50 mM NaCl had greater growth than plants exposed to 100 and 200 mM but was not different from 0 mM. This same pattern is observed of plants inoculated with MH39. A similar pattern is found in LuH78 inoculated plants, however plants exposed to 50 mM showed significantly greater growth than 0 and 200 mM. Neither 100 or 200 mM grown plants were different to the control plants.

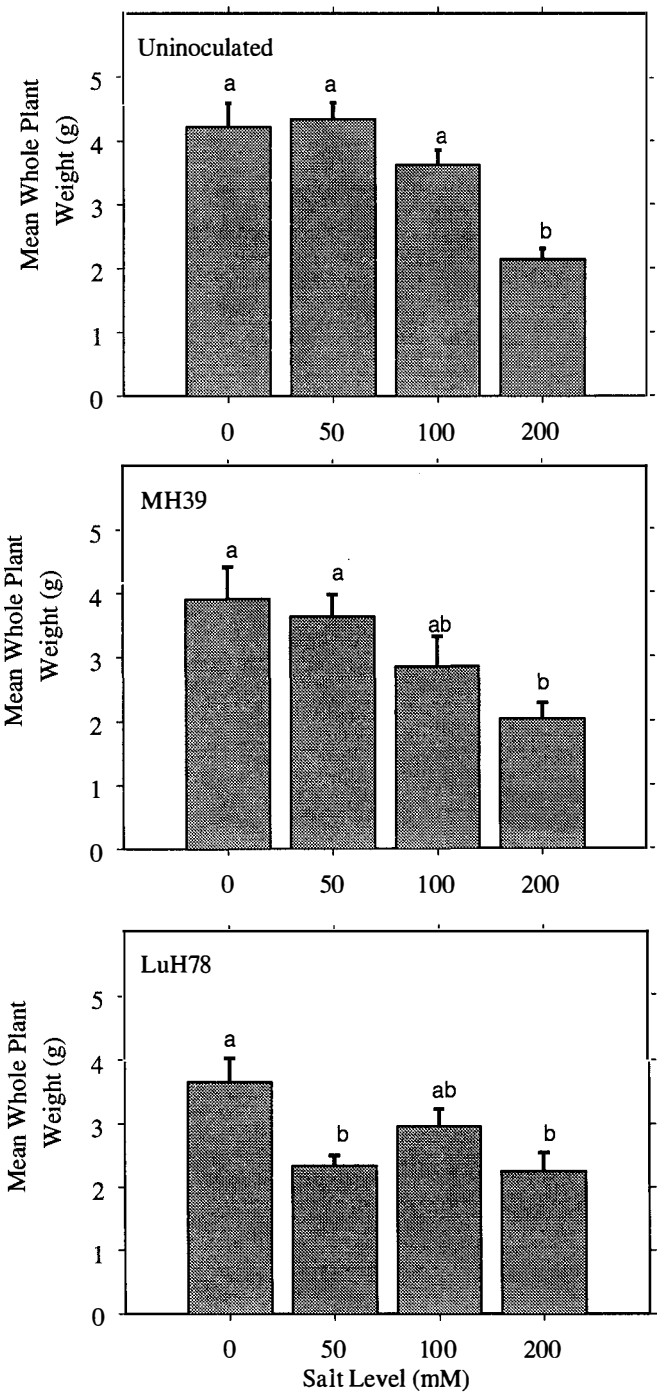

Figure 3.5: Mean whole plant weight of *E. diversicolor* in response to fungal and salt treatment. Error bars indicate standard errors. Values with the same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

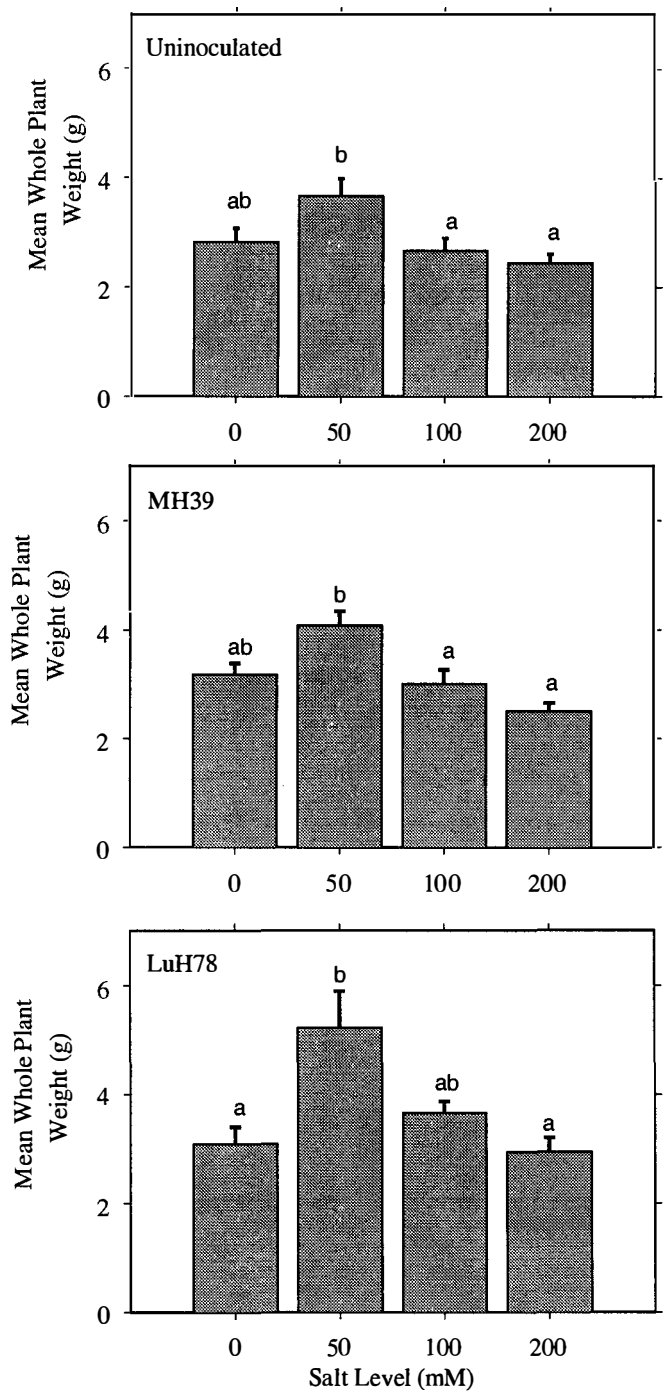

Figure 3.6: Mean whole plant weight of *E. camaldulensis* in response to fungal and salt treatment. Error bars indicate standard errors. Values with the same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

Proline content of plants varied between species as well as between roots and shoots within species. Shoot proline content of *E. diversicolor* was typically lower than that observed of *E. camaldulensis*. For both species fungal treatment significantly influenced proline levels in roots and shoots. Significantly higher root proline values were observed in inoculated *E. diversicolor* exposed to 0 mM salt (Figure 3.7). Lower levels of shoot proline are characteristic of inoculated plants exposed to 50, 100 and 200 mM salt. No significant variation in shoot proline content is apparent in uninoculated *E. diversicolor* ($P = 0.110$).

Uninoculated *E. camaldulensis* showed significant variation between salt treatments with 50 mM groups having higher root proline levels than the remaining salt treatments (figure 3.8a). In contrast MH39 inoculated plants exposed to 50 mM salt had significantly lower levels of root proline than 0 and 100 mM. No significant variation in root proline was found of plants inoculated with LuH78. As with *E. diversicolor*, there appears little relationship between root and shoot proline content of *E. camaldulensis*.

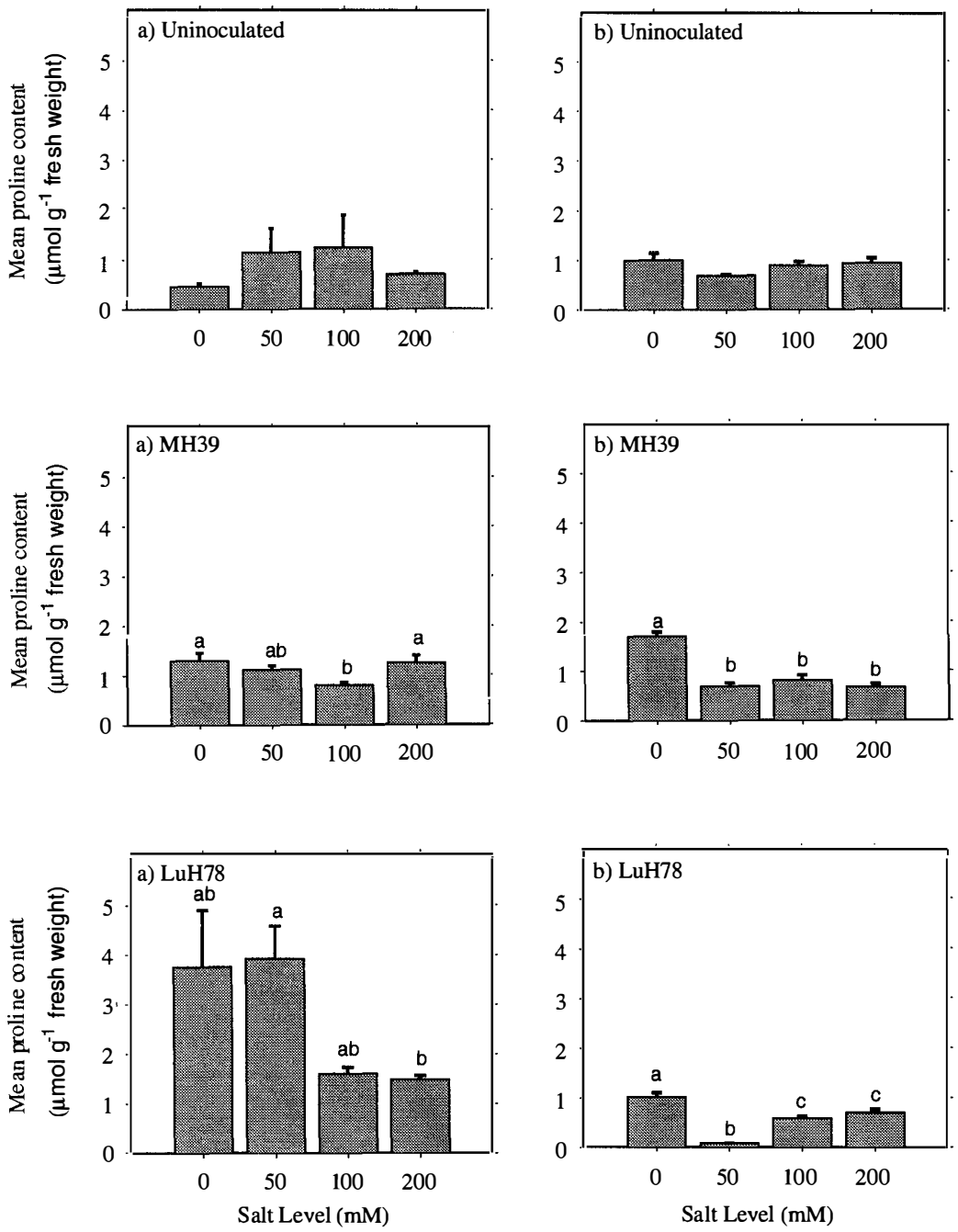

Figure 3.7: Mean root (a) and shoot (b) proline content of *E. diversicolor* in response to fungal and salt treatment. Error bars indicate standard errors. Values with same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

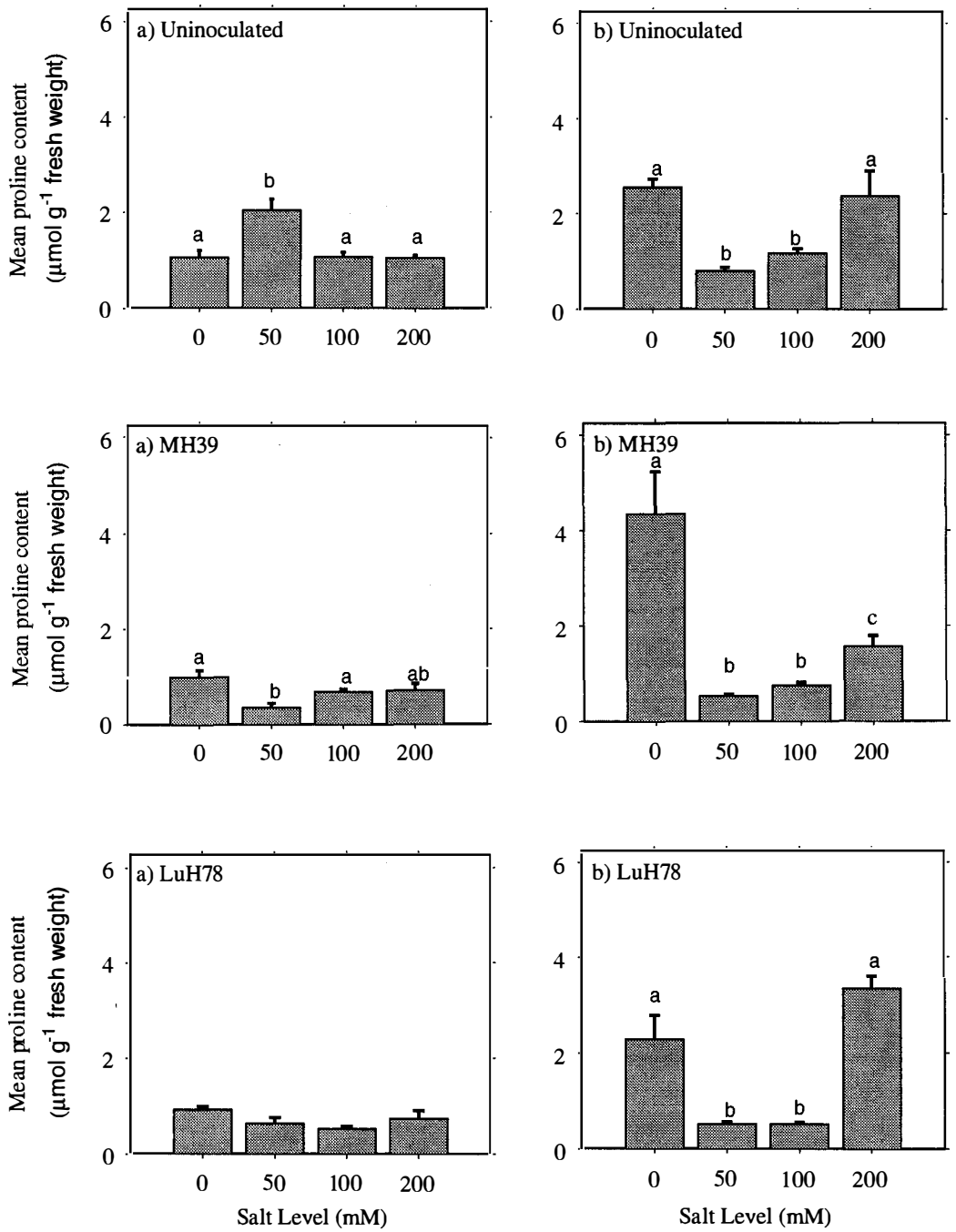

Figure 3.8: Mean root (a) and shoot (b) proline content of *E. camaldulensis* in response to fungal and salt treatment. Error bars indicate standard errors. Values with same letter are not significantly different according to Tukey's multiple range test ($P \leq 0.05$).

3.3.3 Experiment 3

The results of this root analysis are presented in Table 3.6, below.

Table 3.6: Assessment for the presence of hyphae on roots of inoculated and uninoculated *E. camaldulensis* clones. Value given is the proportion of plants having the following level of hyphal development "-" hyphae absent, "+" hyphae present and "++" abundant hyphal development. A total of 94 plants were assessed.

Clone	Salt Level (mM)	Uninoculated			MH39			LuH78		
		-	+	++	-	+	++	-	+	++
C066	0	0.22	0.56	0.22	0	0.44	0.56	0	0.56	0.44
	200	0.33	0.22	0.45	0.77	0.12	0.11	0.12	0.66	0.22
C502	0	0	0.77	0.23	0	0.88	0.12	0	0.34	0.66
	200	0.44	0.56	0	0.12	0.88	0	0.12	0.88	0
C919	0	0	0.44	0.56	0	0.66	0.34	0	0.55	0.44
	200	0.33	0.55	0.12	0	0.66	0.34	0.12	0.44	0.44
C903	0	0	0.77	0.23	0	0.34	0.66	0.33	0.44	0.23
	200	0.55	0.34	0	0.12	0.44	0.44	0.12	0.77	0.11

Statistical analysis of mean whole plant dry weights showed a significant ($P = 0.000$) difference between the four clones. Analysis within clones showed no significant response to inoculation with the exception of C903 which was significant ($P = 0.008$). Two-way ANOVA within each clone identified a significant response of whole plant weight to salinity ($P \leq 0.000$). This pattern is clearly illustrated in figure 3.9.With the exception of C066 and C919 inoculated with MH39 and LuH78, respectively, all other clones had reduced whole plant weights regardless of fungal treatment when exposed to 200 mM salt.

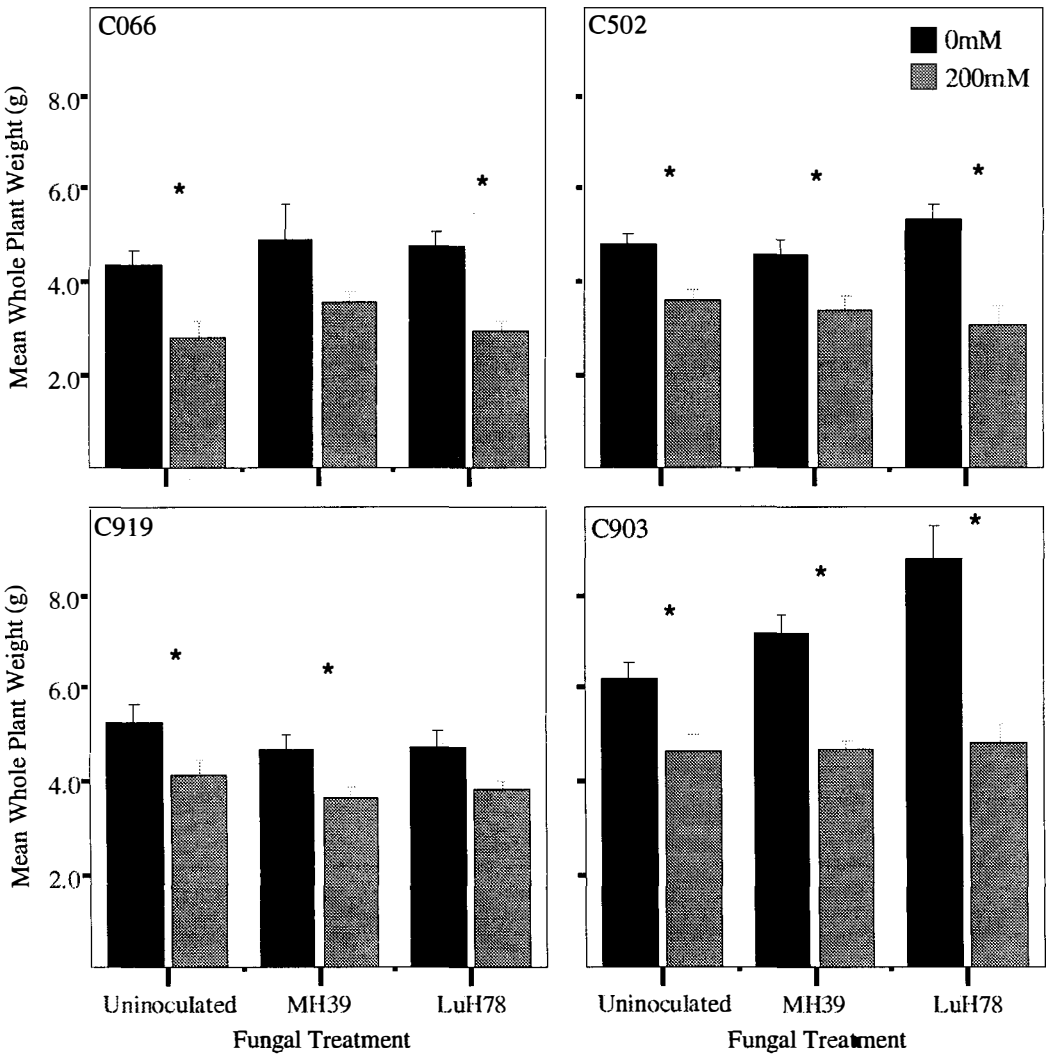

Figure 3.9: Mean whole plant dry weight *E. camaldulensis* clones, inoculated and uninoculated plants grown at 0 and 200 mM salt. Error bars indicate standard error. Asterisk (*) indicates significant differences (Student's t-test) between salt treatments within fungal treatments ($P \leq 0.05$).

Analysis between fungal treatments of individual clones at 0 mM identified a significant response of whole plant weights to fungal inoculation in C903 (Table 3.7). No significant response to fungal inoculation was observed of C066, C502 & C919. Plants of C903 inoculated with LuH78 showed significantly greater growth than uninoculated plants. Similar analysis within clones exposed to 200 mM show no significant differences between fungal treatments (Table 3.8). Both tables indicate results of Tukey's multiple range comparisons where ANOVA statistic is significant.

Table 3.7: Results of one-way ANOVA within *E. camaldulensis* clones, comparing fungal treatments when grown at 0 mM salt. No significant difference between treatments is indicated by "n.s." ($P \leq 0.05$). Where comparisons are significant, results of Tukey's multiple range test are provided where values with the same letter are not significantly different ($P \leq 0.05$).

Clone	Fungal Treatment	Whole Plant Dry Weight	Shoot Proline	Root Proline
C066	Uninoculated MH39 LuH78	n.s.	n.s.	n.s.
C502	Uninoculated MH39 LuH78	n.s.	a a b	n.s.
C919	Uninoculated MH39 LuH78	n.s.	n.s.	n.s.
C903	Uninoculated MH39 LuH78	a ab b	n.s.	n.s.

Table 3.8: Results of one-way ANOVA within *E. camaldulensis* clones, comparing fungal treatments when grown at 200 mM salt. No significant difference between treatments is indicated by "n.s." ($P \leq 0.05$). Where comparisons are significant, results of Tukey's multiple range test are provided where values with the same letter are not significantly different ($P \leq 0.05$).

Clone	Fungal Treatment	Whole Plant Dry Weight	Shoot Proline	Root Proline
C066	Uninoculated	n.s.	n.s.	a
	MH39			b
	LuH78			b
C502	Uninoculated	n.s.	n.s.	a
	MH39			ab
	LuH78			b
C919	Uninoculated	n.s.	n.s.	n.s.
	MH39			
	LuH78			
C903	Uninoculated	n.s.	n.s.	n.s.
	MH39			
	LuH78			

Shoot proline content varied between clones, fungal treatment and salt treatment (P = 0.000, in each case). Comparison of salt treatments within clones identified significant differences of shoot proline in C066, C919 and C903 (Figure 3.10). Fungal treatment was not a significant factor influencing shoot proline levels in these clones, with the exception of C502. This clone grown at 0 mM had significantly lower levels of shoot proline in LuH78 inoculated plants (P = 0.003; Table 3.6).

Clones grown at 200 mM salt did not show significant variation in shoot proline between fungal treatments (Table 3.6). While this was not significant, there is an apparent decline in shoot proline levels in inoculated plants of C066, C919 and in particular clone C903. Comparisons between shoot proline levels of plants grown at 0 and 200 mM salt, generally, indicate significantly higher levels in plants exposed to 200 mM (Figure 3.10). This is also characteristic of C919 and C903, in particular, as well as C066.

Initial analysis of root proline indicates significant differences between clones and fungal treatment (P = 0.008 and P = 0.033, respectively). Clones exposed to 0 mM salt show no significant variation in root proline content between fungal treatments (Table 3.6 & Figure 3.11). However comparisons between fungal treatments within clones exposed to 200 mM indicate significant differences between clones C066 and C502 (see Table 3.7 for results of Tukey's).Differences in proline levels in roots of inoculated clones were not statistically different between 0 and 200 mM salt (Figure 3.11) with the exception of C502 inoculated with LuH78 which had significantly lower root proline at 200 mM salt.

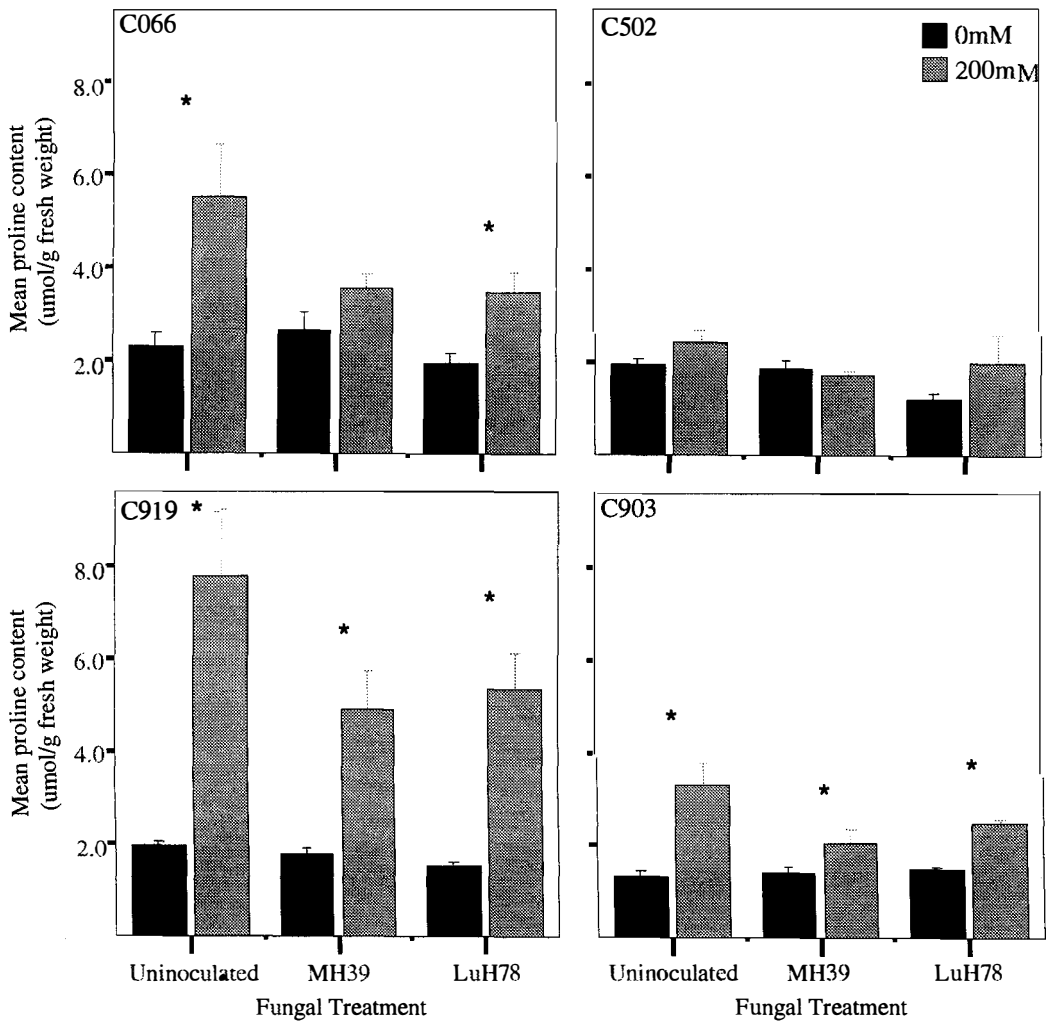

Figure 3.10: Mean shoot proline levels of *E. camaldulensis* clones, inoculated and uninoculated plants grown at 0 and 200 mM salt. Error bars indicate standard error. Asterisk (*) indicates significant differences (Student's T-test) between salt treatments within fungal treatments ($P \leq 0.05$).

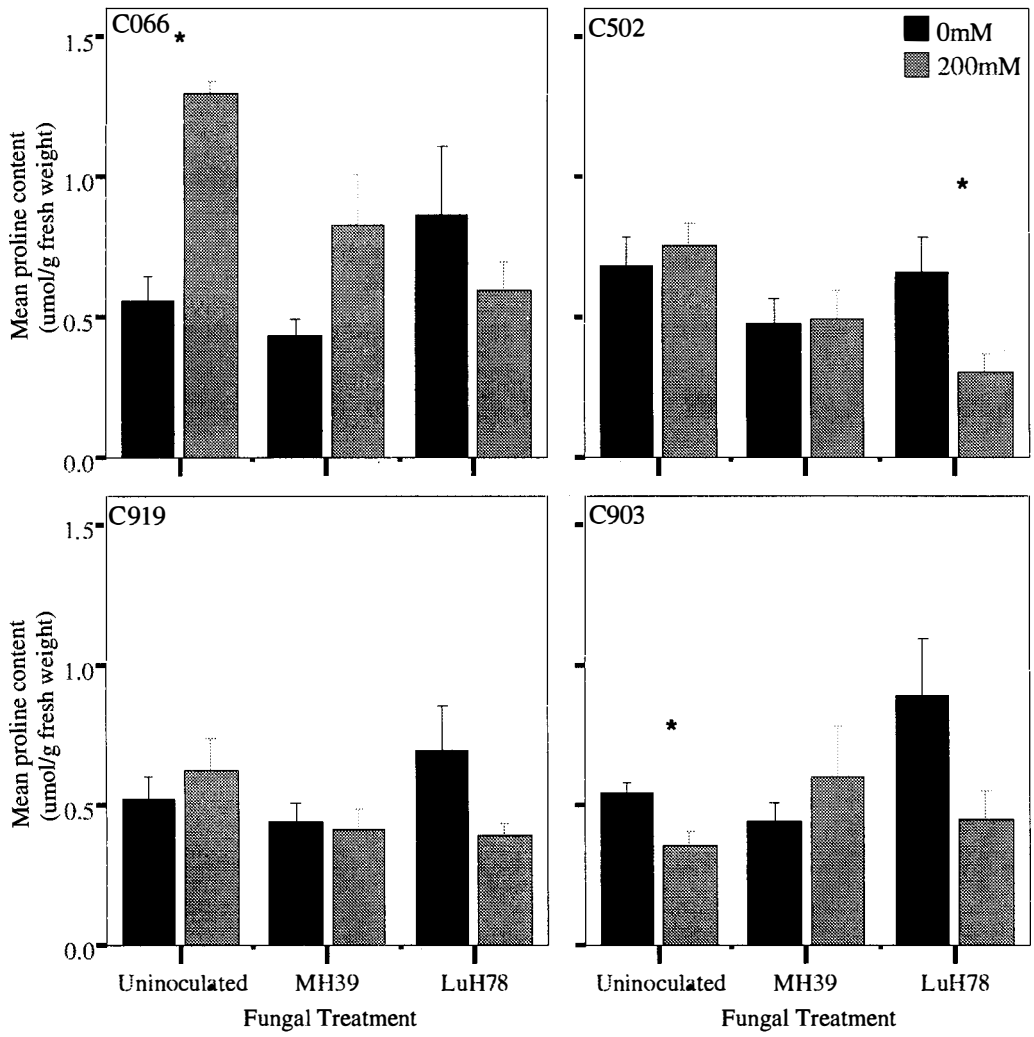

Figure 3.11: Mean root proline levels of *E. camaldulensis* clones, inoculated and uninoculated plants grown at 0 and 200 mM salt. Error bars indicate standard error. Asterisk (*) indicates significant differences (Student's T-test) between salt treatments within fungal treatments ($P \leq 0.05$).

3.4 Discussion

The lack of obvious mycorrhizal structures on the roots of these plants did not allow for appropriate quantitative assessment of the mycorrhizal status of seedlings. The qualitative assessment of hyphal growth has, unfortunately, limitations in assessment for mycorrhizal associations. Close examination of root material identified only a limited indication of an association between the hyphae and root tips. Qualitative analysis of root material has particular disadvantages in these type of studies. Particularly with regard to the identification of associated fungi and the relative dependance of the host and symbiont given the lack of identifiable ECM structures.

3.4.1 Experiment 1

The presence of hyphae in the majority of inoculated root systems suggests spore-based inoculum was effective for the colonisation of experimental plants. Lu *et al.* (1998) applied a comparable number of *Scleroderma* and *Pisolithus* spores to seedlings at planting, which yielded a high percentage of ECM infection in *E. globulus*. The authors reported that by day 65 only 50% of roots sampled were ectomycorrhizal and by day 110 all inoculated seedlings were ectomycorrhizal but often with a low level of infection (<10%). In their conclusion, mention is made of the variability in mycorrhizal infection according to inoculum density and they stress the need to consider differences in compatibility and inoculum viability for ECM production. This same study also found extreme variability in infection rates of *P. tinctorius* with some showing high levels of infection by comparison with no infection.

Despite the lack of true mycorrhizal structures, whole plant growth of both species showed an apparent growth response to inoculation. Further, inoculation of seedlings affected the response; this is reflected in both plant growth and proline content. A decline in whole plant weight of *E. diversicolor* seedlings inoculated with *Scleroderma* spores in response to increased salinity may suggest a negative effect of inoculation with the fungus, as there was no reduction of growth in the controls. However, this reduction did not occur in the *Pisolithus* inoculated plants.

In contrast, uninoculated *E. camaldulensis* seedlings saw a substantial decline (>50% reduction between 0 and 200 mM) in growth with increasing salinity. This reduction is not apparent in the uninoculated seedlings, suggesting the inoculation with mycorrhizal spores increases tolerance of *E. camaldulensis* to soil salinity. The response of both species to salinity is unusual given uninoculated *E. camaldulensis* demonstrated a significant reduction in plant growth whereas uninoculated *E. diversicolor* showed no such response. Typically *E. diversicolor* would be considered a salt sensitive species in comparison to *E. camaldulensis* (Bell, 1999; Kozlowski, 1997) but in this experiment only appeared sensitive in those plants inoculated with spores of *Scleroderma*. In contrast *E. camaldulensis* appeared not to be salt sensitive in inoculated plants.

Proline analysis of root and shoot material identified differences in the relative concentrations between plant species with *E. diversicolor* showing a reduced level of proline accumulation than that of *E. camaldulensis*. The latter produced higher levels of proline in both roots and shoots . Variation in root and shoot proline content occurred between the two eucalypts. *E. diversicolor* had lower proline content in the roots and *E. camaldulensis* had lower proline content in the shoots.

A typical response of proline content was observed in uninoculated *E. camaldulensis* which showed a significant increase in proline with increased salt concentration (Murakeozy *et al.*, 1999). A similar pattern is apparent of *Scleroderma* inoculated seedlings, however, background levels of proline are greater. The significance of increased root proline is unclear and requires further investigation. The increasing trend in proline content of roots of *Scleroderma* and uninoculated plants is in stark contrast to *Pisolithus* inoculated plants. These plants showed a significant reduction in proline content suggesting inoculation with this species of fungus may reduce the requirement for proline as an osmoregulator. It remains that background levels of proline were substantially higher in inoculated plants than in uninoculated plants. The significance of this is, again, unclear but it may be suggested that increased background levels may increase salt tolerance in these plants, should it be encountered.

3.4.2 Experiment 2

Inoculation with mycorrhizal plugs as performed in this experiment may not have been sufficient to enable appropriate development of mycorrhizal structures within the time frame permitted prior to exposure to salt solutions. Peterson and Chakravarty (1991) describe similar procedures for inoculation in growth pouches containing quartz sand material. No indication of the length of time required for ECM development was given However, a number of smaller agar plugs were distributed throughout the soil medium in that procedure.

Plant growth in these experiments were shown to be influenced by inoculation with *P. tinctorius* using agar plugs. It is difficult to discern from this data which fungal treatment affected plant growth. Fungal treatments within plant species responded differently to salinity. *E. diversicolor* showed comparatively lower tolerance to 200 mM in all three fungal treatments. A general decrease in growth with increasing salinity is apparent of *E. diversicolor* at each fungal treatment. In each case, plants at 200 mM showed significantly lower growth than the control plants (0 mM). LuH78 inoculated *E. diversicolor* varied slightly in this pattern with a decrease in growth at 50 mM. These results may suggest *E. diversicolor* is affected by salt concentrations at 200 mM regardless of fungal treatment.

A trend that is evident of *E. camaldulensis* seedlings is that 50mM salt consistently shows higher plant growth than other salt treatments including the control plants. Whilst the difference between 0 and 50 mM treatments of uninoculated and MH39 plants is not significant, it is in plants inoculated with LuH78. This may suggest inoculation of *E. camaldulensis* with this isolate improves plant growth at this level of salinity. Growth of *E. camaldulensis* was not significantly affected by 100 and 200 mM salt treatments at all fungal treatments making conclusions with regard to the application of fungal propagules inconclusive. Certainly increased growth at 50 mM salt of LuH78 inoculated plants is encouraging in this respect. Potentially this response may be associated with the moderately increased nutrition that can be associated with NaCl, particularly Cl^- (Kinraide, 1999; Grattan and Grieve, 1992; Marcar and Termaat, 1990). Sun and Dickinson (1993) found biomass of *E. camaldulensis* was significantly affected by salt concentrations in excess of 150 mM which is in contrast to the results found here. However, in that work biomass measurements were taken after 60 days of exposure to NaCl treatments. Rawat and

Banerjee (1998), on the other hand, found biomass production in *E. camaldulensis* was stimulated by NaCl concentrations up to 160 mM.

Proline levels in both species show little relationship with plant growth as measured in the form of whole plant weights. While variation in proline content in roots and shoots between and within the two plant species is evident, there is no clear indication proline content influences plant growth. Similarly, relationships between roots and shoots within plant species and fungal treatment also indicate no clear relationship. However, proline levels in roots and shoots is of interest in a number of instances. *E. diversicolor* evidently shows significantly higher levels of proline in roots inoculated with LuH78. Plants exposed to 200 mM in this case have lower levels of proline compared to 50 mM NaCl treated plants. Shoot proline content of LuH78 inoculated *E. diversicolor* show a significant decline at 50, 100 and 200 mM. Perhaps proline production in roots is greatly influenced by inoculation with LuH78, which in turn may influence proline production in shoots of *E. diversicolor*. Certainly it is apparent that salinity influences proline production of inoculated *E. diversicolor*.

Very different patterns of proline production in *E. camaldulensis* were observed. A slight reduction in root proline is apparent of inoculated treatments with significant variation between salt treatments observed of uninoculated and MH39 inoculated plants. Whilst these results are significant, they are less dramatic in a quantitative sense in comparison to levels of root proline in *E. diversicolor*.

Of particular interest is the level of proline production in the shoots of *E. camaldulensis*. All fungal treatments showed significantly higher concentrations in plants at 0mM suggesting there is a common factor influencing proline production. The increasing trend in proline concentration from 50 to 200 mM appears more characteristic of expected levels of proline with increasing salt stress (Murakeozy *et al.,* 1999). In each fungal treatment proline levels are significantly higher than 50 and 100 mM treatments but are not statistically different from 0 mM (with the exception of MH39 inoculated plants). From this information it would be reasonable to deduce there is possibly a further stress factor operating in 0 mM plants influencing proline production. The consistency of shoot proline results across each fungal treatment indicates inoculation may not influence proline production to the extent observed with *E. diversicolor*.

3.4.3 Experiment 3

The response to increased salinity was relatively consistent between clones and was evident in plant weights. Plants exposed to 200 mM salt showed reduced growth in all clones regardless of fungal treatment with the exception of an inoculated group within clones C066 and C919. Nonetheless an apparent reduction in plant growth is also observed in these two treatment groups. Plant growth was significantly increased by fungal inoculation in C903, particularly plants inoculated with LuH78. A similar response to fungal treatment was not found between treatments in plants exposed to 200 mM salt. This evidently suggests fungal treatment does not alleviate salt stress in clones as indicated by plant growth.

Direct comparisons between clones is difficult, however indirect comparisons indicate a similar response to salinity of individual clones via plant growth. This is in contrast to the findings of Farrell *et al.* (1996), where significant variation in the response to salinity of clonal lines of *E. camaldulensis* was identified. The reported study identified a high correlation between growth and water uptake ability of individual clones. This may indicate that clones used for this experiment had similar water uptake abilities and similar abilities to deal with salt stress resulting in the regular pattern observed in plant growth. Consideration of this statement is necessary in that these clones were chosen for their relative tolerance levels of salinity. It is possible that 200 mM salt was not high enough in this experiment to identify significant variation in growth between clones. This does not assist in the evaluation of the physiological well-being of the host plant on mycorrhizal symbiosis in saline soil as initially proposed. A greater range in salinity levels above and below 200 mM would be valuable in similar studies with these same clones.

Growth of C903 inoculated with LuH78 increased in the control plants. Similar increases were not observed in the other clonal lines suggesting there may be variability in compatibility of the fungal isolates and the clones used. Given the positive response in growth of this particular clone, and the associated fungal isolate, there appears to be no effect of inoculation in plants grown at 200 mM. This suggests salinity has a significant impact on the functional capacity of ectomycorrhiza in distinct clonal lines of *E. camaldulensis*. Similarly, 200 mM NaCl has a negative impact on the fungal component of ECM. Whilst this suggestion should be treated tentatively, given the absence of mycorrhizal structures, there is an indication that salinity may affect the growth and development of ECM in specific genotypes within this species.

Proline levels in shoots and roots were shown to vary between salt treatments as well as fungal treatments. Proline levels in shoots were greater than in roots which is consistent with the reported results of Hatimi (1999) and Duke *et al.* (1986). There were significant increases in foliar proline concentrations in C502 only. Plants inoculated with LuH78 had lower shoot proline levels grown at 0 mM salt. In general shoot proline appeared very similar within fungal treatments and between clones. This is not the case of plants grown at 200 mM salt, where a general increase in shoot proline is observed of all clones with the exception of C502. Also of interest is that proline levels in this latter clone appeared substantially lower than the remaining clones when grown in 200 mM salt.

Fungal inoculation did not significantly influence shoot proline content in any of the clones. Inoculated plants of C066 and C919 showed slightly reduced levels indicating reduced stress in these plants. This is not significant but may indicate there is a reduced stress response to salinity in these inoculated clones. There appears little discrimination between the fungal isolates used in the experiment where similar results in shoot proline are found for plants inoculated with these two isolates. However, there would appear to be significant variation in shoot proline between the different genotypes of *E. camaldulensis* which may also be affected by fungal inoculation.

The low level of proline accumulation in root samples compared with shoots, suggests shoot proline may provide a better indication of stress in *E. camaldulensis* clones. Generally root proline levels in clones of C066, C502 and C919 decreased in inoculated plants grown at 200 mM salt. This reduction was significant in C066 and C502 with both inoculated C066 plants having lower root proline levels compared to inoculated plants.

Similarly C502 plants inoculated with LuH78 showed lower root proline production. Proline levels in roots between salt treatments were not significant in all but a few groups and did not demonstrate any particular pattern. This may be attributed to the large amount of error associated with mean values.

Certainly inoculation influenced proline levels in specific clones but this pattern was not reflected in plant growth. While there was a positive response to inoculation of one clone at 0 mM, there was no subsequent benefit of inoculation in this clone when grown at 200 mM salt. Alternative concentrations of salt may have yielded a different response to inoculation. Due to physical limitations in experiment size the decision to reduce the number of salt concentrations was made. Ideally the ability to sample a greater number of different salt concentrations may have found there to be a significant response in plant growth to inoculation at lower concentrations of NaCl.

What is encouraging from this experiment is the variability in proline levels between fungal and salt treatments. Hatimi (1999) found proline concentrations in leaves of *Acacia cyanophylla* Lind. to increase in plants with increasing salinity. However, plants that were mycorrhizal showed significantly higher levels of shoot proline than that of uninoculated plants. In contrast, the data from this experiment showed a general decrease in proline levels in specific clones inoculated with ectomycorrhizal isolates grown at 200 mM salt. This may indicate a reduced level of stress in specific inoculated clones. However, these plants grown at 200 mM still maintained higher levels of shoot proline than those grown at 0 mM salt.

Whilst these results are inconclusive in terms of quantitative data relating to mycorrhizal infection, proline data suggest inoculation may reduce the production of this osmolyte in plants grown in a high salt environment. It is also important to mention that this information did not translate directly to plant growth, at least in the case of this experiment.

CHAPTER 4: GENERAL DISCUSSION

The salt tolerance of the ectomycorrhizal fungi *Pisolithus tinctorius* was, in general terms, tolerant of NaCl up to 150 mM. The screening of specific isolates in axenic culture indicated variability in tolerance limits of individual isolates. Variation in growth rates and the sparse growth form of two isolates suggested there is a need for preliminary screening trials to enable more direct comparisons of salt tolerance between isolates that are known to have very similar growth rate and habit. Results of this experiment indicate that there is substantial variation in the salt tolerance of *P. tinctorius* in axenic culture. However, application of the growth response categories described by Hutchison (1990a, b) suggests all isolates examined were at least semi-tolerant of 150 mM NaCl.

An understanding of the underlying mechanisms of salt tolerance is important for the application of ectomycorrhizal technology in forestry and agriculture. It has been suggested by Dixon *et al.* (1993) that many species of fungi reduce internal osmotic potentials via cytoplasmic osmoregulation. The production of organic compounds, particularly proteins, is recognised as a potential avenue for osmoregulation in basidiomycetes (Wilson and Griffin, 1979; Mexal and Reid, 1973). Future studies examining the *in vitro* tolerance of salt requires sampling for protein concentrations of fungal hyphae from colonies grown on media with varied salt concentrations. The mechanisms of tolerance in these isolates was not explored in detail. Preliminary investigation of techniques in protein sampling was carried out, however, the amount of fungal material required to attain reliable and consistent data for assessment of protein concentration was inhibitory in this study but could certainly be applied to future studies.

The translation of *in vitro* salt tolerance of the chosen axenic culture isolates to *in situ* glasshouse conditions is difficult to conclude given the low level of infection in these plants. Experiments utilising axenic culture material identified the isolate LuH78 to consistently influence growth and/or proline levels in inoculated plants in both experiments (although not at 200 mM salt in experiment 3). By comparison MH39 saw only moderate variations in the measured parameters of inoculated plants. Hence it could be concluded from this tentative assumption that *in vitro* salt tolerance is not necessarily an indication of tolerance in soil. It is important to consider the significant functional variability in *P. tinctorius*, the host plant and the interaction between the two entities. These combinations may vary depending on environmental factors. Therefore, it is important not to discount the possible translation between *in vitro* and "in soil" salt tolerance in this and similar experiments.

Glasshouse experiments yielded particular problems with regard to the level of mycorrhizal infection. Potentially there are a combination of factors that may have contributed to the low level of mycorrhizal infection in both *E. diversicolor* and *E. camaldulensis* (seedlings and clones). The most likely problems encountered in this situation includes the length of time allowed for development of ECM, and the time period allowed for sufficient development of the symbiosis. In these experiments, the time period ranged between 6 and 10 weeks prior to the addition of salt nutrient solutions. Horan *et al.* (1988) examined the time sequence of ECM in eucalypts and described the infection process as a matter of days where by day 4 all the anatomical features of the association were present on roots of *E. globulus* inoculated with *P. tinctorius*. This experiment was conducted under well

controlled conditions *in vitro* between plants and fungi that were known to be highly compatible. Comparison of other studies on ECM in eucalypts suggests an average period of 60-70 days is appropriate for the development of a high rate of infection in host plants (Lu *et al.*, 1998; Dixon *et al.*, 1993).

Inoculation of seedlings and cuttings under glasshouse conditions provides challenges in the form of suitable conditions for the development of the symbiosis. For example, it is known that *P. tinctorius* isolates are often dependant on pH (Aggangan *et al.*, 1996) and temperature (Cairney and Chambers, 1997) for an effective rate of infection. It should be mentioned that temperature was not a likely factor in the poor rate of infection in these experiments, as inoculation took place during the warmer months of the year when average temperatures were well above those considered appropriate (Cairney and Chambers, 1997).

Another area that warrants further investigation is the method of applying salt treatments. The addition of salt-nutrient solutions may have inadvertently created excessive soil moisture conditions that are typically unfavourable to the formation of ECM (Mason *et al.*, 2000; Bougher and Malajczuk, 1990). Future studies would benefit from exploration of alternative means of applying the salt treatment. The system used in these experiments had the advantage of introducing salinity incrementally thereby avoiding osmotic shock. The application of salts directly to the soil prior to introducing mycorrhizal plants may induce osmotic shock and inhibit further development of the symbiosis. There is a need to develop new techniques of introducing saline conditions to study its effect on mycorrhizal plants.

Nutrition of inoculated plants may have been sub-optimal for the rapid development of ECM. Both the supply and composition can influence mycorrhizal fungus development in potted plants, particularly in heavily leached potting mixes (Brundrett *et al.*, 1996). Application of nutrient solution to inoculated plants prior to experiments may not have been appropriate for the development of ECM in eucalypts grown in sand culture. Jentschke *et al.* (1999) document an elaborate system for the maintenance of both VAM and ECM in sand culture specifically for experimentation on the effects of heavy metals on mycorrhizal conifers. Future experiments examining the effects of salinity on ECM eucalypts would benefit from the system described in that closer management of soil nutrients is possible. However, it is likely that costs associated with such a system may limit the size of experiments.

Despite the absence of mycorrhizal structures in these plants, in many cases there was an apparent growth response to inoculation. The results for *E. diversicolor* suggests salt tolerance of plant species may be influenced by inoculation with spores of specific mycorrhizal species. Significant reductions in growth in response to increased salinity were observed in *E. diversicolor* inoculated with *Scleroderma*, but this was not reflected in other fungal treatments. Spore inoculated *E. camaldulensis* showed improved salt tolerance compared to uninoculated plants. This would suggest the benefit of mycorrhizal infection may vary between plant species and according to the fungal symbiont.

The time period allowed for development of mycorrhizal infection may have not been sufficient to allow the use of hyphal plugs for inoculation of seedlings and clones. The use of plugs from colonies in axenic culture is not unusual and has provided effective levels of

infection in other studies (Dodd and Thompson, 1994; Heinrich *et al.*, 1988). Both experiments in this study utilising axenic plugs for inoculation of seedlings and cuttings did not show the growth response observed of spore inoculated seedlings. From this it is assumed that inoculation from axenic material requires greater levels of infectious propagules placed in the soil and certainly a greater length of time for hyphal growth and development of the association. Equally, it suggests the use of spore-based inoculum is more effective than plugs for glasshouse experiments.

Inoculation with axenic culture material may be more effective in more controlled conditions such as in tissue culture. The development of the symbiosis can be monitored and introduced to saline axenic or soil culture when it is considered appropriate. This would allow greater control in identifying the physiological status of the symbiosis prior to the application of salt. In addition, it allows greater flexibility in experimental design and may allow for additional factors to be examined. For example, Dixon *et al.* (1993) studied the effects of NaCl on the development of ECM in cultured *Pinus taeda* plants and identified variation in ECM formation by different species of ECM fungi.

The most encouraging aspect that appears to be relatively consistent in all glasshouse experiments is the response of shoot and root proline to both inoculation with mycorrhizal fungi and the subsequent response to salt treatment. Spore inoculated seedlings had greater proline accumulation in plants at lower NaCl concentrations, particularly when compared to uninoculated seedlings. This implies that inoculation with spores of mycorrhizal fungi may significantly influence proline levels in shoots and roots. Inoculation with axenic culture material had a less obvious impact on proline levels in both roots and shoots of *E.*

diversicolor seedlings and *E. camaldulensis* seedlings and clones. However, there were a few exceptions to this generalisation.

Results of proline analysis are important when consideration is given to the osmoregulatory role of proline in many plant species (Hare and Cress, 1997; Greenway and Munns, 1980). It was found by Hatimi (1999) that foliar proline concentration was significantly higher in *Acacia cyanophylla* inoculated with both *Rhizobium* and mycorrhizal fungi regardless of the level of salinity. This similar finding provokes a number of questions with regard to the physiological interaction between plants and mycorrhizal fungi, not only in stress environments, but in non-stress environments also.

4.1 Concluding remarks

This study attempted to evaluate the response of ectomycorrhizal fungi and ectomycorrhizal eucalypts to salinity. The examined isolates of *P. tinctorius* demonstrated tolerance to increased levels of NaCl in agar medium, however, the growth form and habit of individual isolates are variable and future studies of this nature requires preliminary screening of a number of isolates to identify those with similar growth characteristics.

The absence of characteristic ECM structures was particularly disappointing, however, the presence of hyphae and an observed increase in growth and proline response to inoculation suggests the association may have been in the early stages of development. Future experimentation should consider the length of time required for optimal infection in addition to appropriate soil nutrient and soil moisture conditions. The moderate growth

response and considerable response in proline levels of shoots and roots is encouraging of the role of ectomycorrhizas in reducing salt stress in eucalypts. The indication of a reduction in salt stress in inoculated plants suggests the potential critical role of ECM eucalypts in the rehabilitation of salt affected land. Further research on the protective capacity of ECM in saline environments is justified.

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