THE GROWTH RESPONSE OF *EUCALYPTUS GRANDIS X E. CAMALDULENSIS* TO SALT STRESS, ECTOMYCORRHIZAE AND ENDOMYCORRHIZAE DOUBLE COLONISATION

by

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Thesis presented in partial fulfillment of the requirements for the degree of Master of Science in Forestry at the University of Stellenbosch
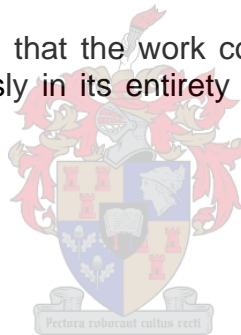
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March 2007

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and has not previously in its entirety or in part been submitted at any university for a degree.

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SUMMARY

The study was undertaken to determine the potential physiological benefits to plants provided by the double colonisation of host plant roots by endomycorrhizal (AM) and ectomycorrhizal (ECM) fungi, when growing under normal and under salt stress conditions. Plants of the *Eucalyptus grandis x E. camaldulensis* clone were grown in a sterile soil with 0 and 75 mM NaCl and with or without infection with the fungi *Glomus etunicatum* (an AM fungus) and *Pisolithus tinctorius* (an ECM fungus). The *Eucalyptus* clone formed both ECM and AM in single and double inoculation. The mycorrhizal symbiosis did not provide any nutritional benefits to the hosts. The double colonisation had no effect on plant growth under normal growth conditions while single colonisations of AM and ECM reduced growth. Double colonisation reduced host plant specific leaf mass by 12% and increased total leaf area by 43% compared with the control under these growth conditions. This colonisation also reduced photosynthesis per leaf area by 29% compared with the control. The reduced photosynthesis of the double colonisation did not result in reduced plant growth because these plants may have had a high total plant photosynthesis because of their large total leaf area. The double symbiosis however did not reduce salt stress when host plants were exposed to 75 mM NaCl, while the AM fungus increased plant dry weight by 13% compared to the control. AM and ECM colonisation in the double colonised roots under salt stress was decreased by 18 and 43% compared to that in plants under normal growth. The reduced colonisation may have reduced the fungi's abilities to be beneficial to the host plant. The double symbiosis is recommended based on the documented positive effects of this symbiosis to plant growth and the considered possible long-term benefits to host plants growing in saline soils.

OPSOMMING

Die studie is onderneem om die potensiële fisiologiese voordele vas te stel wat gasheerplante verkry uit die dubbele kolonisasie van wortels deur endomikorrisale en ektomikorrisale swamme, wanneer plante onder normale toestande en ook onder soutstres groei. Plante van 'n *Eucalyptus grandis x E. camaldulensis* klone is in 'n steriele grond gekweek met 0 en 75 mM NaCl en met of sonder infeksie van *Glomus etunicatum* ('n endomikorrisale swam) en *Pisolithus tinctorius* ('n ektomikorrisale swam). Die *Eucalyptus* klone het beide endomikorrisa en ektomikorrisa gevorm in enkel en dubbele inentings. Die mikorrisale simbiose het geen voordelige voedingstofopname vir die gasheerplante ingehou nie. Die dubbele kolonisasie het geen uitwerking op die plante se groei gehad onder normale omgewingstoestande nie, terwyl enkel kolonisasie van endomikorrisa en ektomikorrisa die gasheerplante se groei verminder het. Dubbele kolonisasie het gasheerplante se spesifieke blaarmassa met 12% verminder en totale blaaroppervlakte met 43% vermeerder in vergelyking met die kontrole onder hierdie groeitoestande. Hierdie kolonisasie het ook die fotosintese per blaaroppervlakte met 29% verminder in vergelyking met die kontrole. Die verminderde fotosintese van die dubbele kolonisasie het nie die groei van plante verminder nie omdat hierdie plante waarskynlik 'n hoë totale plant fotosintese gehad het as gevolg van hulle groot totale blaaroppervlakte. Die dubbele simbiose het egter nie die soutstres verminder toe gasheerplante aan 75 mM NaCl blootgestel is nie, terwyl die endomikorrisale swam die droë gewig van plante met 13% teenoor die kontrole verhoog het. Endomikorrisale en ektomikorrisale kolonisasie in die dubbel gekoloniseerde wortels onder soutstres is met 18 en 43% verminder in vergelyking met plante wat onder normale toestande gegroei het. Die verminderde kolonisasie kon die swamme se vermoë om voordelig vir die gasheerplant te wees, verminder het.

Dubbele simbiose word aanbeveel op grond van die gedokumenteerde positiewe uitwerkings wat hierdie simbiose op die gasheerplante se groei het en die oorweegde moontlike lang-termyn voordele vir gasheerplante wat in brakgronde groei.
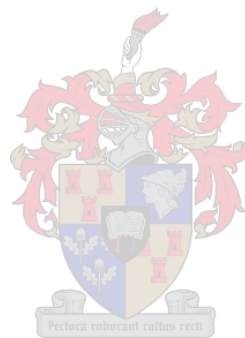
To my mom:

Erica Kavetu Hengari
(1940 –2006)

"Thank you for your love mom. I will always miss you."

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ECM   Ectomycorrhiza/e

AM   Endomycorrhiza/e

VAM   Vesicular Arbuscular mycorrhizal fungi

SLM   Specific leaf mass

Pmax   Photosynthesis

Gs   Stomatal conductance

Dark resp.   Dark respiration

WUE   Water use efficiency

PNUE   Photosynthetic nitrogen use efficiency

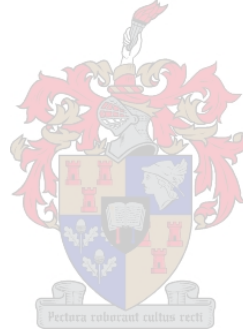PPUE   Photosynthetic phosphorous use efficiency

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CHAPTER 1 GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Forests resources in Northern Namibia play an important role in supporting the livelihood of communities. These resources have always been and still are of great importance for human and animal life, offering protection and shelter and providing timber, medicine, food, firewood and spiritual needs. Human activities, the global climatic change and natural catastrophes affect the forests in Namibia in many visible ways, but there are equally impacted and neglected below-ground organisms. Trees from wet tropical to dry desert environments in Africa grow in symbiosis with both ectomycorrhizal and endomycorrhizal fungi individually or in double symbiosis (Hogberg, 1986; Hogberg, 1992; Bohreg *et al.*, 2001). The presence of mycorrhizal fungi in these environments is influenced by different factors such as rainfall, soil nutrition, availability of suitable host, and soil salinity (Bohreg *et al.*, 2001; Uhlmann *et al.*, 2004a; Uhlmann *et al.*, 2004b). The different endomycorrhizal fungi present in the arid and semi-arid regions of Namibia are presented in **Table 1**. The data contain eight fungi from the semi-arid and one from the arid environment that could only be identified up to the genus level. There are very few reports on ectomycorrhizal fungi in Namibia. The best-known fungus being the edible *Terfezia pfeilii* or "the Kalahari desert truffle" as recorded by Taylor *et al.* (1995). The other documented ectomycorrhizal fungus, *Termitomyces schimperi*, is found on and around termite mounds (Namibia initial national communication on climate change to the United Nations, 2002).

TABLE 1. Endomycorrhizal fungi from arid and semi-arid regions of Namibia (Stutz *et al.*, 2000; Uhlmann *et al.*, 2004a; Uhlmann *et al.*, 2006).

Species from semi-arid environment	Species from arid environment
Acaulospora appendicula	
Acaulospora bireticulata	
Acaulospora dilatata	
Acaulospora foevata	
Acaulospora laevis	*Acaulospora laevis*
Acaulospora nicolsonii	
Acaulospora scrobiculata	
Acaulospora spinosa	
	Acaulospora trappei
Acaulospora tuberculata	
Acaulospora sp.1	
Acaulospora sp.2	
Archaeospora gerdemannii	
Archaeospora leptoticha	
Entrophospora infrequens	
Gigaspora albida	
Gigaspora gigantea	
Gigaspora margarita	
Gigaspora ramisporophora	
Glomus aggregatum	*Glomus aggregatum*
	Glomus arenarium
	Glomus australe
Glomus atunicatum	
Glomus caledonium	
Glomus claroideum	
Glomus clarum	
Glomus constrictum	
Glomus dimorphicum	
Glomus etunicatum	
Glomus fasciculatum	
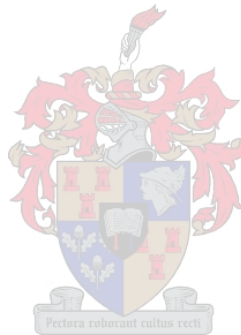
TABLE 1 (*concluded*)

Species from semi-arid environment	Species from arid environment
Glomus geosporum	*Glomus geosporum*
Glomus heterosporum	*Glomus heterosporum*
Glomus hoi	
	Glomus intraradices
Glomus maculosum	
Glomus manihotis	*Glomus manihotis*
	Glomus microaggregatum
Glomus mosseae	*Glomus mosseae*
	Glomus occultum
Glomus reticulatum	*Glomus reticulatum*
	Glomus spurcum
	Glomus sp.1
Glomus sp. 1	
Glomus sp. 2	
Glomus sp. 3	
Glomus sp. 4	
Glomus sp. 5	
Glomus sp. 6	
Paraglomus occultum	
Scutellospora alborosea	
Scutellospora erythropa	
Scutellospora scutata	
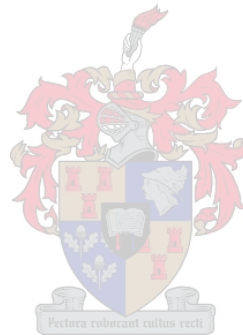
Roots of most healthy plants provide specialised habitat in which various fungi live and obtain all or some of the elements required for growth (Wilcox, 1991). The fungi, in return, absorb nutrients from the soil and provide them to the host plant. The growth forms formed by roots and fungi are termed mycorrhizae. The provision of growth elements (photosynthates) to the fungi result in a carbon (C) cost which can limit the growth of the host plant (Jakobsen and Rosendahl, 1990).

Wilcox (1991) classified mycorrhizal fungi into three broad groups: Ectomycorrhizae (ECM), ectoendomycorrhizae (ECM/AM), and endomycorrhizae (AM). There are also the unclassified mycorrhizae, pseudomycorrhizae, and virulent pathogens. These categories are based on the observation of fungal mycelium in relation to root structures. Vesicular-arbuscular mycorrhizae (VAM), ericoid mycorrhizae and orchidaceous mycorrhizae all belong to the AM group. VAM fungi occur in warm and dry environments with a high turnover of organic matter, while ericoid mycorrhizae occur in cold and wet environments with reduced organic matter decomposition and mineralization activities. ECM fungi form a dense sheath around host roots with limited penetration, while AM grow mostly within the plant roots with few external hyphae. ECM occurs in the intermediate environments having different levels of organic matter decomposition and mineralization. Some trees, such as the genus *Eucalyptus*, form both symbioses (Chen *et al*., 2000).
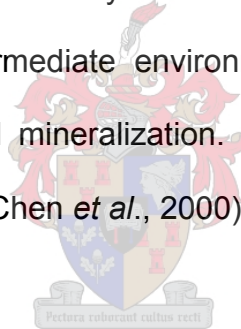
Mycorrhizal fungi enhance host plant growth by improving nutrient uptake by an increased absorbing surface area, by making sparse nutrient sources available to the host plant, and also through excretion of chelating compounds or ecto-enzymes (Marschner and Dell, 1994). The fungal colonisation may also protect roots from soil pathogens and increasing host plant root growth. The practical use of mycorrhizal fungi is also credited to their ability to enhance survival and growth of tree seedlings planted out in harsh environments (Grove and Malajczuk, 1994). It is universally recognised that AM fungi mostly assist host plants with the uptake of phosphorus (P) (Gerdemann, 1975), whilst the ECM fungi are more efficient at nitrogen (N) uptake (Wilcox, 1991).

The ability of mycorrhizae to provide the plant with nutrients depends on:

❑   Availability of the nutrients in the soil

❑   Inability of the plant to obtain the nutrients by other means

❑   Soil moisture

❑   Soil pH

❑   Soil temperature

❑   Interaction with bacteria (i.e. nitrogen fixing bacteria, mycorrhizal helper bacteria)

❑   Interaction with root-inhabiting nematodes and root pathogenic fungi (Krikun, 1991; Wilcox, 1991; Fitter and Garbaye, 1994).

Mycorrhizal fungi are attacked by a number of pathogens that affect their ability to provide plants with nutrients. They must also compete with root pathogenic fungi and bacteria. Management of these fungi are important for successful reforestation and conservation of forest resources (Grove and Malajczuk, 1994).
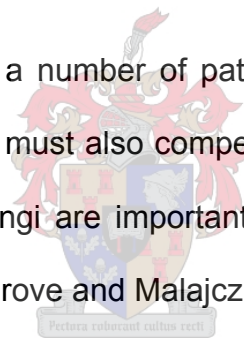
There are no more new territories with endless resources, all the world has been discovered, populated and polluted. The world population keeps rising, although now probably being slowed down by seemingly incurable diseases like malaria and AIDS. The resources of the world are however shrinking and we will have to make-do with unsuitable environments to support human life on earth. It is therefore important to have an understanding of plant growth in stressful environments. It is also important to grasp the relationship between plants that support human life and the micro-organisms that live within their roots, stems and leaves. Jacquard (2004) wrote: "In isolation we are primates; it is encounters that make us human". This analysis can

also be applied to plants, as they could never grow as we observe them now, neither phenologically nor biochemically, if growing in isolation. The understanding of the interaction between plants and soil fungi and their influence on how plants overcome environmental stress will form the central discussion of this document.

Tree species from the genus *Eucalyptus* are widely planted throughout the world. They naturally grow from the latitude 7 ^{o}N to 43 ^{o} 39‘S (Poynton, 1979). It is the preferred genus planted in countries with poor forestry resources because of its hardiness and ability to grow in harsh environments. This genus is generally planted for timber, oils, honey production, firewood and ornamental purpose. *Eucalyptus* grows in symbiosis with mycorrhizal fungi making it possible for them to grow in infertile soils (Grove *et al*., 1996). *Eucalyptus* species, in particular *E. camaldulensis*, are used for afforestation projects in Namibia, mainly for pole production. There is also a potential to start utilizing the old stands for honey production. *E. camaldulensis* grows on alluvial, silty soils of good depth, and can grow on sands or podsols overlying clayey, wet subsoil. The mean maximum and minimum temperatures of its natural habitat are 29 to 35 ^{o}C and 11 to 20 ^{o}C respectively, while the mean annual rainfall ranges from 250 to 625 mm. *E. grandis* grows on deep, well drained yet moist loamy, alluvial deposits but it does not tolerate permanently waterlogged conditions. The mean annual maximum and minimum temperatures of its natural habitat are 29 to 35 ^{o}C and 5 to 6 ^{o}C respectively, while the mean annual rainfall ranges from 1000 to 1800 mm (Poynton, 1979). The afforestation areas in Namibia match the soil and climatic range of the natural habitats of *E. camaldulensis* and *E. grandis*, having sandy soils and an annual rainfall range of 300 to 1000 mm (Mendelsohn and Obeid, 2003). It is possible however that the poor growth of *E. camaldulensis* experienced in

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Namibia is due to insufficient presence of symbiotic ECM and AM fungi. A list of ECM and AM fungi symbiotic with *Eucalyptus* species is provided in **Table 2**.

The combination of numerous factors such as low rainfall, low soil fertility and high temperatures, make tree planting and crop production a difficult task in the highly populated Northern-central regions of Namibia. Desertification is a serious problem in these regions due to the semi-arid climatic environment combined with anthropogenic disturbances. The land-form is a vast alluvial fan that was deposited by the Kunene River in quaternary times when the river drained into the Etosha Pan. These regions have poor soils, being saline and having low nutrients (Moller, 1997). The soils are characterized as solonetz soils, formed by a combination of saline soil low in calcium and high in sodium, a cyclic period of water logging and high potential evaporation. The area has substantial ground water resources, but they are mostly unusable due to their high salinity (Erkkilä, 2001). Tree growth in the North-eastern parts of Namibia is also hampered by low soil fertility and low soil water holding capacity. The water table is however high at places, less than 10 meters deep, causing seasonal water logging. The high water table and salinity levels inhibit tree growth while only allowing grass proliferation. The soils of this area are sandy and poor in nutrient and organic matter content (Rigourd *et al.*, 1999).

TABLE 2. *Symbiotic ectomycorrhizal and endomycorrhizal fungi for* Eucalyptus *species* (Malajczuk *et al*., 1981; Malajczuk *et al*., 1982; Lapeyrie and Chilvers, 1985; Adjoud *et al*., 1996; Chen *et al*., 2000; dos Santos *et al*., 2001; Gange *et al*., 2005).

Ectomycorrhizae	Endomycorrhizae
Agricus xanthodermus	*Acaulopora laevis*
Boletus portentosus	*Glomus etunicatum*
Cenococcum geophilum	*Glomus caledonium*
Cortinarius archeri	*Glomus fasciculatus*
Cortinarius fragilipes	*Glomus intraradices*
Cortinarius microarcheri	*Glomus invermatum*
Cortinarius ochraceus	*Glomus mosseae*
Cortinarius purpurascens	*Glomus pallidum*
Cortinarius radicatus	*Scutellospora calospora*
Cortinarius subcinnamomeus	
Gymnopilus pampeanus	
Hydnangium carneum	
Hymenogaster albellus	
Hymenogaster albus	
Hymenogaster violaceus	
Hygrophorus coccineus	
Hysterangium incarceratum	
Hysterangium inflatum	
Inocybe olivaceofulvus	
Lycoperdon gemmatum	
Macrolepiota procera	
Mesophellia arenaria	
Naematoloma fasciculare	
Octaviana densa	
Pisolithus tinctorius	
Ramaria sinapicolor	
Russula purpureoflava	
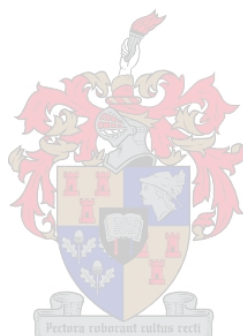
TABLE 2 (concluded)

Ectomycorrhizae	Endomycorrhizae
Scleroderma albidum	
Scleroderma bovista	
Sclerodermia cepa	
Sclerodermia verrucosum	
Tricholoma coarctatum	
Tricholoma pardinum	

An understanding of the physiological response to salt stress of the tree species used for reforestation of these areas is essential for a successful operation. Plant salt tolerance can influence plant species distribution. Salt stress can be in the form of osmotic stress or ion stress. Osmotic stress affects the osmotic potential of plants, affecting plant water relations, while ionic stress affects plant ion balance (Lefèvre *et al.*, 2001). Salt stress causes the reduction of uptake of certain ions such as calcium (Ca^{2+}), potassium (K) and magnesium (Mg^{2+}) (Grattan and Grieve, 1992; Martinez-Ballesta *et al.*, 2004). Salt stress reduces plant growth (Seemann and Critchley, 1985). The reduced plant growth inhibits the utilisation of photosynthates, in turn reducing photosynthesis (Munns, 1993). Salt tolerance is associated with restriction of salt uptake into plant stem and leaves (Zekri and Parson, 1992). Plants also reduce leaf area or drop their leaves to reduce water loss due to salt stress. There is a build up of compatible solutes, such as proline and carbohydrates, in plant leaves as a mechanism to withstand salt stress and maintain turgor (Bradley and Morris, 1991). The high sodium (Na^+) concentration of saline soils can not only injure plants directly but also degrade the soil structure, decreasing porosity and water permeability (Moghaieb *et al.*, 2004).

The aim of the present work was to study the morphological and physiological response of a *Eucalyptus* clone, *Eucalyptus grandis x E. camaldulensis,* to salt stress in combination with AM and ECM fungi colonisation. The plants were obtained from the Sappi nursery at Kwabonabi, South Africa. The two types of fungi exist in Namibia, and plants introduced here are therefore likely to have their roots colonised by these fungi in isolation or in combination. The AM fungus used in this study, *Glomus etunicatum*, has been isolated in Namibia (see **Table 1**). However, no records could be found that the ECM fungus *Pisolithus tinctorius that* was used in this study has been identified in Namibia. This study will provide valuable information about the interaction between the plants and the two types of fungi under normal growth conditions and in a saline soil.

1.2 LITERATURE REVIEW
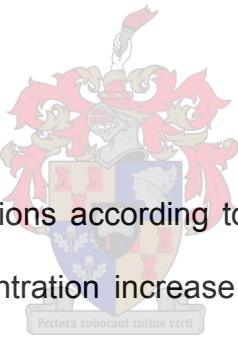
1.2.1 Physiological effects of salt stress

The ability of plants to survive and maintain growth under saline conditions is known as salt tolerance. This variable trait is dependent on many factors including the plant species. The ability of plants to survive under high salt conditions is important for the ecological distribution of plant species and agriculture in semi-arid, arid and salinised regions. Plant growth is generally inhibited by salt stress (Moghaieb *et al.*, 2004). Munns (1993) explains that plant growth is affected because a high build-up of salt kills the photosynthetically active leaves, which in turn affects the supply of carbohydrates or hormones to the actively growing parts.

Salinity is a complex environmental constraint consisting of two main components. These are the osmotic component, due to the decrease in the osmotic potential of the soil solution surrounding roots and the osmotic adjustment in the leaves; and the ionic component. The ionic component is associated with the accumulation of ions that become toxic at high concentrations and to a stress-induced decrease in the cell content of essential elements, such as K^+ and Ca^{2+} (Lefèvre *et al.*, 2001). Pepper plants (*Capsicum annuum*) had a decreased leaf turgor when treated with 60 mM sodium chloride (NaCl) and 60 mM potassium chloride (KCl) (Martinez-Ballesta *et al.*, 2004). The salt treatments had a toxic effect on the plants by affecting their water relations.

1.2.1.1 Mineral nutrition

Plant roots can selectively absorb ions according to their growth requirements. The selectivity decreases with a concentration increase in external ions (Nissen, 1991). The uptake of certain required ions can be reduced by excesses of other ions in the soil solution (Bidwell, 1979). This can cause deficiency symptoms, even though the required ion is abundant in the soil. High levels of Na^+ in the soil solution, for example, induce K^+ and/or Ca^{2+} deficiencies (Grattan and Grieve, 1992). The associated necrotic spots on leaves and leaf abscission can be due to the reduced uptake and transport of Ca^{2+} (Ruiz *et al.*, 1999). Salinity also induces a decrease in the concentration of Mg^{2+} in leaves (Ruiz *et al.*, 1999; Martinez-Ballesta *et al.*, 2004).

1.2.1.2 Photosynthesis and respiration

The increase in osmolality of the nutrient solution with a high salt content result in slower plant growth and a reduced final shoot and root weight and shoot length (Ruiz *et al.*, 1999). The decreased growth causes a build up of photosynthates in leaf mesophyll cells, resulting in feedback inhibition of photosynthesis (Munns, 1993). Salt treatment with NaCl and KCl results in reduced root hydraulic and stomatal conductance and reduced net carbon dioxide (CO_2) assimilation (Martinez-Ballesta *et al.*, 2004). An increase in the salt concentration of the growth medium can increase plant respiration even at low levels of salinity (Schwarz and Gale, 1981). This effect is attributed to the plant's effort to reduce the damaging effects of the salt. Salt stress generally reduces plant photosynthesis (Tattini *et al.*, 1997; Soussi *et al.*, 1999). The salt concentration, the plant species and the developmental stage of the plant, as well as the biotic and abiotic components of the plant's growth environment influence this effect. A study by Curtis and Lauchli (1986), for example, found that exposure of *Hibiscus cannabinus* plants to 75 mM salt stress did not reduce plant photosynthetic rates, although their dry weight was reduced.

1.2.1.3 Effects of salt stress on Eucalyptus *species*

The growth of some *Eucalyptus* species is negatively affected by salt stress (Marcar *et al.*, 2002). The response differs at different salinity levels and between species (Sun and Dickinson, 1993), while the variation may also exist among families within provenances and among provenances (Marcar *et al.*, 2002). The effect of salt stress results in changes in plant survival; plant height, root, leaf and diameter growth;

photosynthetic rates (Sun and Dickinson, 1993; Sun and Dickinson, 1995; Rawat and Banerjee, 1998). The study by Sun and Dickinson (1993) found *E. camaldulensis, E. robusta, E. drepanophylla*, and *E. argophola* to be salt tolerant *Eucalyptus* species.

1.2.2 Mechanism of tolerance

The active uptake of water by plants is done by creating an osmotic gradient that decreases from the soil to the leaves (Kozinka, 1992). Plants growing in saline soils make osmotic adjustments to maintain this gradient to sustain turgor and reduce the detrimental effects of water stress on vegetative and reproductive tissue (Flowers *et al.,* 1991). Many halophytic plants accumulate inorganic ions to a concentration equal or greater than that of the surrounding root solution to maintain the osmotic gradient (Bradley and Morris, 1991). Consequently, the root osmotic potential decreases, which in turn attracts water from the soil and maintains the water uptake stream.
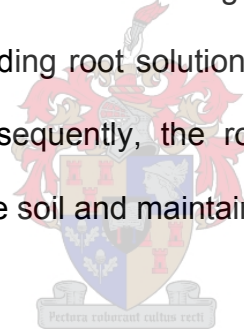
Plants use different mechanisms to limit the effects of salt stress. Morabito *et al.* (1996) suggested that the accumulation level of sodium in the root and shoot could be used as salt tolerance trait for *Eucalyptus* species. They studied the physiological response of two *Eucalyptus microtheca* clones to salt stress and found that although both clones absorbed ions, the more tolerant clone concentrated the sodium ions in the roots to avoid toxicity of the upper plant parts. Salt (NaCl) stressed rice plants (*Oryza sativa)* increase the accumulation of sodium ions in their roots (Lefèvre *et al.*, 2001), limiting sodium accumulation in photosynthetically active leaves. Salt tolerance in citrus species is associated with an ability to restrict the uptake and/or transport of saline ions from roots to shoots (Zekri and Parsons, 1992).

Avoidance is another mechanism used by plants to withstand salt stress. This phenomenon consists of a reduction of water loss via reduction in transpiration rate mainly due to senescence and death of leaves (reduction of leaf area) and reduction of leaf stomatal conductance (De Herralde *et al.*, 1998).

The physiological significance of the accumulation of compatible solutes such as proline and polyamine is still not completely agreed upon. This is because direct evidence for the part played by these solutes during acclimation to stress conditions remains unsubstantiated (Munns, 1993). Nevertheless, identification of intracellular solutes and the importance of the changes induced in their level under stress conditions could be relevant as metabolic traits of interest for breeders concerned with characterization of stress tolerant plant species.

Proline is a compatible solute that accumulates in response to osmotic stress, and its accumulation represents an important adaptive response to salt and drought stress (Morabito *et al.*, 1996; Rentsch *et al.*, 1996; Hong *et al.*, 2000). Proline also functions as (Aspinall and Paleg, 1981; Hare and Cress, 1997; Zhun, 2001):

❑ An energy sink needed for plant recovery after stress

❑ A scavenger for reactive oxygen species produced under stress

❑ A means for reducing the acidity in the cells

❑ Protective agent against denaturation of various proteins in cytoplasm by maintaining their hydrophilic character as it attaches to them

❑ A sink for possible harmful soluble N (storing N from degenerated amino acids)

1.2.3 Endomycorrhizae

Mycorrhizal fungi are symbiotic root colonising fungi, meaning that there is good evidence that the host plant derives some benefit from the association, if only at certain times and under certain conditions (see section 1.2.3.4). These fungi become integrated into the physical structure of the roots, growing in between root cortical cells (Garrett, 1963). The fungi also have a network of external mycelium extending into the soil. The major benefit to the host plant from AM fungi is the provision of a greater absorptive surface for the intake of minerals such as P from the soil (Smith and Read, 1997). The fungi benefit from the association by receiving carbohydrates from the plant.

1.2.3.1 Effects of salinity on endomycorrhizae colonisation

The effect of salt stress does not only influence plant growth, but it also has an effect on the associated plant symbiotic fungi. Salt stress reduces mycorrhizal colonisation of plant roots (Gupta and Krishnamurthy, 1996; Ruiz-Lozano and Azcon, 2000). Tian *et al.* (2004) found that the colonisation rate of *Glomus mosseae* isolates from saline and non-saline soils in roots of cotton plants was reduced with increasing levels of NaCl. Micorrhizal colonisation of cotton plant roots by fungi from saline and non-saline soil was reduced from 46% and 38% respectively, in the absence of salt, to 21% and 15% respectively, at a salt level of 3 g NaCl/kg soil. Cantrell and Linderman (2001) also found that not only does mycorrhizal root colonisation decrease with increasing salt levels, but that the external soil hyphal growth of the fungi is also reduced. It is

therefore clear that the fungi must themselves overcome salt stress before becoming beneficial to their host plants.

1.2.3.2 Effects of endomycorrhizae on salinity tolerance of plants

Salt stress reduces plant growth (Seemann and Critchley, 1985; Morabito *et al.,* 1996). AM fungi can help alleviate plant salt stress (Al-Karaki, 2000; Cantrell and Linderman, 2001). Pfeiffer and Bloss (1988) found that mycorrhizal fungi reduced plant salt stress by reducing the uptake of chlorine (Cl). They also reported that the sodium (Na) concentration of mycorrhizal and non-mycorrhizal plants was equal because the phosphorus (P) concentration of both plants was kept at equal levels. The addition of 100 μg g^{-1} of P decreased the accumulation of copper (Cu), zinc (Zn), potassium (K), sulfate (SO$_4$) and Na. Salt stressed mycorrhizal plants growing in nutrient poor soils should therefore have reduced levels of Na because of the possible higher P uptake of these plants, compared to non-mycorrhizal plants under the same conditions. Giri and Mukerji (2004) reported that salt stressed mycorrhizal *Sesbania* plants had reduced Na uptake and increased P, N and Mg absorption. A study by Poss *et al.* (1985) is in agreement with these findings as salt stressed mycorrhizal plants in soils with low P levels had higher P uptake and improved growth. They however dispute that higher P levels lead to reduced Na uptake, but rather that the faster growing mycorrhizal plants accumulate more Na. The increased salt tolerance of mycorrhizal plants is also attributed to increased CO$_2$ exchange, transpiration rates, stomatal conductance and water use efficiencies (Ruiz-Lozano *et al*., 1996). Mycorrhizal plants further have improved root growth (Cantrell and Linderman, 2001) and improved root cell osmotic adjustments (Feng *et al*., 2002).

1.2.3.3 Endomycorrhizae and plant nutrient uptake

It has been proven that plant roots colonised by mycorrhizal fungi are more efficient in nutrient uptake than non-colonised roots. The major benefit from AM fungal symbiosis is in P uptake (Smith and Read, 1997), although the fungus also assists the host plant with Zn, Cu, nitrogen (N) and K uptake (Marschner and Dell, 1994). The nutrients must first be taken up by the fungal mycelium, transported to the roots, and lastly transferred to the root cells. Plant roots easily deplete the available nutrients in the rhizosphere and root hairs are too short to explore soil further away. The mycelium network in the soil explores areas further away from the rhizosphere and help with the uptake of immobile nutrients such as P. The size of mycelium also enables them to go through small soil pores not easily penetrable by plant roots.

The benefit derived from the symbiosis is most evident when plants grow in soils with low nutrient status. Mycorrhizal plants grow better, having a higher nutrient status, than non-mycorrhizal plants under these conditions (Azcon *et al.,* 2003). This effect is reduced and the fungi can even have a negative effect on plant growth when soil nutrients are sufficient for plant growth.

1.2.3.4 Cost of maintaining endomycorrhizal symbiosis

The manner in which a plant allocates resources expenditure among different organs affects its overall growth performance in a particular environment. Mycorrhizal plants are autotrophic and can grow well without the fungus, provided growth medium

nutrients are sufficient (Smith and Read, 1997). Growth in symbiosis with the fungi, especially when nutrients are low, generally brings about an increased biomass of the mycorrhizal plants compared to the non-mycorrhizal plants. Mycorrhizal colonisation is reduced or impeded when growth medium nutrient levels are high. The reduced colonisation by mycorrhizal fungi at high nutrient levels also causes a reduction in nutrient uptake by mycorrhizal plants compared to non-mycorrhizal plants. The increased growth of mycorrhizal plants can mostly be attributed to increased mineral nutrition (Azcon *et al.,* 2003).

The accrued benefits from the symbiosis however come at a cost to the host plant. Arbuscular mycorrhizal (Endomycorrhizal) fungi receive between 10% and 23% of host plant photosynthetically fixed carbon (Jakobsen and Rosendahl, 1990). In an investigation using ^{14}C supplied to grass colonised by arbuscular mycorrhizal fungi, 3.4% of the ^{14}C initially fixed by the plants was found in the mycorrhizal mycelium 0 – 70 hours after labeling (Johnson *et al.*, 2002). This shows that there is a rapid translocation of ^{14}C-labelled photosynthate to the root and into the fungal hyphae.

The carbon cost differs according to the species of plant and fungus, fungal biomass, the rate of colonisation, the stage of development of host plant, as well as the metabolic activity of the fungus. *Glomus claroideum* resulted in an increased plant growth of *Retama spaerocarpa* seedlings while a mixture of native arbuscular mycorrhizal fungi decreased plant growth (Caravaca *et al.*, 2003). An inverse result from the same study was recorded for seedlings of *Rhamus lycioides*. Burke *et al.* (2003) reported that the colonisation of the salt marsh grass *Spartina patens* by arbuscular mycorrhizal fungi was 26.6% during plant vegetative growth, while it

decreased to 11.5% during dormancy. The higher colonisation during vegetative growth can result in a higher carbon demand and therefore a higher carbon cost from the plant to the fungi compared to that in the dormant period. Snellgrove *et al.* (1982) reported that endomycorrhizal plants transferred 7% more C assimilated to the roots compared to non-mycorrhizal plants.

In a study using clover (*Trifolium repens*), Wright *et al.* (1998) found that mycorrhizal plants had a higher photosynthetic rate than non-mycorrhizal plants. This was so even though the foliar nitrogen and phosphorous content was kept at similar levels. There was also no structural gain to the mycorrhizal plants due to the high photosynthesis, proving that the additional C was used for fungal growth. The increase in photosynthesis was caused by a non-nutritional impact of fungal colonisation upon C assimilation. Mycorrhizal fungi can therefore increase the photosynthetic rate of colonised plants and make use of the additional C, by so doing eliminating any 'cost' to the host plant.
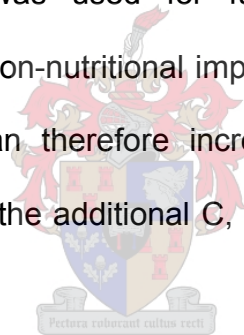
1.2.4 Ectomycorrhizae

ECM fungi are, economically, one of the most important groups of fungi. These fungi form a symbiotic relationship with a plant creating a sheath around the root tip of the plant. The fungus then forms a Hartig Net, which means that there is an inward growth of hyphae (fungal cell growth form) which penetrates the plant root structure. The fungus gains carbon and other essential organic substances from the tree and in return helps the trees with uptake of water, mineral salts, and metabolites (Smith and Read, 1997). It can also fight off parasites, predators such as nematodes and soil

pathogens. Indeed, many forest trees are highly dependent on their fungal partners and in areas of poor soil, could not even exist without them (Francis and Read, 1994). Thus, mycorrhizal fungi must be considered in management of forests.

1.2.4.1 Effects of salinity on ectomycorrhizae colonisation
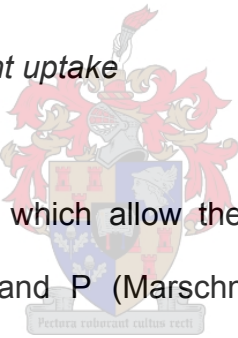
Mycorrhizal fungi growing naturally in soil with high levels of specific elements are expected to be more tolerant towards these elements and to outperform those that grow in soils with average levels of elements. This is however not always the case as indicated by Jones and Hutchinson (1988a). They grew several isolates of ECM fungi, some from a copper (Cu) and nickel (Ni) contaminated site and some from an uncontaminated site, in a solid medium contaminated with Cu and Ni. The fungi from the contaminated site did not grow better than those from the uncontaminated site. Dixon *et al*. (1993) reported that ECM colonisation is reduced by increasing growth medium salinity levels. They found that ECM colonisation of *Pinus taeda* seedlings was reduced after 14 weeks of exposure to 80mM NaCl. Chen *et al*. (2001) found that ECM biomass growth in axenic culture was reduced with increasing NaCl levels up to 200mM.

1.2.4.2 Effects of ectomycorrhizae on salinity tolerance of plants

Inoculation of plants with ECM fungi can improve their ability to withstand soil toxicity of certain toxic elements (Jones and Hutchinson, 1988b). Plants colonised by these fungi will have improved survival rates on sites with toxic levels of certain elements compared to uninfected plants on the same sites. Not all fungi can however equally

reduce the effects of these elements on plant growth, because certain fungi are more effective than others are. Marx (1975) found that *Pinus virginiana* seedlings planted on strip-mined coal sites had a survival rate of 45.5% when infected with *Pisolithus* ECM compared to a 1.5% survival rate when infected with *Thelephora* ECM. In a study by Muhsin and Zwiazek (2002), ECM reduced the uptake of Na while increasing that of N and P to help alleviate salt stress of *Picea glauca*. ECM can increase the uptake of salt while increasing plant growth of salt stressed plants (Routien and Dawson, 1943). The improvement of salt tolerance of plants as a result of ECM fungi can be attributed to several factors, including improved nutrition, improved leaf transpiration and root hydraulic conductance (Muhsin and Zwiazek, 2002).

1.2.4.3 Ectomycorrhizae and nutrient uptake

ECM fungi produce ectoenzymes, which allow the host plants to have access to otherwise unavailable organic N and P (Marschner and Dell, 1994). They also increase nutrient uptake by (1) increasing the nutrient absorbing surface area of the host plants and (2) increasing the host plant transpiration rate and water uptake per unit root length. ECM are more important for the transfer of soil derived N and less so for P to the host plants (Smith *et al*., 1994). A study by Koide and Kabir (2001) however found that *Pisolithus tinctorius* had no effect on the N content of *Pinus resinosa* in nutrient poor soils, while it increased P content. Marschner and Dell (1994) further reported that ECM not only help with the uptake of N and P but also of K, Cu, Zn and many other micronutrients.

1.2.4.4 Cost of maintaining ectomycorrhizal symbiosis

ECM can improve host plant growth in the presence (Muhsin and Zwiazek, 2002) or absence (Ekwebelam and Reid, 1983) of salt stress. This benefit however comes at a cost to the host plants, as the fungi require C to build and maintain biomass and to reproduce. The estimated C-costs to the host ranges from 4 to 17% (Paul and Kucey, 1981; Leak *et al*., 2001). The actual amounts allocated will differ between plant and fungal species involved, the rate of colonisation, the development stage of the host and the fungi (see section 1.2.3.4). The various factors can sometimes result in an even higher allocation of C to the fungi. For example, Wu *et al.* (2002) found C allocation of 24% while Nehls and Hampp (2000) reported a C allocation of up to 30%.

1.2.5 Ectomycorrhizae and endomycorrhizae fungi double symbiosis

The presence of ECM and AM fungi in the same root system has been observed in several plant species (Frioni *et al.,* 1999; Founoune *et al*., 2002; Gange *et al.*, 2005). The study by Frioni *et al.* (1999) however, found that only three out of 23 tree species studied of native tree legumes in Uruguay formed a double symbiosis with ECM and AM fungi. Host plant growth may be negatively affected when the root system forms a double symbiosis of ECM and AM fungi, due to increased C drain from the host plant by the two fungal forms (Egerton-Warburton and Allen, 2001). This can however, be remedied by the natural succession over time of root colonisation by the two fungal forms when occurring in double symbiosis, whereby ECM becomes more dominant as host plants mature (Lapeyrie and Chilvers, 1985; Chilvers *et al*., 1987; Egerton-Warburton and Allen, 2001; Reseder *et al.,* 2004). The negative effect of the double

symbiosis is not true for all plant species and growth conditions, as some reports have presented higher growth for plants forming double compared to single symbiosis (Founoune *et al*., 2002; Lopez Aguillon and Garbaye, 1990).

Chilvers *et al*. (1987) explained the interaction between ECM and AM fungi when in dual symbiosis of plant roots as follows:

❑ AM colonises mature root cortical cells, leaving the root cap zone open for colonisation by ECM;

❑ ECM colonisation form a sheath around the root cap while AM hyphae develop further forward through the inner cortical cells, paralleling the outer ECM development;

❑ AM colonisation is prevented if and when ECM colonise roots first, forming a sheath blocking the AM colonisation sites;

❑ ECM fungi are superior to AM fungi in secondary colonisations due to their extensive hyphal development along and between roots.

The double symbiosis of ECM and AM fungi improves host plant nutrient/water uptake and protection against pathogens (Lopez Aguillon and Garbaye, 1990; Founoune *et al*., 2002; Gange *et al*., 2005).

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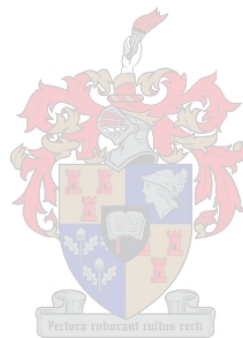
CHAPTER 2 PHOTOSYNTHETIC RESPONSE OF A *EUCALYPTUS* CLONE COLONISED WITH ENDOMYCORRHIZAL AND ECTOMYCORRHIZAL FUNGI

2.1 ABSTRACT

The effect of double mycorrhizal colonisation by ectomycorrhizal (ECM) and endomycorrhizal (AM) fungi on the photosynthetic capacity of a *Eucalyptus grandis x E. camaldulensis* clone was studied. Plant growth, photosynthesis and nutrient levels were analysed. The carbon cost, growth respiration and nutrient utilisation efficiency were also calculated. The *Eucalyptus* clone formed both ECM and AM in single and dual inoculation. ECM reduced AM colonisation by 23% when occurring in the same root system. ECM and AM in single colonisation depressed plant growth while the dual colonisation had no effect. Double colonisation reduced plant specific leaf mass by 12% and increased total leaf area by 43% compared to the control. The double colonisation and ECM reduced photosynthesis per leaf area by 29% and 26% respectively compared to the control. The reduced photosynthesis of the double colonisation did not result in reduced plant growth because these plants may have had high total plant photosynthesis as a result of their large total leaf area. The double colonisation may have been maintained so that the plant can perhaps benefit from one or both fungi when growth conditions became stressful.

Keywords: *Eucalyptus* clone, Double symbiosis, Endomycorrhizal fungi, Ectomycorrhizal fungi, Photosynthesis, Plant growth

2.2 INTRODUCTION

Endomycorrhizal (AM) (Kucey and Paul, 1982; Koch *et al*., 1997; Wright *et al*., 1998) and ectomycorrhizal (ECM) colonisation individually (Colpaert *et al*., 1996; Choi *et al*., 2005) cause an increase in host plant photosynthesis. The increase in photosynthesis however, does not always result in improved plant biomass (Kucey and Paul, 1982; Colpaert *et al*., 1996). While the photosynthetic response of host plants to the AM and ECM individually is well documented, the effect of the double symbiosis on photosynthesis is not.

AM fungi obtain photosynthates from host plants and transfer soil derived nutrients to their host. They also increase host plant resistance to insects and pathogen attacks, and drought tolerance (Smith and Read, 1997). The colonisation generally therefore results in improved host plant growth (Tarafdar and Kuwer, 1996; Rao and Tak, 2001). ECM is also reported to increase host plant tolerance to insect attacks and nutrient uptake (Gange *et al*., 2005), and they improve host plant growth as well (Turjaman *et al*., 2005). Plants grown in double symbiosis with AM and ECM generally perform better than those with single symbiosis (Lopez Aguillon and Garbaye, 1990; Founoune *et al*., 2002). The double symbiosis can however be a big carbon sink for host plant photosynthates and sometimes cause a reduction in plant growth (Egerton-Warburton and Allen, 2001). The effect of the mycorrhizal fungi on host plants will therefore differ for different plant and fungal species and growth conditions.

The genus *Eucalyptus* is reported to form both AM and ECM associations (Lapeyrie and Chilvers, 1985; Gange *et al.,* 2005). AM fungi are found to have a higher initial

colonisation rate compared to ECM fungi when in double symbiosis (Lapeyrie and Chilvers, 1985; Chilvers *et al.*, 1987; Reseder *et al.*, 2004). These studies also found that ECM colonisation however increases over time and surpasses the AM colonisation. ECM fungi are therefore able to offer more benefits to the plant over the long term than AM fungi. Gange *et al.* (2005) found that ECM reduces AM colonisation when growing in double symbiosis in the same root system.

The objective of this study was to determine the photosynthetic response of host plants that had a double symbiosis with AM and ECM fungi.

2.3 MATERIAL AND METHODS

2.3.1 Growth conditions

A sandy soil (pH_{KCl} 7.1), steamed for 35 minutes at $80^{o}C$, was used as the growing medium. Plants of a 3 month old *Eucalyptus grandis x E. camaldulensis* clone, were transplanted into new 1.6 litre plastic pots, and placed in a greenhouse from January to April 2004. The temperatures in the greenhouse varied from $20^{o}C$ to $35^{o}C$, having 12 hour day light. Plants received 100 ml of water a day during summer and every second day during autumn, using overhead sprinklers. No fertilizer was added to the pots and no weeding was done, as there were no weeds present.

2.3.2 Treatments

The experiment was conducted using four treatments and 14 plants per treatment. Four plants were randomly selected from each treatment for the following

measurements: plant height, stem diameter, number of leaves and leaf area, photosynthetic data, nutrient and chlorophyll analysis. Four more plants were selected for determining the mycorrhizal colonisation levels. The treatments were:

❑   Control

❑   Plant root colonisation with endomycorrhizal fungi (AM)

❑   Plant root colonisation with ectomycorrhizal fungi (ECM)

❑   Double plant root colonisation by both the AM and ECM fungi

2.3.3 Mycorrhizal inoculation and analysis

The sterilised soil was inoculated with 5 g of an AM inoculum composed of spores and hyphae of *Glomus etunicatum* in a clay-based granular support substrate. This was placed in six holes, 3 cm deep, around the roots of the seedlings at transplanting. Roots were harvested 120 days after transplanting. Non-woody root segments were cleared with 10% potassium hydroxide (KOH) for 6 minutes at 110°C under steam pressure at 200 KPa in an autoclave. The KOH was then rinsed with distilled water from the root segments and thereafter the roots were acidified with 1% hydrogen chloride (HCl) for 10 minutes. The roots were lightly washed with distilled water and stained with 0.05% aniline blue for 10 minutes in an autoclave at 110°C under steam pressure at 200 KPa. Roots were cut into 1 cm pieces and examined at 400 x magnification under a light microscope. Colonisation was determined according to the method described by Brundrett *et al*. (1994).

Fresh fruiting bodies of *Pisolithus tinctorius* (ECM) were collected from a *Eucalyptus camaldulensis* stand in Stellenbosch during June 2003 and kept at room temperature

for six months to dry. The dry powder from the fruiting bodies was crushed in a container. The spore powder was sampled by dipping an index finger in water and then immersing the tip (1/3 of the finger) into the container. They were then smeared at five spots equally spaced around the roots of the plants at transplanting, using a wet fingertip. Roots were harvested 120 days after transplanting. Root segments of 2 to 3 cm were placed in a Petri-dish containing distilled water and analysed under a binocular microscope for ECM colonisation. The method of analysis was as described by Peterson (1994).

2.3.4 Photosynthesis measurement

Photosynthesis measurements were done by using an infrared-gas-analyzer (IRGA). Measurements were taken at a photosynthetic photon flux density (PPFD) and CO_2 concentration of 1000 µmol m^{-2} s^{-1} and 380 µmol mol^{-1} respectively. The leaf temperature was kept at 25^oC, and the airflow rate in the curvet at 500 µm s^{-1}.

Chlorophyll analyses were performed on leaf discs taken from the same leaves that were used for the gas exchange measurements. Chlorophyll was extracted at 4°C in acetone. The resulting extract was centrifuged at 3000 g for 3 minutes, and the chlorophyll concentration was determined according to the method of Arnon (1949) by measuring the absorbance at 646, 663 and 710 nm in a spectrophotometer.

2.3.5 Plant harvesting and nutrient analysis

Shoot height, stem diameter at ground level and number of leaves were determined at harvest. Soil was carefully washed from the roots and the plants were divided into root, leaf and stem components and immediately weighed to determine the fresh weight. The components were then dried at $80^{\circ}C$ for more than 72 hours and weighed to determine the dry weight. The dried samples were analysed by Bemlab[(PTY)Ltd] (Somerset West, RSA) for root, stem and leaf nitrogen (N), phosphorus (P) and carbon (C) content. Ten plants were also measured at transplanting to determine initial plant fresh and dry weight and shoot height. The leaf areas of the plants were measured with a leaf area meter (Li-cor, model LI-3000, Lambda Instruments Corporation, USA).

2.3.6 Calculation of C-cost and nutrient utilisation efficiency

Daily tissue construction cost (C_t) (μmol CO_2 day^{-1}) was calculated as a product of the tissue construction cost (mmol C gDW^{-1}) and the tissue growth rate (mg day^{-1}). Tissue construction cost was calculated with a modified equation of that used by Peng *et al.* (1993):

$$Cw = [\{C + (kN \times 14^{-1})\} \times (180 \times 24^{-1})] (1 \times 0.89^{-1}) (6,000 \times 180^{-1}) \qquad \textbf{(1)}$$

Where Cw is the tissue construction cost (mmol C gDW^{-1}),

C is the carbon concentration (mmol C g^{-1}),

k is the reduction state of the N substrate (+3 in this study),

N is the organic nitrogen content of the tissue (mol gDW^{-1}),

and 14 is the atomic mass of nitrogen.

The constant (1 x 0.89^{-1}) represents the fraction of the construction cost that provides reductants not incorporated into tissue biomass (Williams *et al.*, 1987) and (6,000 x 180^{-1}) converts units of g glucose gDW^{-1} to mmol C gDW^{-1}.

Growth respiration (µmol CO_2 day^{-1}) was calculated as proposed by Peng *et al.* (1993):

$$R_{G(t)} = C_t - \Delta W_c \qquad (2)$$

Where $R_{G(t)}$ is the growth respiration (µmol CO_2 day^{-1}),

C_t (µmol CO_2 day^{-1}) is the daily tissue construction cost and

ΔW_c is the change in tissue C content. ΔW_c (µmol day^{-1}) was estimated from the product of tissue C content and tissue growth rate.

$R_{G(t)}$ is defined here as the respired carbon associated with the production of new tissue.

Growth respiration ($R_{G(t)}$) was expressed per unit weight of new root tissue or the Growth respiration coefficient, $R_{G(w)}$ (mmol CO_2 gDW^{-1}):

$$R_{G(w)} = R_{G(t)} \times \Delta W_w{}^{-1} \qquad (3)$$

Where $R_{G(t)}$ is the growth respiration and

ΔW_w the rate of increase in root dry weight from initial to final weight at 120 days.

Nutrient utilisation efficiency was estimated using the equation proposed by Koide and Elliott (1989):

$$\Delta C^r \times \Delta P^{r-1} \qquad\qquad\qquad\qquad\qquad \textbf{(4)}$$

Where ΔC^r is total quantity of C accumulated over a period and

ΔP^r is the total P accumulated in the tissue over the same period of time.

The utilisation of N was calculated by replacing its value with P in the equation.

2.3.7 Statistical analysis

Treatments were arranged in a completely randomized design, having 14 plants per treatment. The percentage data were arcsine transformed (Zar, 1984). The difference in photosynthesis, stomatal conductance and mycorrhizal colonisation were separated using a post hoc Student-Newman-Kuels (SNK) multiple test ($P \leq 0.05$) (Super-Anova) (Snedecor and Cochran, 1980). Different letters after each figure in the tables indicate significant difference between treatments.

2.4 RESULTS

2.4.1 Mycorrhizal colonisation

ECM and AM fungi colonised the host plant both in single and double symbiosis. The double fungal inoculation reduced both AM and ECM colonisation by 46% and 24%

respectively compared to the single inoculations (**Table 1**). AM colonisation was 23% lower than ECM colonisation in the double symbiosis.

TABLE 1. *Endomycorrhizal (AM) and ectomycorrhizal (ECM) colonisation percentage of 7 month-old* Eucalyptus *plants grown under greenhouse conditions in a pot soil medium.*

Treatments	AM	ECM
AM	65.75 c	0.00 a
ECM	0.00 a	60.75 b
AM + ECM	35.5 b	46.25 b
Control	0.00 a	0.00 a

Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one column.

2.4.2 Plant growth and C costs

Individual AM and ECM colonisation both reduced plant dry weight by 24% (**Table 2**) compared to the control. The double symbiosis had no effect on host plant dry weight. It however reduced the specific leaf mass and increased total leaf area by 12% and 43% respectively compared to the control. There was no difference in the daily carbon costs (C-costs) between the treatments (**Table 3**). AM and ECM plants both had a 32% and 26% respectively higher growth respiration than the control. They however had lower growth phosphorous use efficiency (PUE) of 21% (AM) and 25% (ECM) than the control. The double colonisation maintained equal levels of PUE as the control.

TABLE 2. Biomass parameters of 7 month-old Eucalyptus *plants grown under greenhouse conditions in a pot soil medium.*

Parameters	Treatments			
	AM	ECM	AM + ECM	Control
Plant DW (g)	22.267 a	22.667 a	33.600 b	29.433 b
Plant FW (g)	64.400 a	69.000 a	89.033 b	79.200 b
SLM (g m^{-2})	88.327 ab	93.293 bc	84.569 a	96.338 c
Leaf area (cm^2)	0.074 b	0.053 a	0.107 c	0.075 b
shoot:root	2.461 b	1.660 a	2.135 ab	1.988 ab

DW = dry weight; FW = fresh weight; SLM = specific leaf mass; AM = endomycorrhiza; ECM = ectomycorrhiza. Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one row.

TABLE 3. Plant growth carbon cost of 7 month-old Eucalyptus *plants grown under greenhouse conditions in a pot soil medium.*

Parameters	Treatments			
	AM	ECM	· Ecto	Control
Daily construction cost (ct)	5014.5 a	4552.8 a	5031.6 a	4577.9 a
Growth respiration coefficient Rg (w)	11.676 b	11.131 b	9.983 ab	8.821 a
Growth N-use efficiency	76.106 a	76.728 ab	94.691 c	88.048 bc
Growth P-use efficiency	1700.1 a	1598.2 a	2060.1 b	2144.9 b

AM = endomycorrhiza; ECM = ectomycorrhiza; N = nitrogen; P = phosphorous. Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one row.

2.4.3 Photosynthetic gas exchange

The double colonisation and ECM reduced photosynthesis by 29% and 26% respectively (**Table 4**) compared to the control. The stomatal conductance and dark respiration of the double colonisation was 47% and 34% respectively lower than that of the control. There was no difference in the chlorophyll content, the water use efficiency and the photosynthetic nitrogen use efficiency of the double colonisation

and the control. The double colonised and ECM plants however had 29% and 32% respectively lower photosynthetic phosphorous use efficiency than the control plants.

TABLE 4. Photosynthetic parameters of 7 month-old Eucalyptus *plants grown under greenhouse conditions in a pot soil medium.*

Parameters	Treatments			
	AM	ECM	AM + Ecto	Control
Pmax	11.213 bc	9.233 ab	8.977 a	12.567 c
Gs	0.235 b	0.185 b	0.110 a	0.206 b
Dark resp.	2.953 bc	2.442 ab	2.073 a	3.127 c
Chl 663	0.517 b	0.398 a	0.467 b	0.537 b
WUE	3.432 a	3.456 a	4.364 b	3.897 ab
PNUE	1.153 a	1.039 a	1.455 b	1.582 b
PPUE	39.377 ab	32.151 a	33.372 a	47.216 b

Pmax = photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$); Gs = stomatal conductance ($cm\ s^{-1}$); Dark resp. = growth and maintenace respiration ($nmol\ CO_2\ g^{-1}\ s^{-1}$); Chl = chlorophyll; WUE = water use efficiency; PNUE = photosynthetic nitrogen use efficiency; PPUE = photosynthetic phosphorous use efficiency;

AM = endomycorrhiza; ECM = ectomycorrhiza. Significant differences ($P<0.05$) between treatments are indicated by different letters. Comparison applies to values in one row.

2.4.4 Plant nutrition

The nutritional content of the different plant components was variable (**Table 5**). ECM root nitrogen (N) content was 14% higher than the control. AM and ECM root phosphorous (P) content was 17% and 28% respectively higher and leaf N was 38% and 22% respectively higher than the control. The stem N content of AM and ECM was 28% and 13% respectively higher than the control. The dual colonisation maintained equal levels of all plant part nutritional content as the control.

TABLE 5. Plant tissue nitrogen (mol N g^{-1}) and phosphorous (mol P g^{-1}) concentration of 7 month-old Eucalyptus plants grown under greenhouse conditions in a pot soil medium.

Parameters	Treatments			
	AM	ECM	AM + ECM	Control
Root N	0.0430 a	0.0489 b	0.0443 a	0.0429 a
Root P	0.0021 b	0.0023 b	0.0021 b	0.0018 a
Leaf N	0.1083 b (1.175%)	0.0957 b (1.340%)	0.0805 a (1.128%)	0.0782 a (1.095%)
Leaf P	0.0032 a (0.098%)	0.0031 a (0.095%)	0.0035 a (0.108%)	0.0031 a (0.096%)
Stem N	0.0419 c	0.0370 b	0.0346 ab	0.0327 a
Stem P	0.0029 a	0.0038 b	0.0038 b	0.0036 b

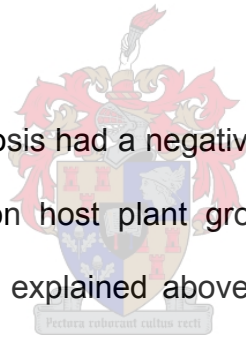AM = endomycorrhiza; ECM = ectomycorrhiza. Significant differences ($P<0.05$) between treatments are indicated by different letters. Comparison applies to values in one row. The percentage nutrient of plant dry weight is given in brackets, for comparison against the norms.

2.5 DISCUSSION AND CONCLUSION

The *Eucalyptus grandis x E. camaldulensis* clone formed both ectomycorrhizae (ECM) and endomycorhizae (AM) in single and dual inoculation in the current study. *Eucalyptus* species are known to form both ECM and AM fungi (Malajczuk *et al*., 1981; Jones *et al*., 1998). ECM fungi are also not host specific within *Eucalyptus* (Malajczuk *et al*., 1982). The *Eucalyptus* species are however more associated with ECM than AM fungi (Lapeyrie and Chilvers, 1985). AM fungi are fast initial root colonisers when growing in the same root system as ECM, although their colonisation levels decrease over time while that of ECM increase (Chen *et al*., 2000). The current study is in agreement with these findings as the AM colonisation was suppressed by ECM when in dual colonisation of the host roots after 4 months' growth.

The reduced photosynthesis of mycorrhizal plants is in contrast with other studies. Ekwebelam and Reid (1983) found that ECM increases host plant photosynthesis,

while Koch *et al*. (1997) found this to also be true for AM fungi. The low photosynthesis of the ECM compared to the control plants in the current study could have been due to the lower chlorophyll concentration in the leaves of these plants (**Table 4**). Dual colonisation caused host plants to have thinner leaves, having a lower specific leaf mass (SLM) than control plants, but a larger total leaf area. The dual colonised plants therefore, although having low photosynthesis per leaf area could have had higher total plant photosynthesis than the control plants. This may have resulted in higher available photosynthates for fungal and plant growth for the host plants. This could be why this fungal combination had no negative effect on plant growth even though they did not offer nutritional or any other benefit to the host plants (**Table 2**).

ECM and AM fungi in single symbiosis had a negative effect on plant growth while the dual colonisation had no effect on host plant growth. The response of the dual colonised host plants could be as explained above. Mycorrhizae generally improve plant growth, especially when plants grow under unfavorable growth conditions (Ibrahim *et al.*, 1990; Baum *et al.*, 2000). The dual colonisation is also found to have improved effects on growth compared to the single colonisation (Lopez Aguillon and Garbaye, 1990; Founoune *et al.*, 2002). The N and P levels of the plants in the current study falls within the recommended levels for optimum growth of young plants of *Eucalyptus grandis* and *Eucalyptus camaldulensis*. For *Eucalyptus grandis* the nutrient levels should be: N = 1.25% to 2.75% of leaf dry weight (DW) and P = 0.075% to 0.200% DW; for *Eucalyptus camaldulensis*: N = 0.95% to 2.05% and P = 0.07% to 0.09% (Schönau and Herbert, 1982; Judd *et al.*, 1996). The plants were therefore

probably not under stress and the fungi could not provide any additional benefit to their host.

Mycorrhizal plants can survive in the absence of the fungi when growing under non-limiting growth conditions. This is however not the case for the fungi, which need host plants for growth (Smith and Read, 1997). The plants therefore have the options to either 1) reject the symbiosis and therefore prevent colonisation, 2) accept colonisation and have increased nutritional and other physiological benefits, or 3) maintain colonisation in the absence of any benefits in return for improved benefits when growth conditions become unfavorable. Double symbiosis in the current study may have increased the total plant photosynthesis compared to the single symbiosis, and the host plants therefore produced enough photosynthates to compensate for the fungal sink. The maintenance of this double symbiosis could be serving to guard host plants against future unfavorable growth conditions. It is therefore recommended to use double symbiosis for mycorrhizal inoculation in nurseries. The total canopy photosynthesis of plants growing in double symbiosis with AM and ECM should be determined in future studies. This will provide a more accurate answer to the effect of this type of symbiosis on host plant photosynthesis.

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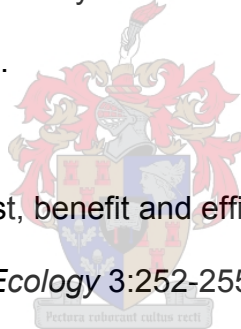
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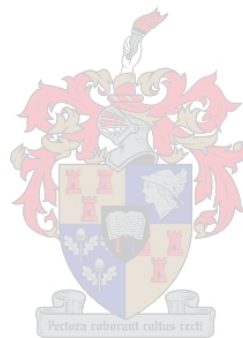
CHAPTER 3 SALT STRESS RESPONSES OF A *EUCALYPTUS* CLONE COLONISED WITH ENDOMYCORRHIZAL AND ECTOMYCORRHIZAL FUNGI

3.1 ABSTRACT

Salt stress alleviation by endomycorrhizal (AM) and ectomycorrhizal (ECM) fungi separately or in double symbiosis of these two fungal forms was studied in a *Eucalyptus grandis x E. camaldulensis* clone. Plant growth, photosynthesis, nutrient levels, and proline concentration were analysed. The carbon cost, growth respiration, and nutrient utilisation efficiency were also calculated. The *Eucalyptus* experienced salt stress at 4 months after an initial exposure to 75 mM NaCl. There was no difference between the treatments in nutritional levels of the plants. AM plants had a 13% higher plant dry weight, correlated with a 16% higher N nutrient use efficiencies, compared to the control plants. Mycorrhizal plants had between 18% to 38% lower photosynthetic rate per leaf area than the control plants. The double symbiosis and ECM fungi did not reduce salt stress of the *Eucalyptus* clone. AM fungi were the most effective at reducing salt stress of this clone. The double symbiosis is however recommended because of different colonisation developments of the two fungal forms and the possible long-term benefits to host plants.

Keywords: *Eucalyptus* clone, Double symbiosis, Endomycorrhizal fungi, Ectomycorrhizal fungi, Salt stress, Plant growth

3.2 INTRODUCTION

Plant growth is generally inhibited by salt stress (Moghaieb *et al.*, 2004). Salinity is a complex environmental constraint consisting of two main components. The first is the osmotic component, due to the decrease in the osmotic potential of the soil solution surrounding roots and the osmotic adjustment in the leaves (Lefèvre *et al.*, 2001; Moghaieb *et al.*, 2004). The second is the ionic component associated with the accumulation of sodium (Na^+) and chloride (Cl^-) ions, which become toxic at high concentrations (Lefèvre *et al.*, 2001). The high build up of salt kills the photosynthetically active leaves, reducing the supply of carbohydrates and hormones to actively growing parts (Munns, 1993). Plant leaves develop necrotic spots before abscission occurs. Salts stress also reduces or inhibits the supply of essential elements from the roots to aerial plant parts (Bidwill, 1979).

Although plants can employ various mechanisms to adapt to salt stress, they can also form root symbiotic associations with endomycorrhizal (AM) and ectomycorrhizal (ECM) fungi, which have been reported to alleviate the toxic effects of salt stress (Ojala *et al.*, 1983; Poss *et al.*, 1985; Ruiz-Lozano *et al.*, 1996; Feng *et al.*, 2002; Giri and Mukerji, 2004; Tian *et al.*, 2004). Although the effects of either AM or ECM on host tolerance to salt stress has been investigated, limited attention has been given to host plants having a double mycorrhizal association with AM and ECM fungi.

Moser and Haselwandter (1983) reported that ECM fungi can accumulate and withstand high levels of heavy metal concentrations, acting as a filter to protect plants from metal toxicity. Dixon (1988) also found that ECM reduced the concentration of

heavy metals in leaves while maintaining a high concentration in roots. *Picea glauca* seedlings under salt stress and colonised by an ECM fungus (*Hebeloma crustuliniforme*) had a reduced sodium (Na) uptake, an increased nitrogen (N) and phosphorous (P) uptake, and higher transpiration and root water conductance compared to the salt stressed non-mycorrhizal seedlings (Muhsin and Zwiazek, 2002). It has been reported that AM fungal symbiosis plays an important role in the alleviation of salinity stress via several mechanisms. In onion plants, AM mediated salt stress tolerance by increasing P accumulation and concentration when soil P was low (Poss *et al*., 1985; Ojala *et al*., 1983). Ruiz-Lozano *et al*. (1996) however concluded that the fungal effects on salt tolerance cannot be attributed to the increase in P uptake alone, but also to increased CO_2 exchange rates, transpiration rates, stomatal conductances, and water use efficiencies. AM can also enhance plant growth under salt stress by improving plant nutrition and stimulating root growth (Poss *et al*., 1985; Ruiz-Lazona and Azcon, 2000; Cantrell and Linderman, 2001). Feng *et al*. (2002) further reported that AM plants might have improved osmotic adjustment in root cells.
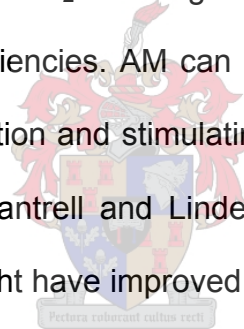
Several *Eucalyptus* species are of great economic importance to many countries such as Brazil, India, Australia, South Africa and Ethiopia (Eldridge *et al*., 1994). *E. camaldulensis* has been planted in the temperate, sub-humid and semi-arid zones of South Africa (Magumba, 1998). In Namibia, *E. camaldulensis* has been planted in the central highlands, the north-western arid (saline) and the north-eastern sub-humid zones (Erkkilä and Siiskonen, 1992). Information on the introduced provenance or provenances is not available, but these introductions were apparently not well adapted to the prevailing sandy and saline soil conditions. This *Eucalyptus* species is still being planted in the northern parts of Namibia (personal observations).

Eucalyptus plants are well known to grow in association with ECM fungi (Lapeyrie and Chilvers, 1985), while their interactions with AM fungi have also received attention (Chilvers *et al.,* 1987). The genus *Eucalyptus* is also reported to form a double association with AM and ECM (dos Santos *et al*., 2001; Gange *et al*., 2005). An understanding of the physiological response of these trees under salt stress to either AM or ECM fungi, as well as to a dual symbiosis with these symbionts will be of great practical value for commercially important trees such as the eucalypts.

The objective of this study was therefore to determine the effect of salt stress on the physiological responses of *Eucalyptus grandis x E. camaldulensis* plants in various symbiotic states with ECM and AM fungi. This was conducted by determining growth, nutrition, photosynthesis, and respiratory carbon costs (C-costs) of host plants during salt stress.

3.3 MATERIAL AND METHODS

3.3.1 Growth conditions

See section 2.3.1 for details on the plant growth conditions.

In addition, 75 mM NaCl (salt) was added with water to the soil of each plant in the salt treatments after transplanting them into the 1.6 litre plastic pots. No extra salt was applied during the 4 months of observation.

3.3.2 Treatments

The experiment was conducted using four treatments and 14 plants per treatment. Four plants were randomly selected from each treatment for the following measurements: plant height, stem diameter, number of leaves and leaf area, photosynthetic data, nutrient and proline analysis. Four more plants were selected for determining the mycorrhizal colonisation levels. The treatments were:

❑   Control

❑   Salt only

❑   Plant root colonisation with endomycorrhizal fungi and salt addition (AM + Salt)

❑   Plant root colonisation with ectomycorrhizal fungi and salt addition (ECM + Salt)

❑   Double plant root colonisation by both AM and ECM as well as salt addition

3.3.3 Mycorrhizal inoculation and analysis

(See section 2.3.3)

3.3.4 Photosynthesis measurement

(See section 2.3.4)

3.3.5 Plant harvest, nutrient analysis and proline concentration

See section 2.3.5 for additional details.

The proline concentration of the leaves was determined with freshly harvested plant material. Four replicates of each treatment were used to determine the proline concentration colourometrically, using the ninhydrin method of Bates *et al*. (1973).

3.3.6 Calculation of C-cost and nutrient utilisation efficiency

(See section 2.3.6)

3.3.7 Statistical analysis

(See section 2.3.7)

3.4 RESULTS

3.4.1 NaCl concentrations

The exposure to 75 mM sodium chloride (NaCl), led to the accumulation of higher levels of sodium (Na^+) and chloride (Cl^-) ions in both root (61% and 77% higher respectively) and shoot (81% and 83% higher respectively) components of non-mycorrhizal plants compared to the control plants. The mycorrhizal plants had from 70% to 122% and 43% to 55% higher Na in their shoots and roots respectively compared to the control plants. These plants also had from 44% to 70% and 26% to 83% higher Cl in their shoots and roots respectively compared to the control plants (**Table 1**).

TABLE 1. *Concentration of salt ions (Na$^+$ + Cl$^-$, $_{mol}$ g^{-1}) in root and shoot components of 7 month-old* Eucalyptus *plants.*

Parameters	Treatments				
	Salt	AM + Salt	ECM + Salt	AM + ECM + Salt	Control
Na					
Shoot	81.5373 b	76.6965 b	83.7035 b	99.9638 c	45.0519 a
Root	143.0533 b	131.0603 b	137.5030 b	126.9291 b	88.5345 a
Cl					
Shoot	0.0233 b	0.0211 b	0.0216 b	0.0183 b	0.0127 a
Root	0.0117 c	0.0090 b	0.0121 c	0.0083 b	0.0066 a

AM = endomycorrhiza; ECM = ectomycorrhiza. Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one row.

3.4.2 Mycorrhizal colonisation

AM colonisation during salt exposure was 62% higher than ECM colonisation (**Table 2**). However, the combination of both AM and ECM inoculation resulted in ECM colonisation levels being maintained, but AM colonisation levels declining by 38% compared to the single AM colonisation.
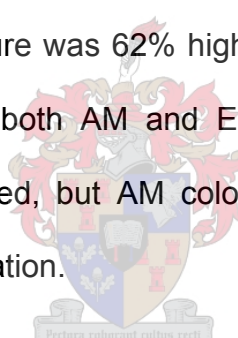
TABLE 2. *Endomycorrhizal (AM) and ectomycorrhizal (ECM) % colonisation of 7 month-old* Eucalyptus *plants.*

Treatments	AM	ECM
AM + Salt	47 c	0 a
ECM + Salt	0 a	29 b
AM + ECM + Salt	29 b	26 b
Control	0 a	0 a

Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one column.

3.4.3 Plant growth and C-costs

During the exposure to 75 mM NaCl, the plant dry weights of the non-mycorrhizal plants were 31% lower than the control plants. This dry weight of the non-mycorrhizal plants was similar to those of the double colonisation and ECM plants under salt stress (**Table 3**). The highest plant dry weights under salt stress were for AM plants, which were 13% higher than the control and between 51% and 67% higher than the other treatments. There were no changes in leaf growth investment, evident in the leaf area and specific leaf mass, between the non-mycorrhizal and control plants (**Table 3**). ECM plants however had a 33% higher leaf area and 10% lower specific leaf mass than the control plants. Carbon (C) costs, determined as growth respiration coefficient ($R_{G(w)}$), was 41% higher in the non-mycorrhizal plants than the control plants. The C-cost of all the symbiotic salt treated plants was similar to that of the control plants (**Table 3**).
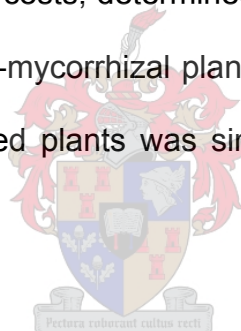
TABLE 3. Biomass and carbon cost parameters of 7 month-old Eucalyptus *plants grown under greenhouse conditions in a pot soil medium.*

Parameters	Treatments				
	Salt	AM + Salt	ECM + Salt	AM + ECM + Salt	Control
Biomass					
Plant DW (g)	20.10 a	33.37 c	22.07 a	20.00 a	29.43 b
Leaf area (cm^2)	0.071 ab	0.079 b	0.100 c	0.067 a	0.075 b
SLM (g m^{-2})	95.84 b	99.24 b	86.63 a	92.12 ab	96.32 b
Growth C-costs					
Growth respiration coefficient $R_{G(w)}$ (mmol CO_2 gDW^{-1})	12.423 b	9.108 ab	10.490 ab	8.683 a	8.812 a
N + P use efficiency					
Growth N-use efficiency	78.75 a	94.81 c	76.49 a	87.01 b	81.89 a
Growth P-use efficiency	1814.04 a	2197.99 b	1924.18 a	2028.93 ab	2031.99 ab

DW = dry weight; SLM = specific leaf mass; AM = endomycorrhiza; ECM = ectomycorrhiza. Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one row.

3.4.4 Plant nutrition and proline levels

The efficiency of N utilisation for plant growth of AM plants during NaCl exposure was 16% higher than the control plants and between 9% to 24% higher than the other treatments (**Table 3**). These plants also had 21% and 14% higher growth P utilisation efficiency than the non-mycorrhizal salt stressed and ECM plants respectively. The high growth N and P utilisation efficiency of AM plants concurs with their increased growth (**Table 3**), which was 13% higher than the control and between 51% and 67% higher than the other treatments. The leaf proline concentration of the non-mycorrhizal plants was 12% higher than the control, although this was not statistically significant. The proline concentration of these plants was however from 30% to 51% higher than the other treatments (**Table 4**). Exposure to 75 mM NaCl caused no difference in root or shoot N and P nutrition of non-mycorrhizal plants compared to the control. The presence of mycorrhizas had no significant effect on the N and P nutrition of the host plants during NaCl exposure. The double symbiosis had the lowest leaf proline concentration, which was 25% lower than the control and between 9% and 33% lower than the other treatments (**Table 4**). There was a positive correlation between the proline concentration and the growth respiration coefficient with $r^2 = 0.79$ (**Figure 1**).

3.4.5 Photosynthetic gas exchange

During 75 mM NaCl exposure, the non-mycorrhizal salt stressed plants had the highest photosynthetic gas exchange rates (Pmax). Mycorrhizal plants had between 18% and 38% lower photosynthetic gas exchange rates per leaf area than the control plants (**Table 5**). The stomatal conductance (Gs) remained unchanged amongst all

the treatments (**Table 5**). There was no difference in the photosynthetic water-use efficiency (WUE) between the non-mycorrhizal plants (**Table 5**). The WUE among the mycorrhizal plants was variable (**Table 5**).

TABLE 4. Shoot and root nitrogen (mol N g^{-1}) and phosphorus (mol P g^{-1}) content, leaves proline concentration (mmol g^{-1}) of 7 month-old Eucalyptus *plants.*

Parameters	Treatments				
	Salt	AM + Salt	ECM + Salt	AM + ECM + Salt	Control
N					
Shoot	0.1267 a	0.1139 a	0.1193 a	0.1038 a	0.1109 a
Root	0.0452 a	0.0430 a	0.0446 a	0.0436 a	0.0429 a
P					
Shoot	0.00604 a	0.00691 ab	0.00694 ab	0.00802 b	0.00671 a
Root	0.00214 b	0.00186 a	0.00192 ab	0.00197 ab	0.00193 ab
Proline					
Leaf proline concentration	0.0104 c	0.0076 b	0.0080 b	0.0069 a	0.0093 c

Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one row.
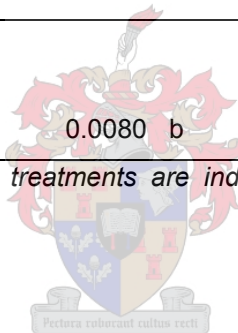
TABLE 5. Photosynthesis parameters of 7 month-old Eucalyptus *plants grown under greenhouse conditions in a pot soil medium.*

Treatments	Pmax	Gs	WUE
Salt	15.133 c	0.197 a	4.567 c
AM + Salt	7.757 a	0.169 a	3.577 bc
ECM + Salt	10.343 a	0.172 a	3.430 b
AM + ECM + Salt	8.333 a	0.170 a	2.679 a
Control	12.567 b	0.206 a	3.897 bc

Pmax = photosynthesis per leaf area (μmol CO_2 m^{-2} s^{-1}); Gs = stomatal conductance (cm s^{-1}); WUE = water use efficiency; AM = endomycorrhiza; ECM = ectomycorrhiza. Significant differences (P<0.05) between treatments are indicated by different letters. Comparison applies to values in one column.

3.5 DISCUSSION AND CONCLUSION

The 75 mM salt level used in this study induced salt stress in plants and concurs with previous reports (Rawat and Banerjee, 1998; Gibberd *et al.*, 2002; Marcar *et al.*, 2002). During salt stress, the higher AM colonisation compared to ECM was due to the more rapid rate of AM development during single inoculations. This is in agreement with other studies that found AM fungi to be faster initial root colonisers than ECM fungi (Chilvers *et al.,* 1987; dos Santos *et al*., 2001). However, during the dual symbiosis of AM and ECM, the constant ECM levels at the expense of AM development in the current study (**Table 2**), may be related to C-cost or the different rates of colonisation during salt stress. AM fungi are reported to have a higher initial colonisation rate compared to ECM fungi when in double symbiosis, but ECM colonisation increases over time and surpasses the AM colonisation (Lapeyrie and Chilvers, 1985; Chilvers *et al*., 1987; Reseder *et al*., 2004). These authors also suggest that the development of a sheath by ECM fungi around the growing root cap, itself a strong carbohydrate sink, provides it with fewer carbohydrates.

The enhanced levels of the Na^+ and Cl^- ion concentrations in the mycorrhizal plants of the current study (**Table 1**) indicate that the AM and ECM symbionts did not prevent the uptake of salt from the root environment, and that salt tolerance involved other mechanisms. The effect of mycorrhizal colonisation to host performance during salt stress was evident in the growth of the *E. grandis x E. camaldulensis* clone. ECM fungi did not reduce salt stress for host plants (**Table 3**), as the total plant dry weight of ECM plants did not differ from that of the non-mycorrhizal salt stressed plants. AM

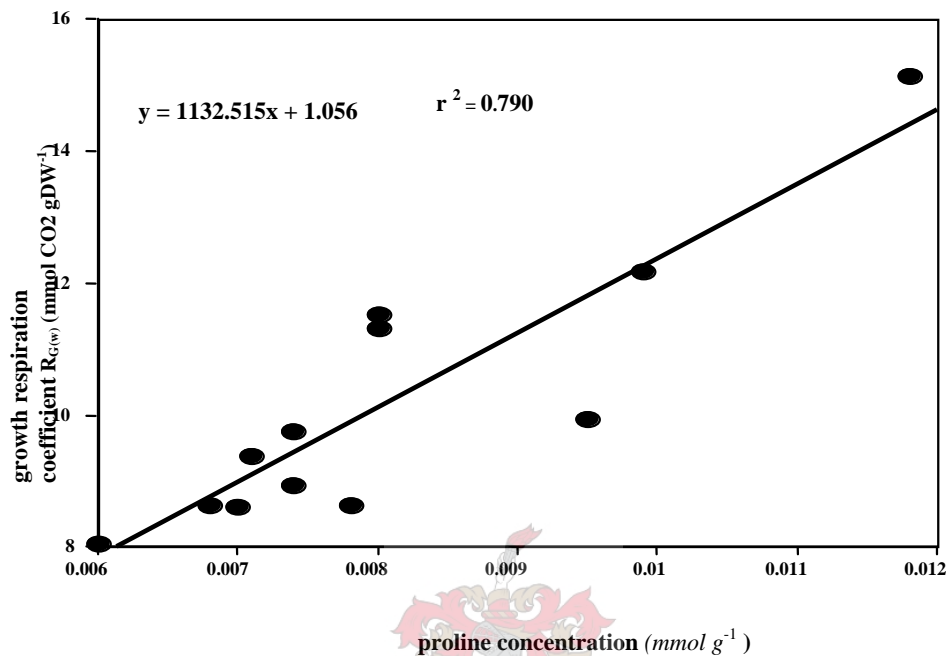fungi however reduced plant salt stress by improving the nutrient use efficiency of these plants (**Table 3**).

FIGURE 1. Linear correlation between the growth respiration and proline concentartion of 7 month-old Eucalyptus plants grown under greenhouse conditions in a pot soil medium.

The improved N and P use efficiency for the growth of salt stressed host plants in the presence of AM fungi may be related to the ability of this symbiont to acquire N and P at lower C-cost to the host, than the other symbiotic partnerships with ECM and AM-ECM combination. Yanai *et al*. (1995) reported that nutrient supply to host plant via mycorrhizae is influenced by the rate of nutrient transport through the fungal hyphae and the rate of transfer from the hyphae to the plant roots. They concluded that AM fungi would therefore have a more efficient nutrient transfer from fungus to plant roots than ECM fungi. This is because AM fungi, unlike the mostly external growing ECM fungi, predominantly grow and form structures (such as vesicles, arbuscules and

hyphae) in between and inside root cells. These structures improve the transfer of nutrients and other elements between the fungi and the host plant.

In non-mycorrhizal salt stressed plants, the reduction in plant growth concurs with the documented negative effect of salt stress on plant biomass (Seemann and Critchley, 1985; Soussi *et al*., 1999; Moghaieb *et al*., 2004). In these plants, the response to salt stress was evident in the increase in proline levels (**Table 4**) and the elevated C-costs of respiration (**Table 3**) and photosynthetic rates (**Table 5**). The accumulation of proline under saline conditions is considered to be an adaptation of plants against salt stress (Stewart and Lee, 1974; Rentsch *et al*., 1996; Hong *et al*., 2000). Exposure of plants to salt stress causes an increase in respiration levels (Kelavkar *et al*., 1993; Tiwari *et al*., 2002). Suzuki *et al*. (2005) found that exposure of *Bruguiera sexangula* to 150 mM NaCl salt stress resulted in a 1.4-fold increase in respiration at 24 h after treatment. The significant positive correlation between proline concentration and the growth respiration coefficient in the current study indicates that there is a significant C-cost of synthesizing the compatible solute (**Figure 1**). A high plant proline concentration is positively correlated to plant respiration (Garcia-Mauriño *et al*., 2005). A study by Curtis and Lauchli (1986) also found that a 75 mM salt stress reduced plant weight but did not reduce the photosynthetic rates of *Hibiscus cannabinus* plants. The findings of the current study are however still in conflict with other previous work showing reductions in plant photosynthesis and stomatal conductance due to salt stress. It should be noted that these reductions were found at excessive salt concentrations of 200mM NaCl or higher (Tattini *et al.,* 1997).

These findings indicate that non-mycorrhizal salt stressed plants incurred a high C-cost to adapt to salt stress, which may have caused the reduction in plant growth. In contrast, AM fungi minimized the effects of salt stress without raising proline levels of host plants, or causing any increases in the host plant growth respiration and photosynthetic gas exchange, compared to the non-mycorrhizal plants.

In conclusion, AM fungi in this study proved to be the most beneficial symbiotic state for salt stress resistance of the *Eucalyptus grandis x E. camaldulensis* clone. Afforestation of saline soil environments should make use of mycorrhizal fungi to improve plant growth and survival rates. The choice of the type of mycorrhizal fungus must take into consideration the prevailing environmental conditions and the succession of the type of mycorrhizal colonisation over time. This is based on the colonisation data produced in this study and the documented double colonisation succession (Chilvers *et al*., 1987; Reseder *et al.*, 2004). Long-term research must be conducted to study the effect of the double symbiosis on stress alleviation of *Eucalyptus* species.
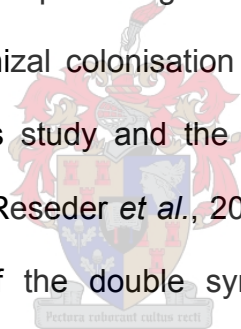
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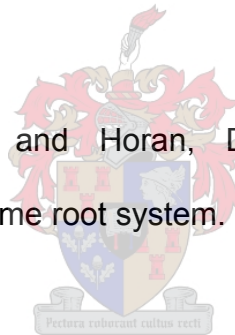
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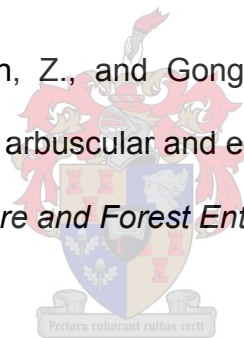
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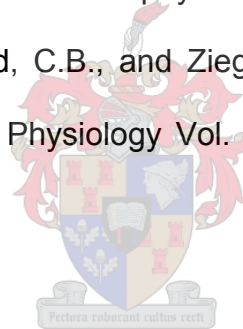
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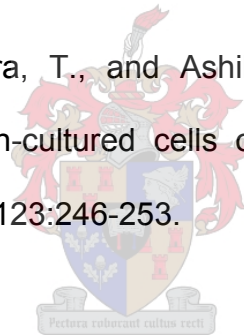
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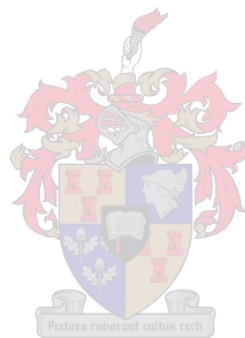
CHAPTER 4 GENERAL DISCUSSION AND CONCLUSION

4.1 GENERAL DISCUSSION

Almost all plants of the world grow in symbiosis with mycorrhizal fungi (Smith and Read, 1997). Ectomycorrhizal fungi (ECM) grow a large mycelium network into the soil, which expands the root absorption surface of the host plants and improves nutrient and water uptake (Olsson *et al.*, 1996). Endomycorrhizae (AM) or more often refered to as Arbuscular mycorrhizae, are important for plant growth and productivity improvement as they also increase nutrient uptake, especially phosphorus (P) (Gerdemann, 1975). The absorption and translocation of nutrients by the AM and ECM fungal mycelium to the host requires energy provided by the host through the supply of photosynthates to the fungi (Martin *et al.*, 1987). The colonisation of plant roots by mycorrhizal fungi is essential for plant growth under adverse conditions.
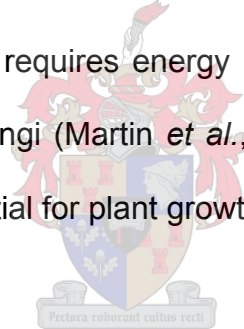
The current study found that single colonisation with either AM or ECM mycorrhizal fungi reduced host plant growth when N and P levels are optimum for growth. This is in agreement with other studies that found that host plant symbiosis with, for example, AM fungi can cause a reduction in plant growth when the nutrient supply is not limiting growth (Hall *et al.*, 1984; Azcon *et al.*, 2003). This growth suppression can be transitory depending on the supply of the limiting nutrients.

The dual colonisation had no effect on the host plants' growth under the current conditions. This form of symbiosis has been reported to increase growth compared to the single symbiosis of AM or ECM (Lopez Aguillon and Garbaye, 1990; Founoune *et*

al., 2002), depending on plant species and growth conditions. The double symbiosis can however also reduce growth by imposing a large carbon (C) sink, thereby reducing available C for host plant growth (Egerton-Warburton and Allen, 2001). The results of the current study indicate that this type of symbiosis did not have a negative effect on the carbon supply for host plant growth. Total leaf area of the double colonised host plants was between 43 and 101% higher than the other treatments (Chapter 2, **Table 2**). This meant that these plants had a larger surface area available for capturing light for photosynthesis, and therefore possibly had higher total plant photosynthesis than all the other treatments. This would be so despite the low photosynthetic rate per specific leaf area of these plants (Chapter 2, **Table 4**). Reduced plant leaf area generally results in reduced plant growth (Gange *et al*., 2005). A study by Wright *et al*. (1998) found that mycorrhizal fungi of *Trifolium repens* compensated for their carbon cost to the host plant by increasing the host plants' photosynthetic rate. Dosskey *et al.* (1990) also found similar results for the ectomycorrhizal fungus (*Rhizopogon vinicolor*) and *Pseudotsuga menziessii* (Douglas fir) seedlings. The increased host plant photosynthesis however had no effect on growth, which did not differ from that of non-mycorrhizal plants. These findings are in agreement with the current study.

Salt stress reduces plant growth (Moghaieb *et al.*, 2004). The use of either AM or ECM fungi in forestry and agriculture can increase the ecological zones for tree planting. Mycorrhizal symbiosis enables host trees to be more resistant to environmental stresses while maintaining and/or increasing their growth (Auge *et al*., 1986; Smith and Read, 1997; Caravaca *et al.*, 2002). Both AM and ECM fungi can help improve host plant survival rates in saline environments (Feng *et al*., 2002; Giri

and Mukerji, 2004; Tian *et al*., 2004). The use of mycorrhizal fungi for tree planting in these environments is therefore recommended.

The double colonisation and the individual ECM symbiosis did not reduce host plant salt stress in the current study. This could have been because these symbioses were large sinks for photosynthates, while not reducing the detrimental effect of salt stress to the host plant. The fungal colonisation levels of these plants were relatively low (Chapter 3, **Table 2**) and therefore probably unable to provide benefits to the host plant. It has been confirmed in several studies that there are optimum levels of mycorrhizal fungal colonisation below which the fungus becomes parasitic (Ruehle and Brendemuehl, 1981; Sung *et al*., 1995; Mortimer *et al*., 2005). These optimum levels are influenced by several factors such as plant and fungal developmental stages; growth medium nutrient levels; temperature; light; water; and plant and fungal species involved. Salt stress has a negative effect on mycorrhizal colonisation of host plant roots (Gupta and Krishnamurthy, 1996; Cantrell and Linderman, 2001). The ECM fungus and the AM/ECM fungal combination had to overcome the salt stress effect before becoming beneficial to their host plants.

The observed result of the effect of ECM and the double symbioses on host plant salt stress alleviation is in contrast with other studies that found mycorrhizal colonisation reduced salt stress (Al-Karaki, 2000; Muhsin and Zwiazek, 2002). Their studies are however mainly referring to the effect of individual AM or ECM colonisation. In the current study the individual AM colonisation reduced host plant salt stress, which is in agreement with these other studies. The AM colonisation was 62% higher than the

ECM colonisation and therefore possibly at a level allowing it to be more beneficial to the host than the ECM fungus, as discussed above.

There is interaction between mycorrhizal fungi and other soil organisms, and between the different mycorrhizal fungi. This interaction can be beneficial or detrimental to the growth of the fungi. The fungi are affected differently at different stages of their growth. The interactions are presented in **Table 1** and they range from destruction of the mycorrhizal fungal spores by parasitic fungi to stimulation of mycorrhizal fungal growth by mycorrhizal helper bacteria (Fitter and Garbaye, 1994). There is also interaction between AM and ECM fungi when occurring in the same root system, that influence the dominance of one type of fungi over the other during the different plant and fungal development stages (Chilvers *et al*., 1987). AM fungi are reported to be more aggressive at the initial stages of colonisation than ECM fungi when occurring in the same root system, while ECM colonisation increases over time and surpasses the AM colonisation (Lapeyrie and Chilvers, 1985; Reseder *et al.*, 2004). The colonisation level of host plant roots by mycorrhizal fungi can also change as host plants age and between different seasons (Robinson, 1971; Harvey *et al*., 1978; Burke *et al*., 2003). AM colonisation of the salt marsh grass *Spatina patens*, was found to change seasonally from 26.6% during vegetative growth to 11.5% during dormancy (Burke *et al*., 2003). All these factors must be considered when deciding on the use of mycorrhizal fungi in forest management.

The results of the current study were obtained under a relatively controlled environment. It should be noted that field conditions in which plants have to grow, would be very different from conditions in this study. Caution must therefore be

exercised when using these results for practical decision-making. The soils of the planting areas in Namibia may already contain the identified fungi indicated in Chapter 1 (**Table 1**). The available fungi of the specific area should be screened from literature and from the field and the host-fungus specificity determined likewise. It is possible that, due to the competition between fungi to colonise the plant roots, the indigenous fungi could replace the introduced fungi over time. Mason *et al.* (1983) reported that there is a difference between early stage fungi, that colonise seedlings, and late stage fungi that grow in association with mature plants. The succession of these different types of fungi should be considered when selecting fungi for inoculation.

TABLE 1. Summary of impacts of soil biota on different stages of the life cycle of mycorrhizal fungi (Fitter and Garbaye, 1994).

Life cycle phase	Process	Effect	Responsible Group
Spore population dynamics	grazing	dispersal	small mammals
		death	invertebrates
	parasitism	death	chytrids, amoebae
Germination	chemical	inhibition	bacteria
Colonisation	interactions	stimulation	bacteria
Internal hyphal spread	uncertain	inhibition	fungi, nematodes
External hyphal growth	grazing	disconnection	invertebrates
Sporulation	unknown	stimulation	bacteria

The inoculation of plants with mycorrhizae in the nursery can still prove to be beneficial for plant growth rather than waiting for available fungi to colonise plant roots in the field (Grove and Malajczuk, 1994). A study by Muro *et al.* (1999) found that *Acacia tortilis* seedlings with additional mycorrhizal inoculation had improved growth compared to those only colonised by fungi available in the field. Thus inoculation of plant roots with symbiotic fungi in the nursery can avoid the delay of the colonisation

by available fungi in the field and insure that plants survive harsh conditions that may occur at the time of planting. This is especially true for Namibia, as tree planting is done during the summer rainy season when frequent dry and hot days occur. The inoculation with mycorrhizal fungi of nursery plants in Namibia could possibly improve the current poor survival rates experienced for new plantings (personal observations). No literature could be found on studies done about the effects of mycorrhizal symbiosis on the growth of trees in Namibia. An investigation of this kind should be conducted to offer possible solutions to the current tree planting difficulties faced in Namibia.

There are several methods for mycorrhizal inoculation in nurseries: soil from natural forests; spores; mycorrhizal seedlings; and pure culture of mycorrhizal fungi (Mikola, 1973). The most economically viable option for Namibia is to inoculate plants by using soil from under established healthy mother trees growing in similar environments as where trees are to be planted. This is due to the lack of technical knowledge in the country to produce pure fungal cultures. This should be done with care as root pathogens and parasites can be introduced with the soil. The use of exotic plants for the rehabilitation of degraded land in Namibia may require that pure culture of the appropriate fungi be imported for inoculation of these plants. Mikola (1973) recommends that the success of inoculation using pure fungal culture can be improved if the planting medium is sterilised and by using a large amount of inoculum. This can give a competitive advantage to the introduced fungi over the natural fungi, especially when plants are planted out in the field.

The use of mycorrhizal fungi in Namibia is further necessitated by the fact that tree planting is done in soils reported to have low nutrient and organic matter content (Rigourd *et al*., 1999). Mycorrhizal plants are reported to grow better than non-mycorrhizal plants in soils with low nutrient status (Azcon *et al*., 2003). The mycorrhizal symbiosis improves host plant resistance to environmental stresses and helps increase growth (Auge *et al*., 1986). The northern regions of Namibia, especially north of the Etosha National Park, are charactarised as having poor saline soils (Moller, 1997). These adverse conditions in Namibia necessitate the use of mycorrhizal fungi to improve plant survival in the field. The raising of plants in Namibian nurseries should therefore include the inoculation of plants with appropriate mycorrhizal fungi. An alternative to inoculating nursery plants would be to change the current method of land preparation, which includes ploughing and burning of residual plant materials, as these are reported to reduce the ability of indigenous fungi to colonise plant roots (Harvey *et al*., 1980). Salt stress affects both fungal and plant growth, as noted in Chapter 1. It is therefore still important to reduce the salt levels of the planting areas to benefit fungal and plant growth. Salt levels can be reduced by leaching soils; applying mulch to reduce transpiration and evaporation, and therefore reducing the upward movement of underground salt water; and application of gypsum to replace Na^+ with Ca^{2+} in the soil exchange complex (Bresler *et al*., 1982). The best option must be determined by field observation under different environmental conditions and economic considerations.

4.2 GENERAL CONCLUSION

This study has confirmed that mycorrhizal fungi can be parasitic when the host plant growth environment is not limiting growth. The double symbiosis (AM and ECM), although not limiting growth, offered no additional benefits to the host plant under the favorable growth conditions. This symbiosis increased the total host plant photosynthesis and therefore compensated for the carbon drain of the fungi on the host plant. This symbiosis however had a negative effect on plant growth when under salt stress, which is attributed to the low colonisation levels observed. The negative effects of the different symbiosis combinations observed under the current growth conditions could change and become positive over time. This could happen when plants are transplanted into the field where soil nutrients are normally lower than in nursery soils; and when fungal colonisation levels increase to levels that allow the fungi to be beneficial to host plants. The double colonisation is recommended for plants growing under the adverse conditions encountered in many parts of Namibia. This recommendation is based on the documented positive effects of mycorrhizal fungi on host plant growth, and the possible long-term benefits to host plants growing in saline soil considered in the current study.

The results of the current study are based on young plants growing under a controlled environment in a green house for 4 months. It is important that long-term studies be done to verify these results, while taking into consideration the different developmental stages of the fungi and the host plant and the other natural variables encountered under field conditions. The long-term studies should also determine the actual total canopy photosynthesis of host plants with a dual symbiosis of AM and

ECM, and the long-term effect of the dual symbiosis on host plant salt stress alleviation.
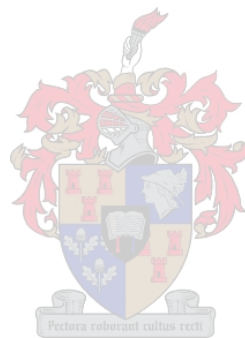
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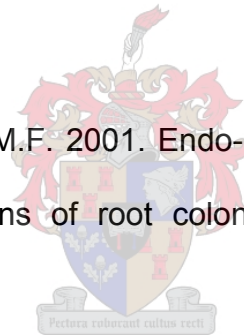
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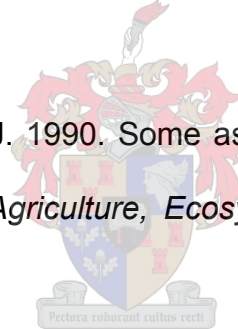
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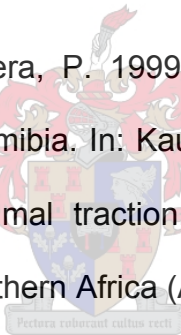
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