THE EFFECT OF VESICULAR-ARBUSCULAR MYCORRHIZA ON THE GROWTH OF TWO INDIGENOUS GRASS SPECIES *THEMEDA TRIANDRA* AND *TRACHYPOGON SPICATUS* GROWN ON COALMINE SPOIL TOPSOIL

by

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Abstract

The main project was an assessment of the effect that colonization by five different Vesicular-arbuscular mycorrhiza (VAM) cultures have on the growth of the indigenous grasses *Themeda triandra* and *Trachypogon spicatus*, when grown on coalmine topsoil.

With unamended topsoil, VAM showed the ability to significantly increase the growth of the grasses compared to non-VAM control plants. The amount of effect varied with the VAM inoculum culture type, with a VAM culture originally from the Cape Flats being the most effective. In a second trial, soil fertilized with nitrogen, potassium and low concentrations of phosphate (P) was used. Again VAM displayed the ability to improve grass plant growth. The increase in P caused the Large spore inoculum to become the most effective. This indicated that different VAM cultures are inhibited to different degrees by an increase in phosphate fertilization.

The low level of VAM infection, in both trials, seemed to preclude most of the VAM associated nutrient uptake control.

Varying reports have been published on the effect of fertilization on VAM infection and colonization. In an attempt to further elucidate the role of fertilizer in VAM inhibition, rhizosphere soil from a long term fertility trial near Witbank, S.A. was sampled. Amcoal environmental services fertilized forty-two plots with varying concentrations of nitrogen, potassium, phosphate and lime to assess the growth of a variety of grasses. The trial had been maintained for ten years before sampling was completed for this project. Samples from each plot were taken from the rhizosphere soil of the most prominent grass (*Digitaria eriantha*). VAM spores were extracted from all the samples and five different types of spores were identified and counted for each sample. By comparing spore counts from each plot, the effect that the fertilizer regime had on the VAM on that plot could be assessed.

Variation in the concentrations of nitrogen (N) and potassium had no significant effect on VAM colonization. Very low concentrations of N could not be assessed as all plots had been initially top dressed with nitrogen fertilizer. Phosphate (P) fertilizer concentration had a marked effect on spore concentrations. There was a significant increase in spore concentration as P levels were increased from zero P fertilization to 80kgs P/ha. Further increase in P to $\geq$160kgs P/ha resulted in a significant decrease in spore concentrations. From this it would appear that a low level of soil P is needed to give maximum VAM colonization and further increase in soil P causes VAM inhibition. Lime ameliorated the VAM inhibition caused by high concentrations of P.

Increase in P caused spore concentrations of low abundance propagules (LAP) too decreased more rapidly than high abundance propagules (HAP). In high P soils VAM with LAP would eventually be eliminated from the system resulting in a decrease in VAM diversity.

A project was attempted to use the recently developed Randomly Amplified Polymorphic DNA in conjunction with the Polymerase Chain Reaction (RAPD PCR) techniques to identify

different VAM families. The technique causes the amplification of segments of DNA which can be visualized by gel electrophoresis and staining. Band patterns formed can be related to the VAM of origin and hence used in identification of that VAM. An attempt was made to amplify DNA from a single spore in this manner which would, in conjunction with morphological observations, make identification of VAM easier and more accurate. Problems with either releasing the DNA from the spores, or substances in the spore inhibiting the PCR reaction made obtaining band patterns difficult. After many PCR attempts, varying extraction methods and PCR conditions, no repeatable results could be obtained and work on this project was discontinued.

Preface

I hereby certify that the experimental work in this thesis was the result of my own investigation under the guidance of Dr W. A. Cress in the Department of Botany, University of Natal, Pietermaritzburg. Non of the work completed has been submitted towards a degree at any other university. Where use has been made of work completed by any other party, it has been duly acknowledged in the text.

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CHAPTER 1

1. INTRODUCTION

Symbiosis, a state occurring when two organisms live in intimate contact, has until recently been considered as more of a rarity than a normal situation. The forms of symbiosis are very diverse and were first formally described by A. deBary (1887). A summary of the relationships is shown in Table 1.1.

Table 1.1 Types of symbiosis between organisms (after Allen, 1991)

SPECIES 2	SPECIES 1		
	+	0	-
+	MUTUALISM	COMMENSALISM	PARASITISM
0	COMMENSALISM	NEUTRALISM	AMENSALISM
-	PARASITISM	AMENSALISM	ANTAGONISM

The effect of a symbiosis on a species can have one of three outcomes :-

I Positive (+) which indicates that a species derives a benefit from the association.

II Negative (-) which indicates that the association is detrimental to the species.

III No effect (0) the association is neither detrimental nor beneficial to a species.

Interactions between two participating species are complex and with the same two species may vary from mutualism to parasitism, dependent on the prevailing conditions (deBary, 1887).

Mutualistic symbiosis where both partners benefit would intuitively be the most advisable and it is indeed of ancient origin. Mutualism probably originated in symbiotic relationships between prokaryotes which ultimately evolved into complex life forms such as present day eukaryotic cells. The mitochondria and protoplasts of these eukaryotic cells are the result of evolutionary integration of prokaryotic organisms (Margulis and Bermudes, 1985). This indicates the potential value of a mutualistic relationship to the participating symbionts.

A common and widespread type of mutualistic symbiosis is the mycorrhizal association. There are a number of types of mycorrhizal associations as shown in Table 1.2 after Allen (1991).

The mycorrhiza range from ectomycorrhizae through ectendomycorrhizae to endomycorrhizae [vesicular arbuscular mycorrhiza (VAM)]. Ectomycorrhizae are characterized by the effect they have on the root structure. They form a fungal sheath around the outside of the root and modify the epidermal layer of infected root tissue. Hyphae growing between the epidermal cells form a nutrient exchange network called the Hartig net. A schematic diagram Figure 1.1 after (Harley and Smith, 1983) of an ectomycorrhizal root shows the changes as compared to a non-mycorrhizal root. The root structure of the mycorrhizal plant is distinctly different from the non-mycorrhizal plant in the epidermal area, but the hyphae do not infect the cortical layers of the root (Harley and Smith, 1983)

Endomycorrhizae (VAM), Table 1.2 demonstrate no distinct effect on the structure of the roots. VAM infect the roots by penetrating the epidermis using an appresorium-like structure and then ramify through the cortical layers of the root. The hyphae grow between the cells along the cell walls. Endomycorrhizae or VAM are characterized by the arbuscules that they form inside the root cells and vesicles formed between root cells, which are visible only when stained. No hyphal sheath forms in VAM (Friese and Allen, 1991).

In ectendomycorrhizae the infection type ranges from ectomycorrhizal like infection with a distinct sheath and only small amounts of hyphae penetrating the cortical cells, to

endomycorrhizal like infections where the sheath is very limited (Allen, 1991).

Endomycorrhizae or vesicular-arbuscular mycorrhiza (VAM) have been demonstrated in the fossil record to have formed symbiotic associations with plants from the time plants first

Table 1.2 **Types of Mycorrhiza** (after Allen, 1991)

PENETRATION OF ROOTS	TYPE OF MYCORRHIZA	EFFECT ON ROOTS
NO PENETRATION OF CORTICAL CELLS	**ECTOMYCORRHIZAE**	SHEATHED ROOTS
	E strain	
	Arbutiod	
	ECTENDOMYCORRHIZAE	
	Ericiod	
	Monotropoid	
	Orchid	
	ENDOMYCORRHIZAE	
PENETRATION OF CORTICAL CELLS	Vesicular/Arbuscular	UNSHEATHED ROOTS

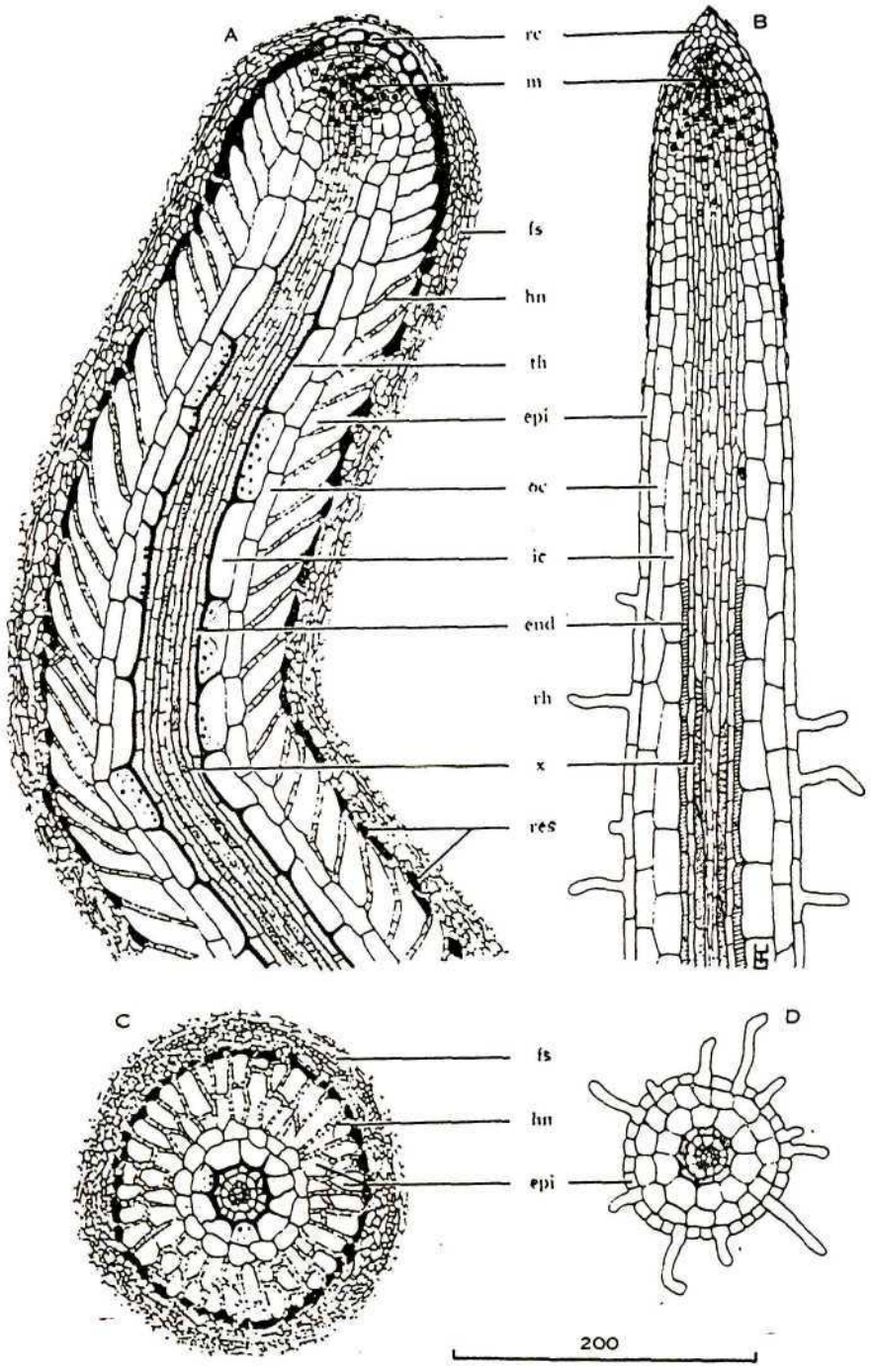

Figure 1.1 **Schematic diagram comparing ectomycorrhizal and non-mycorrhizal roots.**

After Harley and Smith (1983). rc = root cap, m = meristem, fs = fungal sheath, hn = Hartig net, th = thickened layer, epi = epidermis, oc = outer cortex, ic = inner cortex, end = endodermis, rh = root hair, x = xylem, res = cell of root-cap complex in the fungal sheath.

began to colonize the land (Pirozynski and Mallock, 1975). For example Stubblefield (1985) suggested that fossil chlamydospores from the lower Devonian of the Palaeozoic Era are of VAM origin. It is possible that VAM co-evolved with terrestrial plants when they first colonized the land. The VAM symbiont was responsible for the absorption of the very low levels of certain nutrients (eg. phosphorous) from the early soils. Some of these nutrients were then passed to the plant in exchange for fixed carbon products thus allowing the survival of both symbionts under these nutrient limiting conditions (Pirozynski and Mallock, 1975; Pirozynski, 1981).

Growth of natural grasses of the South African grasslands on minespoil soils has been a problem for the reclamation of strip mined sites. *Trachypogon spicatus* and *Themeda triandra* are among the grass species that usually predominate in mature grasslands. The term 'terminal grasses' is used to indicate their predominance towards the climax of the successional process leading to the mature grassland. Heavy fertilization does not favour the growth of these grasses and the possibility of allowing succession to eventually form a natural mature grassland seems to fail. This process takes an exceedingly long time to occur and often results in the loss of topsoil before the successional process can result in the takeover of the terminal grasses (Roux, 1969). *Trachypogon spicatus* and *Themeda triandra* are both mycorrhizal grasses, but the symbiosis is adversely affected by fertilization. The possibility that inoculation with mycorrhizal flora could help the growth of these grasses was postulated. This could be tested by comparing the growth of the two grass species on control minespoil soil and minespoil soil inoculated with a number of different mycorrhizal flora. If any of the mycorrhizal flora caused a significant growth increase in the grasses, there would be a possibility that it may help with minespoil reclamation.

Two problem areas of mycorrhizal research were identified. Firstly, what effect high phosphorus fertilization had on the VAM fungal associations and secondly, the difficulty of identifying VAM species.

To see if fertilization had any effect on VAM colonization level and species diversity, a set of long term fertilizer trial plots would be sampled to compare the VAM spore populations of the different treatments.

The DNA stored in a small number of VAM spores could possibly be used to identify the VAM species using the RAPD's (randomly amplified polymorphic DNA) technique to PCR (polymerase chain reaction) a number of DNA fragments which could be subjected to Agarose gel electrophoresis (section 3.2.2). The band pattern formed could be analysed in a similar way to the established RFLP (restriction fragment length polymorphism) technique, to identify the spore species by the DNA band pattern that the RAPD technique will produce.

A great deal of literature has been produced on the subject of mycorrhizal symbiosis, with VAM associations only recently being accorded the status they warrant. To deal with the literature review effectively, the topic was divided into eleven sub-sections. Those topics are listed below.

1. Vesicular-arbuscular mycorrhizal colonization, development and anatomy.
2. Physiological and biochemical aspects of vesicular-arbuscular mycorrhizal infections.
3. Ecology of vesicular-arbuscular mycorrhiza and related subjects.
4. Growth enhancement by vesicular-arbuscular mycorrhiza.
5. Phosphorous uptake and use by vesicular-arbuscular mycorrhizal fungi.
6. The effect of vesicular-arbuscular mycorrhiza on uptake of minerals other than phosphorus.
7. Water relations in vesicular-arbuscular mycorrhizal plants.
8. Multi species associations of vesicular-arbuscular mycorrhiza, their hosts and other rhizosphere organisms.
9. Uses of vesicular-arbuscular mycorrhiza in erosion and reclamation work.
10. Fungicidal suppression of vesicular-arbuscular mycorrhiza.
11. Genetic and transformed plant associated topics.

1.1 Vesicular-Arbuscular mycorrhizal colonization, development and anatomy

There are three distinct types of infection propagules :-

I. Spores

II. Runner hyphae

III. Root fragment hyphae

I. Spores are thick walled resting structures which are generally soil born, but are capable of dispersal by wind and animals to distances of a few kilometres (Allen, 1991). They are multi-nucleate, containing many thousands of nuclei (dependent on species) and range in size from less than 100μm in some *Glomus spp.* to 500μm in *Gigaspora spp.* Spores form the major criteria for identification of VAM fungi, their size, colour, shape, wall thickness, wall layers and ornimentation, as well as type of attachment to the hyphae are indicative of their species. Full description is beyond the scope of this literature review, however the most recent information on spore identification can be found in Schenck and Perez (1990).

Germination of spores leads to one or more germ tubes forming, which grow in response to root exudates, toward the root. Although the signal mechanism for this chemotropic process has not been fully elucidated, carbohydrates (Same *et al.*, 1983; Thomson *et al.*, 1990) and isoflavonoids (Nair *et al.*, 1990) have been proposed as the signal molecules. In contrast to most other researchers, Becard and Fortin (1988) working with Ri T-DNA (*Agrobacterium rhizogenes*) transformed carrot (*Daucus carota* L.) roots, found that *Gigaspora margarita* germination tubes showed negative geotropism. This upward growth was not affected by roots nearby, unlike the generally accepted attraction of the germtube to the roots described by Koske (1982). There are a number of possible reasons for the apparent anomaly between the observations of Koske (1982) and Becard and Fortin (1988). Becard and Fortin (1988) worked with transformed roots that may have an altered physiology, therefore not producing the signal that attracts the germtube to the root. If the germtubes are normally attracted to host roots, then the signal molecule may be formed in the shoot area, therefore would not be produced in the Becard and Fortin (1988) system.

Becard and Piche (1989) postulated that a root generated signal molecule initiates germination and germtube elongation. The authors demonstrated that spore germination and germtube elongation was initiated by the close proximity of potential host roots. Removal of these roots after germination caused germtube elongation to cease before the germtube reached 30mm. If potential host roots remain in the proximity of the germinated spore the germtube can extend to a length of 400mm before growth stops. This indicates that the signal molecule must be unstable, easily degradable or in very low concentration, as removal of the roots from the spore proximity rapidly resulted in the cessation of germtube growth (Becard and Pechi, 1989). Spores are multinucleate, which allows for the formation of up to ten germtubes if spurious germination occurs. Nutrient depletion of spore reserves was postulated as the limiting factor in the possible number of germtubes formed (Koske, 1981). The cessation of germtube growth after spurious germination was postulated to reduce spore nutrient store depletion, thus allowing for future germination when host infection was possible (Becard and Piche, 1989). Spore nutrient considerations also play a part in root infection, since the infection rate decreases with the distance of the inoculum source from the root (Becard and Piche, 1990).

The rhizosphere width for mycorrhizal infection is defined as the maximum distance from the root where spore germination can be initiated resulting in infection of the root. The width is thought to be related to the maximum distance that root exudates can disperse through the soil, to cause spore germination. A method for measuring the rhizosphere width was developed by Smith *et al.* (1986). This method indicated that the rhizosphere width varies with the species of VAM used for inoculum. After 10-12 days, clover (*Trifolium subterraneum* L.) had a rhizosphere width of 2.5-6.5 mm with mixed VAM inoculum, but the rhizosphere width with *Glomus mosseae* was 8.9-13.2 mm.

When the germtube contacts the root, at a distance of 3.5 -4.5cm behind the root tip an appressorium is formed and a chemical signal is apparently generated. This signal causes branching of the hyphae into fine hyphae (2-3μm diameter) near the appressorium which form multiple infection sites (Friese and Allen, 1991). This was not the case in the work of Becard and Fortin (1988), as fan like branching only occurred after five days and often well away from the appressorium. The appressorium was described by Emmett and Parbery (1975) as having a

capacity to adhere to a host's surface and the ability to germinate and penetrate the host.

II. Runner hyphae grow out from infected roots, are thick walled (2-3μm) and have uneven diameter of 10-15μm. The hyphae are coenocytic and can grow in two ways:-

a. Hyphae grow along the root epidermis towards the root tip, to cause secondary infection in the same root.
b. Hyphae grow out away from the root in search of another root to infect. This root may be from the same host, but the hyphae can infect the roots of a second host.

Runner hyphae generally only form one infection site, but two or three infection sites generated by one runner hyphae have been observed (Friese and Allen, 1991). These hyphae are implicated in the formation of hyphal bridges between adjacent roots of the same plant. Runner hyphae have also been implicated in the formation of bridges between roots of different plants, though the extent of this type of bridge network is unknown (Camel *et al.*, 1991). These inter-plant bridges (if formed) have great significance in the possibility of inter-plant nutrient transfer, which may aid in the survival of weaker plants and growing seedlings (Newman, 1988). Runner hyphae can grow up to 90 mm away from the roots and are likely to be the proginators of the appressorium like globose swellings on the end of the hyphae seen by San Antonio (1987).

III. Root fragment hyphae grow from pieces of detached root, with the internal hyphae sending out a number of infection hyphae which coil around each other like a rope. They grow out towards any potential host root system. When in close proximity to the roots, root exudates are thought to cause the hyphal network to separate into single hyphae, causing a multiple infection network ending in appressoria (Friese and Allen, 1991).

How long does it take for infection to occur? This is variable, dependent on the inoculums potential and the plant and fungal species. Assuming a soil inoculum potential high enough to give rapid infection, infection can take place within two to fourteen days. Onion plants (*Allium cepa* L.) inoculated with *Glomus deserticola* only took three days to become infected,

whereas with *Glomus mosseae* twelve days were needed (Afek *et al.*, 1990).

The infection time and level can be modified by soil conditions. The level of chemicals in the soil, such as phosphorus, can cause slower infection and lower levels of infection as P increases. Boron on the other hand has been found to increase infection level (Dixon *et al.*, 1989) Variations in pH (Arines *et al.*, 1988), temperature (Allen, 1991) and soil water content (van Duin *et al.*, 1989) can also cause variation in infection level. Soil conditions appropriate for one VAM/plant symbiotic relationship may not be acceptable for other VAM associations (Abbot and Robson, 1991). A great deal of variance in VAM infection level was demonstrated with different growth media, using the same plant/VAM symbiosis (Caron and Parent, 1988; Douds and Schenck, 1990). This implies that correct VAM/plant associations must be chosen for the soil conditions.

A high level of VAM inoculum in the soil is important in low nutrient soils often associated with poor subsistence farmers. Here good crop rotation practices can be of great benefit. Plants that become extensively mycorrhizal in one growing season, can produce large quantities of VAM infection propagules. These propagules can greatly help a subsequent crop that requires a high VAM inoculum level to become effectively mycorrhizal (Douds and Schenck, 1990). A high level of inoculum also aids early VAM infection, benefiting fast growing seedlings and latter improving plant nutrient status, to cope with flowering and seed formation (Fitter, 1989). Improvement of colonization can be achieved by increasing boron intake (foliar spray), which enhances root permeability, possibly due to inhibition of indole-acetic acid oxidase (Dixon *et al.*, 1989)

The last of the types of external hyphae distinguished by Friese and Allen (1991) was the absorptive hyphae. This type of hyphae is solely responsible for nutrient uptake and does not appear to cause any infection of roots. Adsorptive hyphae are aseptate (Allen, 1991), multinucleate (Garriock *et al.* 1989) and grow outward from the root dividing dichotomously to form a fan like network. The primary hyphae are thick walled (3μm) with a diameter of 10-20μm. Five to eight dichotomous divisions of the primary hyphae, decreases the wall thickness of the hyphae to less than 1μm and the diameter to only 2μm. The hyphal length of these

terminal branches is 4-7cm (Friese and Allen, 1991). These networks last for five-seven weeks but viability of the network decreases from an initial 100% to 15% after five weeks (Schubert *et al.*, 1987).

VAM are not considered to have an effect on the root anatomy, however recently Fusconi et al. (1994) found that VAM infected leek (*Allium porrum* L.) roots had larger apical diameters. This was partly due to a greater number of meristematic cells, and partly due to the increased size of the cortical cells

The internal structures of VAM symbiosis are internal hyphae, arbuscules and vesicles. About two days after the appressorium attaches to the epidermis of the roots, it germinates producing hyphae that enter into the root and grow along the boundries between cells. The initial dormancy of the appressorium was thought by Becard and Fortin (1988) to be a time when the appressorium is preparing for hyphal entrance into the host. The authors postulated that recognition processes, or enzyme preparation to penetrate the host cell wall, were being performed during this period. One to several infection hyphae can germinate from the multinucleate appressorium, with Bonfante-Fasolo (1984) distinguishing two types of hyphae. Intrarcellular hyphae which are 3-7μm in diameter (Abbot, 1982) and are formed from the infection hyphae. They form in the outer cortical layers and penetrate cell walls forming hyphal coils which invaginate the cell membrane. This type of hyphae penetrates from cell to cell as it grows. The plant cell wall thickens in the region of the appressorium and also where the hyphae penetrate the cells, in response to the invasion. A second type of hyphae recognised by Bonfante-Fasolo (1984) is the intercellular hyphae which run along cell walls between the cortical cells. They range in diameter from 2-6μm dependent on VAM species and can run along cell boundries for 3mm. Hyphal division maps cell boundries giving a complex pattern around cells. These hyphae penetrate the cell walls and form arbuscules which are hand like hyphal projections that invaginate the plant cell membrane. The walls of the internal hyphae vary among different VAM species. Some are thought to have two layers while others only one, but this can vary within the same species with different hosts (Bonfante-Fasolo, 1984).

Becard and Fortin (1988) described the infection differently, with the spore being

responsible for fungal nutrition during appressorium formation, penetration and internal hyphae growth. The VAM only becomes independent of the spore when the first arbuscule forms, after which point the spore and appressorium are no longer needed by the colonizing VAM. In VAM/plant associations that form coils rather than arbuscules this changeover point has not yet been established. Growth and branching of the internal hyphae appears to occur only after the spore dependance is broken, this indicates that arbuscules are the site of exchange of host and VAM nutrients (Becard and Piche, 1990).

Arbuscules were one of the original criteria for membership of the Endogonales [now classified as Order Glomales (Schenck and Perez, 1990)] (Gerdemann and Trappe, 1974). They are formed when the internal hyphae penetrate a cell wall. The cell wall region around the penetration thickens reacting to the penetration, but the hyphae enters with little further reaction (Becard and Pitche, 1989). After penetration the hyphae is similar to the internal hyphae, but rapidly divides forming a glove like structure, the arbuscule. The hyphal branches formed are progressively smaller with the end branches being only 0.5μm in diameter and the wall thickness decreasing concommitantly. At these end branches, all VAM species appear to have the same hyphal structure (Bonfante-Fasolo, 1984). The arbuscules contain numerous nuclei, are very vacuolated and contain numerous polyphosphate granules, indicating their role in phosphorous transfer (Capaccio and Callow, 1982). Arbuscule volume covers as much as 10-20% of the cell volume, while the cell protein content of infected cells increased from around 3% to above 10% (Alexander *et al.*, 1989).

The life span of an arbuscule is four -five days, before the cytoplasm is withdrawn and the arbuscule collapses into a fist like clump, the plant cell however, does not die (Cox and Tinker, 1976). The cell can in fact be re-penetrated to form new arbuscules while the degenerating arbuscule remains visible (Alexander *et al.*, 1989).

The second criteron for membership of the Endogonales (Glomales) is the formation of vesicles, though not all members of this order form vesicles and some only in certain hosts (Schenck and Perez, 1990). Vesicles are intercalary or terminal swellings of the internal VAM hyphae that generally result in globose bodies, but can have varying shapes which are dependent

on the VAM the species involved (Bonfante-Fasolo, 1984). The formation of vesicles seems to develop in the same manner as chlamydospores (Mehrotra, 1993), with the wall being trilaminate with layers of differing electron density (Kinden and Brown, 1975). The cytology of vesicles is not well studied, they contain large numbers of nuclei, glycogen granules and later become very vacuolated. Mature vesicles are engorged with lipid droplets and more vesicles form in older or dying roots. This indicates that they probably have a role as resting organs forming infective root segments (Bonfante-Fasolo, 1984; Friese and Allen, 1991).

VAM have a very diverse structure which can vary with both the host and the soil conditions (Boyetchko and Tewari, 1990). This makes identification of VAM species very difficult, leaving only the spores as a satisfactory criteria for identification. More work is needed in assessing the effects that host and soil have on both VAM physiology and anatomy.

1.2 Physiological and biochemical aspects of vesicular-arbuscular mycorrhizal infections

Because VAM have not as yet been cultured *in vitro,* the study of physiological and biochemical processes associated with VAM fungi are extremely difficult. The main problem is to distinguish whether a process is occurring in the fungus or is being induced in the plant by the fungus. It has become evident that VAM fungi do not merely alter plant host conditions by simply increasing the plant's P status, but play an active role in modifying some plant physiological and biochemical functions (Dakessian *et al.*, 1986)

The role of P in driving the symbiosis is one of the reasons why plants readily allow the infection by VAMs, but what do VAMs receive in return ? One of the prime nutrients sought by the VAMs are fixed carbon compounds usually in the form of soluble carbohydrates (CHOs). The level of soluble carbohydrates in the roots correlates to the amount of VAM colonization in the roots, with exudates of CHOs attracting germinated hyphae to the plant roots (Same *et al.*, 1983). This would imply that high P levels would reduce soluble CHOs in the roots, thereby reducing VAM colonization at high P levels. Amijee *et al.* (1990) showed that high P levels do

not reduce root soluble CHO levels but that aborted entry points of VAM hyphae increased. The authors made the assumption that high P levels in some way change the root anatomy making it resistant to VAM colonization.

Thomson *et al.* (1990) stated that some of the reduction in length of root infected by VAMs on high P soils is a result of reduction in secondary infection. The main form of soluble CHO found in VAMs appears to be trehalose. This was shown by Schubert *et al.* (1992) using the *Glycine max / Glomus mosseae* symbiosis. When high P levels were used to reduce VAM colonization, the amount of total soluble CHOs in the roots was not affected, but the level of trehalose was reduced. In light deprived plants, a reduction in total CHOs and trehalose occurred which was concomitant with the reduction in colonization by VAMs. This implies that the trehalose is synthesised by the VAM from the CHOs provided by the plant. Temperature also has an effect on the CHO levels in VAM plants . In non-VAM plants the CHO level increased with temperature from 15° to 25°C where plant growth was the best. VAM inoculation moderated the adverse effects of high soil temperature and allowed better growth and CHO content at higher temperatures (Borges and Chaney, 1989). However, the total CHO level was always lower in the VAM inoculated plants. This could be due to the inability to separate all the VAM hyphae from the soil. Although low temperatures are not conducive to VAM activity (Bledsoe *et al.*, 1990), VAM can help with plant growth in chilling conditions. Charest *et al.* (1993) showed that maize (*Zea mays*) plants infected with *Glomus mosseae* grew better at 10°C than did non-mycorrhizal plants.

Other compounds affecting VAM symbiosis are iso-flavonoids which appear to act as signal molecules for VAM hyphal growth and colonization. Two iso-flavonoids were shown to be produced in P-stressed clover plants (*Trifolium repens*) and were effective in concentrations of only 5 mgl^{-1} (Nair *et al.*, 1990).

Fatty acids (FAs) are main storage compounds of soybean (*Glycine max*) roots and infection by a *Glomus sp.* caused a change in root fatty acid content. Five types of FAs were found in VAM infected roots that were not found in non-VAM roots one of which [16:1(11C)] (no name given), was assumed to be the main storage FA in VAM vesicles. Non of these five FAs

were found in the aerial section of the plants, but there was a more subtle change in the shoot leaf and seed FA content of VAM colonized plants. This change was accentuated by concomitant infection with *Bradyrhizobium*. Pacovsky and Fuller (1988) on showing this effect, suggested that the FA change in the seed may have an effect on seed quality which would need further assessment.

A number of enzymes are associated with VAM infection and arbuscule formation. Phosphate is thought to be transported as polyphosphate granules by protoplasmic streaming in VAM hyphae from distant rhizosphere regions to the arbuscules in plant root cells. Acid phosphatase is one of the enzymes induced at arbuscule formation. It is responsible for the hydrolysis of the polyphosphate granules which releases P for cross membrane transport to the plant cell (Dodd *et al.*, 1987).

VAM have demonstrated the ability to reduce pathogenic attack from other fungi on their hosts. Giovanetti *et al.* (1991) showed that in tobacco plants infected with *Glomus monosporum*, subsequent infection by the pathogen *Thielaviopsis basicola* (black root rot) was markedly reduced. The levels of the two amino acids arginine and proline were greatly increased over those of control plants, when subjected to a *T. basicola* challenge. Proline is expressed in greater amounts under various stress conditions and may be responsible with arginine for a change in the plant cell membrane protein structure that inhibits pathogen attack. The proline reaction is transmitted to regions other than those directly colonized by VAM. This provides some protection from pathogen infection in areas of the root that are not infected by the VAM.

A physiological process of the VAMs may be one of the reasons for the increased uptake of P in VAM colonized plants. Knight *et al.* (1989) demonstrated that the level of CO_2 in the soil immediately surrounding the VAM infected roots was higher then in non-VAM plants. This increase in CO_2 due to the fungal metabolism causes a localized decrease in pH due to the formation of carbonic acid. The fixed P is solubilised to a greater extent than in the control and so more is made available for VAM uptake and transport to the plant.

Mycorrhizal arbuscules have a number of similarities to the nodules of *Bradyrhizobium*.

Some of the polypeptides associated with the arbuscules are cross reactive with antisera raised from the nodules of *Bradyrhizobium* (Wyss *et al.*, 1990). Some of these polypeptides are associated with the transport of nutrients across the plant and fungal /bacterial membranes, while others are thought to be signal and control proteins (Wyss *et al.*, 1990). The soluble polypeptides (named mycorrhizins after their namesake nodulins) could well be responsible for the change in root cell growth patterns noted by Barea and Azcon-Aguilar (1982).

1.3 Ecology of vesicular-arbuscular mycorrhiza and related subjects

Conflicting results have been reported under field conditions as to whether VAM infections are mutualistic (Sanders and Fitter, 1992a). Sparling and Tinker (1978) reported no VAM instigated growth increase in grassland, whereas Hayman and Mosse (1979) did find a significant yield increase. It would appear that soil conditions determine what type of relationship VAM may form and that this controls where they may thrive.

VAM are ubiquitous, forming associations with most plants and having a very large range of habitats (Harley and Smith, 1983). Some plants do not form any mycorrhizal associations with most of these being Crucifereae, Cyperaceae and Proteaceae. Estimates are that 70-90% of all plants form some kind of mycorrhizal association (Allen, 1991).

Bledsoe *et al.* (1990) performed a ecological survey in the Canadian Arctic region and even in these harsh conditions found some plants that were ectomycorrhizal, whilst some others were ericoid. No VAM or VAM spores were found in the soil at this latitude indicating that VAMS do not grow in permanently cold climates. At a slightly lower latitude in an Ontario maple forest most of the trees were VAM infected, while others were infected with ectomycorrhizae. Most of the herbaceous plants were VAM infected and a number of plants were found to have the orchid type of mycorrhiza. In this region the VAM colonization was very seasonal from spring to late summer again indicating that VAM do not like cold or that the host dies back in cold weather (Brundrett & Kendrick, 1988). Temperate ecosystems have an abundance of VAM infected plants, but infection levels vary widely. This indicated to Fitter (1989) that in this system

the VAM may only be beneficial to the plants at certain periods of their growth when nutrient uptake (especially P) is in greatest demand. The effect of VAM on prairie grassland was found to be very variable and dependent on season and plant species. Fire allowed VAM plants a competitive advantage over non-VAM species (Hartnett *et al.*, 1994).

In warmer climates VAM are more profuse as shown from the Californian serpentine grassland (Mediterranean climate) surveyed by Hopkins (1987). Even annuals are heavily colonized by VAM in this region and only two species were found to be non-mycorrhizal while 98% of the herbaceous plants were VAM infected. Moving to the tropics, in Kenyan Savanna grassland all five grass species studied by Newman *et al.* (1986) were mycorrhizal. Here, water shortage causes a seasonal dieback of grasses and consequently VAM while VAM increase coincides with new root growth in the rainy season.

Colonization by VAM even occurs in forest canopies, where epiphytic *Piperaceae* species are facultatively mycorrhizal. Although normally non-VAM, they can be colonized when the inorganic nutrient level is low, commonly in developing plants. The high level of inorganic nutrients in the moisture of the canopies generally means that VAM colonization is unnecessary (Maffia *et al.*, 1993).

VAM are present even in harsh climates such as the semi-arid mallee community in Australia. In a survey of 93 plant species from 37 families found in the community, 84 were VAM infected and some showed both VAM and ectomycorrhizal infection (McGee, 1986).

Mycorrhiza are in fact particularly suited to growth in low nutrient soils as shown by the work of Benjamin *et.al.* (1988). These authors assessed VAM infection of little bluestem (*Schizachyrium scoparian*) from a sand prairie with low nutrient soil, along an increasing nutrient gradient to closed forest. VAM infection was far highest in the low nutrient sand prairie region.

Possibly the lowest nutrient levels are found in the reclamation of sand dunes. In Singapore, dredged sea sand was deposited on a reclamation site to form dunes which consequently contained no mycorrhizal propagules. The initial plants grown on the dunes were

non-mycorrhizal and growth was sparse with ground cover being poor. In slightly older regions of the reclamation site, the plants had become mycorrhizal and these seemed to be replacing the non-mycorrhizal plants. Growth of the VAM plants was better and the ground cover was increased, reducing dune erosion (Louis, 1990).

Of the 15 halophytic plants species tested in salt marshes, all showed seasonal VAM infection. Areas of the marsh containing non-VAM plants were on low lying land often flooded by salt water for extended periods, which indicated that the flooding rather than the salt was responsible for lack of VAM infection (Van Duin *et.al.*, 1989). VAM are even found in the drier regions of floating wetland mats (predominantly *Typha* species), but not in the permanently wet sections (Stenlund and Charvat, 1994). Colonization by VAM does not seem to occur in permanently waterlogged regions. A transect from drier soil down a river bank showed that *Casuarina cunninghamiana* VAM colonization, decreased as the soil became more permanently wet. Roots protruding into river water were always non-mycorrhizal (Khan, 1993).

Mycorrhiza are thus ubiquitous, with VAM being found everywhere plants are found except for very cold climates and very wet soils.

What then is the percentage of plants that are mycorrhizal ? This is variable dependent on the conditions prevailing for example, high levels of phosphate fertilizer and erosion of topsoil reduce mycorrhizal colonization (Jasper *et al.*, 1987; Sylvia and Neal, 1990). The season also dictates the VAM colonization level, with plants having higher colonization in summer than winter. The highest level of colonization in grasses was found to be at flowering, when nutrient uptake is highest (Lopez-Sanchez and Honrubia, 1992).

Looking at surveys conducted to assess the number of mycorrhizal plants in a system, Birch *et al.* (1988) found that of 60 plant species, 44 were always mycorrhizal, 3 were variable and 15 were non-mycorrhizal. In this system 78% of plants were mycorrhizal. McGee (1986) in a Australian melee community assayed 93 species from 37 families and of these 84 were mycorrhizal (90%). Herbaceous plants in Hopkins (1987) study showed that 98% were mycorrhizal.

As VAM are so diverse and colonize so many different plants, it would appear that they must have some benefit, otherwise some of the plants would react as they do to pathogens.

1.4 Growth enhancement by vesicular-arbuscular mycorrhiza

Although growth enhancement by VAM may play an important role in species survival, most of the work on growth enhancement by VAM has centred on commercial crops. Fertilizer of any type is usually expensive, so anything that can reduce the fertilizer levels needed for plant growth must be an advantage to farmers. This is of special importance in third world countries where soils are often low in nutrients and high level fertilization is beyond the incomes of most subsistence farmers. It was shown (section 1.3) that VAM can colonize most plants. They can also colonize most crop plants, with tomato, maize and onions commonly used as host plants to amplify VAM (Schenck, 1984).

Both greenhouse and field trials comparing growth of mycorrhizal to non-mycorrhizal plants have been completed on a number of crops. Aziz *et al.* (1990) demonstrated a distinct growth and fresh weight increase in pineapples colonized by *Glomus aggrigatum.* Little bluestem *(Schizachyrium scoparium)* bushes (Meredith and Anderson, 1992), papaya (Mohandas, 1992) and strawberries (Chevez and Ferrera-Cerrato, 1990) showed both growth and nutrient uptake increases when colonized by various *Glomus spp. Glomus aggrigatum*-infected palmorosa plants displayed a two-fold growth and three fold biomass increase (Gupta and Janardhanan, 1991). Fredeen and Terry (1988) showed that in the soybean/*Glomus fesiculatum* symbiosis, low P VAM plants had higher biomass than high P fertilized plants. The nodulation and leaf surface area of these VAM plants was also greater than the high P fertilized plants. Cocoa seedlings colonized by a *Gigaspora spp.* had equivalent growth to non-VAM high P fertilized seedlings (Hashim and Raga, 1986). Similar growth increases in VAM plants occurred in a number of hardwood trees in Quebec, when only a basal fertilization regime was instituted (Corke *et al.*, 1992).

Growth increases by VAM infected plants can also be maintained in adverse conditions. Mycorrhizal guayule *(Parthenium argentatum)* grown in saline soils with low fertilization levels

had comparable growth rates to high P-fertilized non-mycorrhizal plants, but had a reduced Na uptake (Pfeiffer and Bloss, 1988). Perhaps the most spectacular growth increase was shown by *Stylosanthes quianensis* inoculated with *Acaulospora serbiculata*. In its third year of growth with low P and Ca fertilization, *S. quianensis* provided a 5129% growth increase over non-VAM controls. An added advantage was that the normally acid soils were ameliorated by the symbiosis. This process normally takes large amounts of lime to increase the pH to a point where aluminium toxicity no longer affects the plants. Other VAM species were not as effective in this system (Lambais and Cordoso, 1990).

In an unusual aspect of VAM behaviour, *Ranunculus adoneus* trees had increased development of infection with *Glomus tenue*, long after reproduction and peak growth of the plants had occurred (Mullen and Schmidt, 1993). The authors indicated that this caused a phosphorus build up in the plant, which allowed for early growth and flowering the next season. *Ranunculus adoneus* trees did not have to wait for thawing to allow nutrient uptake, hence giving the trees a competitive advantage over plants needing to wait for the thaw to start growing.

Not all VAM associations are beneficial, as demonstrated by Hung *et al.* (1990). Working with sweet potatoes, of the nine VAM associations assessed, a range of responses from growth increase to growth suppression was observed. These authors found that the maximum growth increase caused by the VAM symbiosis occurred at the seedling stage, after which the advantage was less noticeable.

In one of the largest trials completed, 19 crop plants were assessed for VAM enhanced growth (Khasa *et al.*, 1992). The following crops showed a high level of dependence on their VAM symbiosis to achieve good growth on low fertility soils:- African yam beans, wild mung beans, *Acacia leacaena*, onion, sweet potato, tomato and cassava. Of the 19 crops tested, 16 showed increased colonization by VAM with higher inoculum levels.

The time honoured principle of crop rotation also increases VAM inoculum levels and diversity. Ellis *et al.* (1992) demonstrated this using the rotation of soybean and sorghum. In monoculture systems of either crop, the level and diversity of VAM inoculum was much lower

than in the rotational system. This increase in diversity and inoculum level caused by crop rotation would be an advantage to crops that need high levels of inoculum (Khasa *et al.*, 1992). to become effectively mycorrhizal. In the sorghum/soybeans rotation, VAM colonization of sorghum was high, providing high inoculum levels for the next soybean crop. These authors stated that a good arable soil has a high VAM diversity and in a test of arable soil found 26 species.

One of the major points to be illustrated by growth trials on VAM symbiosis is that despite being able to infect many plants, a VAM species is usually more specific to one type of plant. This implies that a number of VAM should be tested in a trial, to see which provides best plant growth while still being able to withstand competition in the field (Borowicz and Fitter, 1990; Chavez and Ferrera-Cerrato, 1990). Despite correct symbiotic pairing being a major factor in increasing plant growth, VAM diversity is still essential. Different VAMS can be effective at different times of the year and under different conditions. To achieve optimum growth from a crop it is therefore important to make use of different VAMs when they are the most effective, so a high VAM diversity is needed (Ellis *et al.*, 1992).

Borowicz and Fitter (1990) also simulated grazing pressure in their trial, by clipping *Lotus corniculatus L.* showing that VAM colonized grass recovered better from this disruption than did non-VAM plants. These authors also emphasized that VAM affectivity is more important than infectivity when assessing growth trials.

Turnau *et al.* (1992) working in a Polish forest, found that when the soil was enriched with P.K.N. fertilizer the plant diversity decreased at a concomitant rate to the decrease in VAM diversity. *Vaccinium myrtillus* disappeared completely, while non-mycorrhizal plants (mainly annual "weeds") proliferated. This was considered economically disadvantageous to the forestry industry.

A perceived problem with greenhouse growth trials is that the sterilization process may modify the soil used for growing the control plants. Both Cerligione *et al.* (1988) and Sanders and Fitter (1992b) tested sterilized and unsterilized soil for growth potential and found no statistical difference between the treatments. Microbial replacement after soil sterilization also had

no statistical effect on growth potential of plants in the soil (Sanders and Fitter, 1992b).

In some of these growth trials, the effect of fertilization levels, especially phosphorus has been mentioned. The advantages of VAM colonized plants growing in low fertility soils, especially with regards to P uptake will be discussed in section 1.5.

1.5 Phosphorous uptake and use by vesicular-arbuscular mycorrhizal plants

The prime advantage of VAM infection to a plant in low nutrient soils is thought to be the increased rate of phosphorus (P) uptake (Bell *et al.*, 1989). Soluble P is rapidly leached out of the soil if it is not take up by plants and thus becomes limiting to plant growth (Allen, 1991). Phosphorous is considered to be the most important nutrient for plants to extract from eroded and misused soils, with nitrogen and lime next in importance (Habte and Aziz, 1991). The advantage of VAM colonization has been postulated to revolve solely around the enhanced uptake of P. Bass (1989) is one of the exponents of this theory. Despite demonstrating that VAM infections increase P uptake markedly in low P soils, he ignores a number of VAM benefits not due to increased P uptake. Uptake of minerals other than P (section 1.6) and water relations (section 1.7) demonstrate that VAM have an effect on many other plant activities that cannot be attributed solely to enhanced P uptake.

Jakobsen (1986) showed that VAM increase plant P uptake in low P soils to levels associated with plants grown on high P soils. That it was the VAM causing the increased uptake of P was demonstrated by Wellings *et al.* (1991) who showed that there is an initial linear correlation between VAM colonization and plant P uptake levels. This linearity does not hold at high colonization levels or in older plants. McGonigle and Fitter (1988) compared P uptake to unit root length colonized and found this to be non linear in older plants. They postulated that older plants can acquire a surfeit of nutrients compared to the rapidly growing young plants. This causes the VAM association to diverge from mutualism towards parasitism and so less P is transferred to the plant.

A strange phenomenon was described by Manjunath *et al.* (1989), who discovered that non-VAM plants use P more efficiently than do VAM colonized plants. This implies that the non-VAM plants are using all the P as they absorb it, while the VAM plants absorb more than they can initially use. In non-VAM plants P will therefore become limiting in poor soil conditions long before it limits VAM plants. Ikombo *et al.* (1991) demonstrated this principle on cowpea (*Vigna unguiculata (L)* Walp.) plants, where the VAM plants showed no signs of P deficiency at a fertilization level of 10 kgs P/ ha. The non-VAM plants needed 240 kgs P/ha for comparable results. A similar scenario was depicted by Habte and Manjunath (1987) using *Glomus fasiculatum* infecting Acacia (*Leucaena leucocephala)* trees. Bell *et al.* (1989) showed that the VAM plants in their trial had maximum growth at 20 kgs P/ha, while non-VAM plants again needed 240 kgs P/ha for similar growth. With *Tetraclinis articulata* infected with *Glomus mosseae*, the optimum P level for maximum growth was found to be 6mg P/ kgs soil (Diaz and Honrubia, 1993).

The advantage that VAM plants have in P absorption at low concentrations, could be due to the greater affinity of their P absorbing site, allowing for lower levels of P to be absorbed by the VAM plants (Cress *et al.*, 1979). Increasing the P fertilization to high levels (100 kgs P/ha) does not help the uptake of P by VAM infected plants, as high P levels cause inhibition of VAM colonization. Hence low levels of P are needed by VAM colonized plants, as indicated by Bell *et al.* (1989), or very high levels of 240 kgs P/ha for non-VAM plants (very expensive fertilization). The exact level at which P starts to cause VAM inhibition is not totally clear, as different VAM species seem to have varied tolerances to P. Some of the species of VAM tested could tolerate fairly high P levels (above 50 kgs P/ha) but colonization by most was greatly reduced at this level. A second problem with high P levels is that VAM diversity decreases as the species unable to tolerate high P levels are eliminated from the system (Lamar and Davey, 1988; Waterer and Coltman, 1988; Turnau *et al.*, 1992). Martensson and Carlgren (1994) used diaspores to indicate the level of VAM inoculum in the soil during a long term field fertilization trial. High P decreased diaspore counts within the first year, but cessation of fertilization only caused an increase in diaspores after 5-14 years.

To prove that it is the VAM hyphae that are responsible for most of the P uptake in the

symbiotic associations, Li *et al.* (1991a) used a two compartment growth chamber with the compartments separated by a 40μ filter. The plant roots in the inner chamber could not penetrate the filter, although VAM hyphae could. Analysis of the P used in the outer chamber indicated that the VAMs were responsible for 76% of P uptake by the symbiosis compared to a non-VAM control.

An inexpensive method of providing P fertilization is using rock phosphate (calcium phosphate). For non-VAM plants it is not suitable, as it is only sparingly soluble and therefore large quantities are needed for good plant growth. In the case of *Leucaena leucocephala* there does not seem to be an optimum level of rock phosphate for maximum growth, the more added the more growth. With VAM colonized plants, rock phosphate increased both colonization levels and P uptake compared to non-VAM plants. A growth plateau was reached where further rock phosphate addition resulted in no further plant growth increase in the VAM-colonized plants. It appears that the VAM hyphae can either solubilize rock phosphate, or are able to absorb P from the very low soil concentrations of soluble P from rock phosphate. The main advantage of rock phosphate is that it is only sparingly soluble at a pH greater than 4 and so is not easily leached from the soil (Manjunath *et al.*, 1989).

Thomson *et al.* (1992) demonstrated that some VAM species are more tolerant to high P levels than others. Although this could be of benefit in agriculture, the authors found no evidence to suggest that some of the more effective VAM could be bred for higher P tolerance. By fertilizing a field and observing what happened to the VAM population, Johnson (1993) attempted to test the hypothesis that P fertilization supports VAM that are poorer mutualists. Fertilization caused four VAM species to decrease in number, while *Glomus deserticola* increased. Growth trials on big bluestem grass (*Andropogon gerardi*), using the fertilized and non-fertilized soils as inoculum were conducted. These indicated that fertilized soil inoculum caused less growth and inflorescence formation in the big bluestem than did the non-fertilized soil inoculum. Johnson (1993) assumed that this indicated that fertilization selects for poorer mutualists, however this author considers that all that was proved was that *Glomus deserticola* is not the best VAM symbiont for big bluestem grass. Other soils and plants could give different results. This can be illustrated by work done by Brejda *et al.* (1993) on *Andropogon gerardii*

infected separately with *Glomus deserticola* and an indigenous inoculum. Fertilization of each caused the *G. deserticola* to rapidly decrease in colonization ability, while it did not affect the indigenous VAM. These findings are opposite to those of Johnson (1993).

To make the most of any commercial venture, a cost:benefit analysis must be completed, as was done for a sorghum crop inoculated with *Glomus fasciculatum* (Raju *et al.*, 1990). The analysis indicated that the most cost effective P level was that which was just above the level where signs of P depletion were evident.

As soil conditions differ with type (calcareous, clay etc.) and pH, so the type of phosphate absorbed by plants changes. In acid soils phosphate is taken up mainly as aluminium phosphate, whereas in calcareous soils the uptake is as calcium phosphate (Young *et al.*, 1986). Phosphate uptake is increased by VAM in both these cases. Only VAM colonized plants were able to absorb P as ferric phosphate and *Glomus fasciculatum* greatly increased this type of P uptake. This again indicates that the VAM species must be selected for the prevailing conditions (Young *et al.*, 1986).

Soil P levels are not the only factor to affect VAM infection of plants. Sylvia and Neal (1990) in a combined nitrogen (N) and P level trial, demonstrated that when N levels become limiting the suppression of VAM by high P levels was greatly reduced. The authors indicated that this was probably due to a lowered resistance of the N stressed plants to VAM infection.

From the information collected it would seem certain that VAM have a major effect on plant P interactions at low P levels. This is however, not the only plant nutrient affected by the VAM symbiosis.

1.6 The effect of vesicular-arbuscular mycorrhiza on uptake of minerals other than phosphorus

VAM are also responsible for the uptake control of a number of other minerals. The

mineral second most affected by VAM colonization is zinc (Zn) (Faber *et al.*, 1990), but its increased uptake is only noticeable in nutrient deficient soils where Zn is at low concentrations. This may be the reason why Lu and Miller (1989) found that VAM have little affect on Zn uptake, when compared to benomyl (non-VAM) treated control plants. Zinc is taken up most in early plant growth and during flowering (Faber *et al.*, 1990). These would be the times when a study of Zn uptake would give the most noticeable results especially in low Zn soil.

In pigeonpea plants growing in low Zn soils, Zn uptake initially increases exponentially with an increase in VAM colonization (Wellings *et al.*, 1991). Interestingly, this research showed that *Phytophthora infestans* (a fungal pathogen) reduced the Zn uptake in high Zn soils (possibly by chelating the Zn) to levels not toxic to the plant. An odd case where a pathogen can help its host. Introduction of such a virulent pathogen into a system would not be considered appropriate by this author, as it is pathogenic to a number of crops.

Kothari *et al.* (1991) showed that VAM are responsible for the Zn translocated (as well as Cu and P) from distant hyphae to the root system. This translocation of Zn is also implied in the work of Kucey and Janzen (1987) where VAM plants have increased P, Zn and Cu levels. The increase uptake is enhanced in plants grown in larger pots, which implies that the VAM can exploit a larger soil volume than can plant roots alone. As the VAM hyphae penetrate more of the soil in the larger pots than do the non-VAM plants roots, translocation via the hyphae to the roots gives the VAM plants a greater uptake.

Another of the most important nutrients is nitrogen (N). This can be available in the soil, for plant nutrition, as either NH_4^+ or NO_3^-. VAM show no sign of being nitrogen fixing, but they do contribute to the nitrogen uptake of the symbiosis. Azcon *et al.* (1992) showed that in low P soils, nitrates were a better N source than ammonium ions for VAM colonized plants. Non-VAM plants in low P soils grew better when fertilized with ammonium ions. In high P conditions non-VAM plants had the best growth with a 50% NH_4^+ / 50% NO_3^-.

To demonstrate that it is the VAM hyphae that take up the NH_4^+ and not that VAM merely enhance the ability of roots to absorb NH_4^+, Johansen *et al.* (1992) performed a

compartmentalized growth trial. The inner of the three compartments of the trial container held the mycorrhizal plant, which was separated from the second compartment by a nylon mesh which allowed only hyphae to penetrate. The soil in the second compartment was to reduce any mass flow of NH_4^+ from the outer compartment to the inner root compartment. Finally the outer compartment contained soil mixed with the ^{15}N labelled NH_4^+. This compartment also contained N-serve, which acts as a nitrification inhibitor reducing the formation of the highly mobile NO_3^- ion . Their experiment showed that $^{15}NH_4^+$ was transferred to the roots by the hyphae. Further experiments indicated that ^{15}N was not significantly translocated to hyphae in another non-^{15}N containing compartment. This implies that plant to plant transfer of N may not occur, but further work is needed to clarify this implication.

Haystead *et al.* (1988) attempted to show that in a VAM colonized system, ^{15}N fixed by clover was transferred to companion grasses via a common hyphal network. In a compartmental experiment they showed that ^{15}N was transferred from the clover to the grass, but could not show that the VAM network was common to both plants. Hamel *et al.* (1991) working with maize and soybean plants (*Glycine max*) suggested from their findings that ^{15}N transfer from soybean to maize did not occur at the symbiotic interface. They suggested that soybean ^{15}N secretions are rapidly taken up by absorptive hyphae from nearby VAM colonized maize plants and that no significant translocation via hyphal bridges occurs. Most of this work was done with young plants that rapidly utilize any N taken up. If the N fixing plants were mature, the work may have had a different outcome. The principle of hyphal bridge transport is as yet unproved.

Iron, another major cation required by plants, is also affected by VAM. Cress *et al.* (1986) demonstrated that VAM increase the iron uptake rate of the grass galleta [*Hilaria jamusii* (Toor)] as compared to non-VAM control plants. These authors suggested that the VAM hyphae produce siderophores that cause chelation of non-available Fe allowing the VAM to absorb it and then transport some to the plant. Increased Fe uptake in VAM colonized plants was also recorded by Kucey and Janzen (1987) in field beans; Raju *et al.* (1987) in sorghum and Xinshu and Runjin (1990) in apples.

One interesting study by Graham and Syvertsen (1989) showed that chlorine (Cl) uptake

is increased by VAM (*Glomus intraradices)* infection of orange (*Citrus sinensis)*, but that little enhancement of salinity tolerance occurred. This is in contrast to the stress tolerance enhancement demonstrated by Castle (1987). At 12.5 mM NaCl, *Glomus fasiculatum* infected mung bean plants had both increased proline and total sugar levels, both before and during flowering. However, with 25mM NaCl proline was only higher at flowering, which implies that the VAM can enhance nutrition sufficiently in saline conditions to improve the stress response (Jindal *et al.*, 1993).

The uptake of many minerals is increased by VAM infections with P, potassium (K), magnesium (Mg), sulphur (S), calcium (Ca), manganese (Mn), iron (Fe), Cu, and Zn all showing this effect (Raju *et al.*, 1987). This enhanced uptake was not solely due to the plant growth increase (Pacovsky, 1986; Raju *et al.*, 1990b), as VAM and non-VAM plants of similar size were also analysed. VAMs do not merely increase the uptake of minerals, they also show control over some mineral assimilation. In the case of Mn where some soils show levels toxic to plants, inhibition of plant growth would occur. In trials with high Mn soils, non-VAM controls did show signs of Mn toxicity, but in VAM plants Mn uptake and transport were reduced. The VAM plant's root and shoot had lower Mn levels than the controls (McGee, 1987; Arines *et al.*, 1989) as was the Mn level of *Glomus mosseae* infected soybean (*Glycine max*) plants lower leaves in a study by Bethlemfalvay and Franson (1989). These VAM plants showed no signs of Mn toxicity. The authors attributed this reduction in Mn uptake to VAM plant root exudations, but other minerals are not affected in the same way making this theory speculative.

Manjunath and Habte (1988) found that the copper (Cu) level in the roots of VAM infected plants was very much higher than in the shoots, whereas levels in the control plants were more evenly distributed. The authors hypothesised that this may have an adverse effect on VAM plants at lower than optimum soil Cu levels. They assumed that leaf Cu concentrations could suffer due to the reduced translocation of Cu from roots to shoots. Copper translocation from distant VAM hyphae to plant roots was demonstrated by Li *et al.* (1991b) in a compartmentalized pot trial, where Cu was only accessible to the VAM hyphae. These authors also indicated that a higher phosphorus level in the plants is responsible for a more even root:shoot distribution of Cu in the plants.

One of the problem variables in soils is pH, as acid soils tend to reduce the mineral uptake and hence growth of plants. VAMs have a beneficial effect, as VAM colonized root systems are effective over a wider pH range than plant roots alone. This was shown by Raju *et al.* (1988), where VAM plants had good growth and mineral uptake at pH 4.5, which remained constant as pH increased to neutral. In non-VAM controls, the low pH caused reduced growth which only reached VAM plant levels at pH 5.8.

Another topical compound is carbon dioxide (CO_2). Increase in CO_2 causes an increase in the VAM colonization of *Pascopyrum smithii* and *Bouteloua gracilis*. Increase in temperature had the opposite effect as did increase in soil moisture. A simulation of possible climatic change due to increased CO_2, with temperature rising 4° and precipitation decreasing, indicated that VAM colonization would decrease. The possibility that increased temperature may increase precipitation would be even more detrimental to VAM colonization (Monz *et al.*, 1994)

1.7 Water relations in vesicular-arbuscular mycorrhizal plants

Conclusive research on this topic seems to be in flux with authors reporting conflicting results.

It appears likely that VAM increase transpiration in infected plants. Transpiration increases were shown by Allen and Allen (1986) in *Agropyron smithii*; Auge (1989) in *Rosa hybrida* and Runjin (1989) in apples (*Malus hupehensis*). However contradictory evidence was reported by Davies *et al.* (1987) in *Rosa multiflora* where the author showed a decrease in transpiration rate of the VAM roses. Graham *et al.* (1987) working with Carrizo citrange (*Poncirus trifoliata* L.) and sour orange (*Citrus aurontium* L.) showed no difference in transpiration rates between VAM and non-VAM plants. Auge and Duan (1991) suggested that the problem with early work in this field relates to the level of infection of the plants. Greater than 50% of the plant needs to be colonized before transpirational differences can be noticed. These authors suggested that a large quantity of external VAM hyphae are required to increase water transport to the roots and hence to the leaves for increased transpiration. The reasons for this increase in transpiration are thought to be :-

I. Higher stomatal conductance (Hardie, 1985; Bethlenfalvay *et.al.*, 1987; Auge, 1989).

ii. Greater hydraulic conductivity of roots and shoots (Hardie and Leyton, 1981)

iii. Chemical or hormonal signalling (Levy and Krikom, 1980; Allen, 1991; Auge and Duan, 1991)

Auge (1989) suggested that the increased stomatal conductance is due to the lowering of the osmotic potential of the leaf cells adjacent to the stomatal guard cells. This allows water to pass into the guard cells, maintaining their turgor pressure and so keeping the stomata open longer. The reduction in osmotic potential of the leaf cells may be due to greater hydraulic conductance of roots and shoots, caused by the larger amount of water absorbed and transported to the leaf by VAM plants. (Hardie and Leyton, 1981).

Solute concentrations in the symplast of well watered VAM infected roots are lower, giving a lower root turgor than non-infected roots. But in drought conditions VAM infected roots maintained a greater turgor over a range of hydration levels than did non-VAM roots. This was not due to either osmotic adjustment or alteration in cell wall elasticity, but to increased water percentage and amount of osmotic solutes in the symplastic cells. Below full turgor VAM infected roots can thus absorb water better than non-VAM roots (Auge and Stodola, 1990).

Water uptake in this process is dependent not only on the roots and the fungal hyphae, but also on the soil particle size and water holding capacity. A strong relationship exists between the soil water potential at a plant's wilting point and the growth enhancement of mycorrhizal plants (Dakessian *et.al.*, 1986). This enhanced growth is due to the exploitation of lower soil water potentials, hence giving VAM plants a lower water potential at which wilting occurs, compared to non-VAM plants (Hardie and Leyton, 1981). Soil particle size and holding capacity are also affected by VAM colonized roots. Water soluble soil aggregates seem to improve the water holding capacity of sandy to loam soils, whilst improving drainage in clay soils. Formation of water soluble aggregates is greater in VAM roots than either non-VAM roots or VAM hyphae alone, possibly due to differential slaking (Thomas *et al.*, 1993). *Glomus mosseae* colonizing soybean (*Glycine max*) increased aggregation in loam soil by 400%, while clay soil aggregates

were increased by 50%, thus the soil quality was improved by VAM infected roots (Bethlenfalvay and Barea, 1994).

Water uptake, increased root and shoot conductivity and prolonged stomatal opening, were not found to be solely related to the increase in phosphorus nutrition of VAM plants. Some other VAM association dependent control was thought to be responsible for the enhanced water relations (Dakessian *et al.*, 1986; Auge and Duan 1991). Chemical and hormonal signals created by VAM associations are not well understood. The hormones responsible for the control of water relations in the plant are either unidentified or have functions not yet fully elucidated (Saab and Sharp, 1989; Zhang and Davies, 1990). Among its many other functions abscisic acid (ABA) has been shown to be implicated in stomatal closing. Its exact function is not known, but active ion channel transport of calcium and potassium from the guard cells due to ABA has been postulated (Davies *et al.*, 1990). As VAM plant leaf cells have lower turgor, this exodus of osmotically active ions causes stomatal turgor loss before turgor loss occurs in other leaf cells, hence closing the stomata (Auge and Duan, 1991). In VAM plants the water uptake by the VAM hyphae from areas unavailable to plant roots is thought to lead to more water being taken in by VAM plants, thus maintaining high root cell turgor. As ABA is only produced by the roots of drought stressed plants, these stomata will close later than those of non-VAM plants. In pot cultures, there is little area of the pot that is not exploited by VAM hyphae. In this situation, water uptake by these hyphae may lead to dehydration of VAM plants before non-VAM plants, implying that they are less drought tolerant. This could lead to the disparity of some workers demonstrating that mycorrhizal plants are less drought tolerant than non-VAM plants (Auge and Duan, 1991). Another possible contender for the effect on water relations shown by VAM infected plants is a "positive inhibitor" postulated by Gowing *et al.* (1990), but this inhibitor has not yet been elucidated.

Transpiration is not only altered when the soil water content is reduced but also when light is decreased. The stomata of VAM infected plants close much more rapidly as light and therefore photosynthesis is decreased, reducing the transpiration loss. When photosynthesis has decreased the need for transport of nutrients by the xylem vessels is less essential therefore less transpiration is needed to drive the water flow in these vessels (Allen and Allen, 1986). An

adequate explanation for this has not been found, but has again been related to ABA levels.

Not all workers deem VAM beneficial to plants. Osonumbi *et al.* (1991) showed that VAM-infected *Acacia* and *Albizia* trees were less drought tolerant than non-VAM trees. They also stated that the lowering of drought tolerance in VAM-infected plants, could be used as a measure of the mycorrhizal dependence of the legumes they tested. Michelsen and Rosendahl (1990) suggested that the improved nutrition of VAM infected *Acacia* trees in low P soils may aid drought resistance. Though VAM plants show increased transpiration and may therefore be subject to more rapid wilting, their permanent wilting percentage and ability to recover from drought stress are enhanced (Runjin, 1989).

Additional research is required to further elucidate the effect that VAM have on the water relations of plants.

1.8 Multi-species associations of vesicular-arbuscular mycorrhizae their hosts and other rhizosphere organisms

The VAM plant association is not isolated from the rest of the rhizosphere flora and can interact in various ways with other organisms, to improve nutrient uptake. Two of the most important nutrients that must be made available to the plants are phosphorus and nitrogen. VAM have already been shown to increase P uptake while it is common knowledge that *Rhizobium* in legumes can improve N status by fixing atmospheric nitrogen. A logical research direction was therefore to employ both symbionts in the same plant to see if they would act additively.

Ames *et.al.* (1987) performed a split root experiment on cowpea (*Vigna unguiculata*) where one half of the *Rhizobium* infected roots were also infected with VAM. Nodule formation was not affected in these plants but their dry weight was increased over non-VAM controls. A similar result was reported by Kucey and Bonetti (1988). Here the authors showed that the fungicide/bacteriostat Captan, commonly applied to seeds had negligible effect on the associations. The type of phosphate added was shown to affect the system with rock P giving best

growth (Azcon-aguilar *et al.*, 1986). Hicks and Loynachan (1987) showed that VAM increased both nodulation and nitrogen fixation and that the nodule occupancy was different to a non-VAM plant of equal P status. The effect of VAM on the association with *Rhizobium* was not due solely to the increased P uptake (Ames *et al.*, 1987; Hicks and Loynachan, 1987). The tripartite association gives sufficient nutrient uptake and growth benefits for plants to show a degree of dependence on the association. This dependence is directly related to the level of growth and nutrient uptake enhancement afforded by the VAM and *Rhizobium* (Pacovsky *et al.*, 1991).

The first years crop of a legume (*Hedysarum coronarium)* infected with VAM and *Rhizobium,* behaved similarly to a high P fertilized crop in increasing the N fixation. The second year crop showed that not only do the VAM improve P uptake, but also ammonium ion uptake and translocation from the rhizosphere soil to the plant (Barea *et al.*, 1987). The effect of improved nutrition on carbon dioxide fixation afforded by both symbionts has been shown to be additive. This increase is not parallelled by fertilization with high levels of P and N (Brown and Bethlenfalvay, 1988). For such a system to show optimal legume growth, the two symbionts must be co-selected for the plant as strains of both symbiont have a degree of host selectivity (Thiagarajan *et al.*, 1992).

Other important multi-species bacterial associations also occur with VAM and various soil bacteria. Perhaps the most important is with phosphorus solubilizing bacteria. The solubilized P generated by the phosphorus solubilizing bacteria can be taken up rapidly by VAM plants, while VAM may also help in the process of solubilization (Kucey, 1987). Piccini and Azcon (1987) suggested that P solubilizing bacteria increased the growth of both VAM and non-VAM plants but that VAM plants had shown the greatest increase. Shivaram *et al.* (1987) noticed that VAM increase the P and N nutrient availability to, and growth of, both plant species in a mixed grass-legume field. In contrast Lee and Bagyaraj (1986) found that P solubilizing bacteria had little effect on the shoot dry mass of VAM infected tomato plants.

VAM also enhanced the persistence of *Azospirillum*, a nitrogen fixing bacterium, which may increase the nitrate content of the soil, which then becomes available to mycorrhizal plants (Pacovsky, 1989). Not only do VAM plants seem to encourage nitrogen fixing bacteria, they

produce conditions favourable to the growth of bacteria antagonistic to both fungal plant pathogens (eg: *Fusarium* spp.) and bacterial pathogens (eg: *Pseudomonas)* (Secilia and Bagyaraj, 1987). This is probably the reason for the observation made by Vančura *et al.* (1989) that VAM plants had a different microflora than non-VAM plants. Soil bacteria have different effects on different VAM/plant associations hence the growth enhancement is variable. Some bacteria such as *Klebsiella pneumoniae* may increase the infectivity and spore germination capabilities of the VAM (Will and Sylvia, 1990).

In the same vein but moving to fungi, yeast cultures co-inoculated with VAM, increased both VAM colonization and plant dry mass (Singh *et al.*, 1991). Conversely *Bipolaris sorokiniana* inhibits VAM colonization of plants (Thompson and Wildermuth, 1989) possibly in a mutually exclusive manner similar to that caused by nematodes.

VAM help to reduce nematode infestations in plants and Carling *et al.* (1989) postulated that this was due to the improved P nutrition of VAM plants making them more resistant. However, Ingham (1988) demonstrated that nematodes will not infest regions of plant roots that have already been infected by VAM. This infection is mutually exclusive as VAM cannot infect regions already infested by nematodes. High levels of infection by VAM reduces the area available to nematodes thus reducing infestation.

An interesting negative association is between VAM plants and bettongs (a marsupial) which search out and eat VAM sporocarps formed after fires. This greatly reduces the propagule density of VAM in the region of the fire thus reducing the selective advantage of VAM dependent plants (Claridge, 1992).

VAM can form multi species infections in the same plant (Allen, 1991). Boudarga *et al.* (1990) has shown that by infecting ectomycorrhiza forming plants with VAM first, the plants can form both associations in the same plant. The VAM and ectomycorrhizae coexist and their advantages to the plant are additive.

1.9 Uses of vesicular-arbuscular mycorrhiza in erosion and reclamation work

Reclamation of disturbed and eroded soils has proved to be both a difficult and costly operation. In order to determine if VAMs can help in the revegetation of these lands, clarification of the effects of erosion and disturbance processes on the soil and microflora were needed.

Mild erosion of sloping ground, was shown by Day *et al.* (1987) to reduce the amount of VAM colonization and propagule density even though ground cover appeared to have been unaffected. This VAM reduction indicated that a large portion of the VAM propagule bank is close to the surface. A byproduct of the erosion was that deposition of soil and eroded VAM propagules on the foot slopes altered the soil type, causing a VAM diversity shift.

The study of natural erosion is extremely time consuming, necessitating the use of simulated erosion. Evans and Miller (1988 and 1990) demonstrated that tilling disturbance of previously zero tilled soil cores (transferred to plastic cylinders) had a deleterious effect on maize plants (*Zea mays*) growing in them. A decrease in P and Zn uptake indicated lower VAM effectivity in the disturbed system, compared to non-tilled controls. Tilling did not affect P and Zn uptake of non-VAM maize controls. The authors considered that the disruption of the external hyphal network of the VAM was responsible for the decrease in nutrient uptake.

To determine how long it took to negate the effects of soil disturbance, Fairchild and Miller (1990) prepared soil cores which had been artificially disturbed and planted them with maize. Using a three week cycle, half the soil cores were disturbed and replanted with maize, while the control cores were replanted without soil disturbance. After three cycles, the P uptake of the maize growing in control soil cores was significantly higher than in the disturbed soil cores. The authors proposed that soil disturbance caused a breakdown in the mycelial network of the VAM symbionts thus reducing their efficacy in the symbiosis. Infection rates and levels were greatly reduced in the disturbed cores, indicating that infection from hyphae of established VAM

infected plants may be of importance in the rapid infection of seedlings. When annual plants that colonise disturbed land have a similar mycorrhizal flora to the native plants, revegetation with the native plants can give a rapid shift to native rhizosphere conditions. This can take as little as five months, as in the work of Nelson and Allen (1993) with *Stipa pulchra*. To speed up the process, some weed control measures may be necessary.

Jasper *et al.* (1987) also found that soil disturbance reduced VAM infectivity and found that in stored soil, VAM propagules numbers decreased with time stored. Revegetation of these soils only resulted in an increased VAM spore bank after about 4-5 years. Revegetation is only possible if soil fertility is high enough, therefore soil amendment may be needed. This was the case on an iron mine site, where low P amendment was needed to facilitate the growth of mycorrhizal *Acacia pyrifolia* (Jasper *et al.*, 1988).

Soil disturbance not only occurs from weathering processes, fire acts as a disturbance to the soil. Fire does not only destroy the aerial vegetation, as VAM mycelial networks and spore banks are also adversely affected. A reduction in VAM colonization due to fire was noted by Klopatek *et al.* (1988), with dry soil being affected more than wet soil. Other forms of soil disturbance are caused by animals such as gophers (*Thomomys talpoides*), which bring subsoil to the surface, thus reducing the amount of VAM propagules in the soil of gopher mounds (Korde and Mooney, 1987).

Habte (1989) attempted to simulate severe erosion in an oxisol by incremental removals of surface soil, after which the density of VAM propagules remaining in the soil was assessed. Removal of less than 7.5cm of soil resulted in a reduction in VAM propagules without adversely affecting VAM colonization ability or symbiotic effectiveness. In fact VAM activity was stimulated, possibly due to the removal of an antagonistic biotic factor, present in this top layer of soil. As more than 7.5cm of soil was removed, VAM colonization and effectiveness was successively reduced. After 30cm of soil had been removed VAM activity practically ceased and could only be effectively restored by VAM propagule replacement and amendment of the soil with nutrients. Phosphorus addition was essential in this process (Habte *et al.*, 1988; Habte 1989).

Attempts to revegetate mine spoils have met with mixed success. Revegetation of a phosphate minespoil, using various native woody plants (pre-inoculated *with Glomus etunicatum*) showed no improvement in growth or survival over the non-VAM controls (Sylvia, 1990). As control plants had become VAM infected these conclusions were not valid. The author considers more work was needed. Stahl *et al.* (1988) had difficulty in revegetating coalmine spoil soil. The native VAM population was effective in colonizing the Wyoming big sagebrush (*Artemisia tridentata* subsp *wyomingensis)* in the native undisturbed soil. However, the characteristics of the reclamation soil had changed so much that colonization by native VAMs was very limited. Lindsey *et al.* (1977) had partial success when attempting to rehabilitate a coalmine spoil. Rabbitbrush (*Chrysanthamnus nauseosus*) showed increased growth and survival with VAM (*Glomus fasiculatus)* inoculation, but the VAM had no effect on maize (*Zea mays)* or fourwing saltbrush (*Atriplex canescens).* Contaminants such as heavy metals can necessitate the selection of both plant and VAM species for revegetation, as the soil change makes it impossible for the native vegetation to survive (Pfleger *et al.*, 1994)

An important feature indicated by the work of Loree and Williams (1987) was that early topsoil replacement on reclamation sites allows plants to become VAM colonized more rapidly than sites not topsoiled. The growth rates and VAM propagule density of the topsoiled sites approached that of adjacent rangeland within five to seven years. Working with *Artemisia tridentata.* Allen *et al.* (1993) found that fresh non-sterile soil inoculum was the best inoculum for improving plant growth. VAM spores or bacterial flora inoculum alone had little effect on plant growth.

If VAM are to be used in reclamation work, methods of amplifying them must be used. Al-Raddad (1995) showed that a series of plants must be tested to see which gave the best spore production for the VAM species to be amplified. The time of harvest must also be selected to achieve optimum spore yield for the symbiosis. Not only does the plant and harvest time affect the VAM growth, the potting mixture must also be selected for optimum VAM growth (Estaun *et al.*, 1994).

In order to improve revegetation methods for disturbed land it is necessary to understand

successional processes occurring on disturbed land. An original hypothesis(Roux, 1969; Allen, 1991) was that on disturbed land non-mycorrhizal annual plants with extensive root systems initially invaded the land. These plants were rapidly able to take up the nutrients made available in the disturbed land and thrive. As these nutrients were exhausted plants with large root systems together with mycorrhizal infections were thought to utilize nutrients more efficiently and so succeed the annuals, which then decline in number. As the nutrient base of the soil diminished, plants that were increasingly mycorrhizal became dominant.

To test this hypothesis Allen and Allen (1988) planted a VAM free subsoil plot with *Salsola kali* (pioneer annual non-mycorrhizal plant) and a number of mycorrhiza forming grasses (*Agropyron spp.*). Inoculation of part of the system (with native mycorrhizal flora) accelerated the succession from *S. kali* to *Agropyron spp.*, with the number of the pioneer annual decreasing by up to a half. The grasses did not respond as expected to this situation, as their density and growth rate decreased. The authors considered that the annual plants protected the grasses from the harsh wind conditions, exposure to which caused an decrease in grass plants. They found that inoculation with VAM actually set the successional process back, instead of accelerating it as had been found by Janos (1980).

In May 1980 a natural disaster provided a whole mountain of disturbed land to study the succession process, this being the eruption of Mount St Hellens, in the United States. After the eruption, Allen *et al.* (1992) found that three distinct areas were formed on the mountain:-

A. Pumice plain: where 100m of landslide material and more than 20 meters of sterile pumice covered the oldsoil.

B. Blast zone: where 45cm of blast and tephra deposits covered the land.

C. Ashfall regions: with ash carried from the eruption covering the land 8-15cm deep.

Rapid re-development of both VAM and ectomycorrhizal plants had occurred in this region by 1982, as was expected after two years. This re-colonization was facilitated by the exposure of seedlings to the VAM propagule bank available in the oldsoil below the ash. In the high ashfall areas this infection was increased by the action of gophers (*Thomomys talpoides*)

bringing old soil to the surface.

The blast zone also re-colonized rapidly with both VAM and ectomycorrhizae, due to gopher activity, erosion exposing the old soil propagule bank and roots growing down into the oldsoil region. The ash/soil VAM propagule density had stabilized by around 1985.

The most interesting area was the pumice plain. Here the facultative VAM plants *(Lupinus lepidus)* were first to re-establish. A single specimen found in 1981 formed a clump of 40,000 individuals by 1989. These plants first became VAM infected in 1983 with the introduction of rodents that carried VAM propagules into the area. *Lupinus lepidus* numbers decreased to 8,300 by 1990, but by this stage 27 other facultative VAM plant species had established. The revegetation of the pumice plane was very localized and VAM activity was very variable during this first ten years after eruption. Willows (*Salix sitchensis*) growing near a spring, had not become ectomycorrhizal after five years of growth, but incomplete ectomycorrhizae were found on pines establishing on the pumice plain. One reason proposed for the ectomycorrhizae taking longer to establish than VAMs, was that ectomycorrhizae need a higher organic content in the soil (Read, 1984). A more likely reason is that the ectomycorrhizal spores that infected the pines are monokaryotic and don't form proper ectomycorrhizae. Dikaryotic hyphae are needed to form clamp connections and also an effective Hartig net encasing the roots. (Wong *et al.*, 1989). The chance of two compatible spores from infected forests 5km away germinating next to each other, mating and hence forming a clamp connection, were considered by Allen *et al.* (1992) to be fairly low. So ectomycorrhizal infection was not as rapid as the rodent propagated infection of VAM.

A full successional theory has not yet developed and considerably more work is needed to fully understand the revegetation of badly disturbed land (Allen *et al.*, 1992).

1.10 Fungicidal suppression of vesicular-arbuscular mycorrhiza

Vesicular-arbuscular mycorrhiza are generally considered to be of benefit to the plants

that they colonize especially in low nutrient soils. Many of the systems and chemicals used to remove plant pathogens from the soil also have a deleterious effect on the fragile VAM/plant symbiosis. Fumigation with metataxyl is often used to kill root pathogens, especially when preparing trial plots for use as controls, but VAM associations are also destroyed. After fumigation re-inoculation with VAM is necessary to increase plant growth (Afek *et al.*, 1991). These authors found that solarization was preferable for experimental purposes as no lasting effects occurred. Solarization is the process whereby the soil is covered with plastic thereby increasing the soil temperature, to effect sterilization. Fumigation can also lead to other problems. In fumigated tamarillo (*Cyphomandra betacea*) plants, Cooper (1987) found that root knot nematodes severely depressed plant growth. This decrease could be offset by inoculation with VAM which cause a reduction in nematode infestation.

Sulphur dioxide (SO_2) is also commonly used to fumigate soils but has the side effects of acidifying the soil if used in high doses. Low levels of SO_2 cause a decrease in VAM populations (Clapperton *et al.*, 1990) and probably caused the deleterious effect on a mixed grass prairie adjacent to industries producing sulphur based emissions. These emissions caused a reduction in VAM and an acidification of the soil both of which cause a reduction in plant growth (Clapperton and Parkinson, 1990). Pepper plants (*Capsicum annuum* L.) showing signs of collapse due to root rot, were fumigated with methyl bromide which caused fumigation-induced stunting. This stunting was attributed to VAM death caused by the fumigation. As the soil was very P-sorptive, P levels high enough to provide full growth of peppers could not be reached without regular addition of high levels of soluble P to the irrigation water. This problem could only be solved efficiently by re-infecting the peppers with VAM which increased the plants P uptake (Hass *et al.*, 1987).

Some of the chemicals used on crops can effect the VAM associations, for instance the herbicide cyanazine (which inhibits photosynthesis) has an effect on VAM at high doses. This was thought to be due to the reduced growth of the plants caused by the herbicide, as low levels of herbicide have little effect on the VAM association. With low levels of herbicide the VAM colonized plants showed less deleterious effects (Garcia-Romera *et al.*, 1988).

Fungicides such as chlorothalonil (used to reduce fungal root pathogens) have considerable effect on the VAM symbiosis, as the fungicides are not pathogen specific. The level of this fungicide should not be greater than 50μg/g soil if VAM enhanced P uptake is to remain unaffected (Aziz *et al.*, 1991). Both benomyl and captan decrease VAM growth which can be detected by the decrease in metabolic activity associated with the growth decrease. After fungicide application, a decrease in the metabolic rate of the VAM was detectable (by a succinate dehydrogenase assay) within a few days of treatment (Kough *et al.*, 1987).

The fecundity of VAM colonized plants can be tested using benomyl. *Vulpia ciliata* plants in two field sites in eastern England were treated with benomyl. The first site had a high level of VAM colonization and plant fecundity decreased concomitantly with the decrease in VAM colonization, due to the benomyl. The second site which had a low VAM colonization had an increase in fecundity. This was probably due to the increased plant growth due to the reduction in pathogens, offsetting any reduced growth due to the decrease in VAM. This work indicated that random use of fungicides may well have detrimental rather than beneficial effect on plants, especially when no severe disease problem is evident (Carey *et al.*, 1992). When benomyl is used as a fungicide on mycorrhizal crops, to reduce fungal pathogens, it can cause temporary reduction in VAM colonization. This reduction is most felt by seedlings when growth is rapid and nutrient stores are low (Borowicz, 1993). The effect this would have on successional processes, where seedling growth decrease could result in reduced competitiveness is open to speculation.

The time of application of fungicides can have profound effects on the VAM population, as was demonstrated by Vijayalakshmi and Rao (1993). Treatment of *Sesamum indicum* L. with copper oxychloride ('Blitox') or carbendazim thirty days after sowing, greatly reduced VAM colonization. However, when the soil was drenched with either Blitox or Captan before sowing, VAM colonization was slightly enhanced.

Benomyl can be effectively used in trials to set up non-VAM controls, where soil sterilization might cause changes detrimental to plant growth. Benomyl also leaves most microflora intact, producing a more natural control (Fitter and Nichols, 1989).

1.11 Genetic and transformed plant associated topics

Genetic variations of both plant and VAM symbionts can cause differences in the effectiveness and infectivity of VAM fungi for their host. Field soil experiments by Sanders and Fitter (1992a) using a mixed VAM inoculum to inoculate various plant species, did not prove or disprove the hypothesis that multiple infections of a single plant can occur. This was due to the fact that an increase in spore number of only one VAM species could be detected over the experimental period. This did not disprove the multiple infection hypothesis, but showed that only one infecting species of VAM sporulated in the time period.

As the morphology of VAM hyphae varies with host species, studies of root infection cannot show decisively that multiple infection of a host has occurred. What this work did show was that a degree of selectivity was present in the field soils, as *Plantago lanceolota* caused an increase in *Glomus constrictum* spores while *Holcus lanatus* and *Rumex acetosa* increased the number of *Aculospora laevis* spores. The authors indicated that VAM respond differently depending on the host species. This could result in selective pressures that would favour a specific host-VAM association.

Genetic differences between cultivars of the same plant can have significant effects on the capability of VAM to infect the plant. Using three pea cultivars *(Pisum sativum* L.), inoculated with three different VAM species, Estaun *et al.* (1987) found that although the infectivity of the three cultivars to the VAM was similar, the effect of the VAM on the growth of the cultivars varied widely. Similar result were obtained with soybean [*Glycine max* (L) Merr.] cultivars where there was a distinct difference in the infectivity of VAM for certain of the cultivars, although this was not related to effectiveness (Heckman and Angle, 1987). Half-sib families of alfalfa (*Medicago sativa* L.) also showed significant differences in colonization by *Glomus versiformus.* There is a potential to improve the colonization of alfalfa by plant siblings selection (Lackie *et al.*, 1988). Kesava *et al.* (1990) demonstrated a similar result with groundnuts (*Arachis hypogaea* L.) where there was a linear relationship between increase in VAM colonization and pod yield. The significant differences in VAM colonization between the groundnut cultivars indicates a possibility to improve pod yields by selective breeding for high VAM colonization.

The effect of VAM species can also affect the growth of a specific plant as was demonstrated by Riech (1988), where five VAM species were used to inoculate an apple cultivar. *Glomus intraradices* and *Glomus epigaeum* gave the greatest plant dry weight of the five VAM species used.

Interestingly, selection of maize *(Zea mays)* for resistance to fungal pathogens has had the adverse effect of also reducing the plant's ability to form VAM associations. This leads to the plant needing a larger root system and causes slow maturing of the pathogen resistant plants (Toth *et al.*, 1990). Further work is required on the effect that this VAM decrease has on yield and P requirements of pathogen resistant plants. Modern varieties of wheat suffer from a similar problem, in that hybridization of the four ancestral genomes, has eliminated the dependence of wheat for VAM. Of the four diploid ancestral genomes three had VAM dependent strains whereas modern day tetraploid plants have only one VAM dependent genome. This indicates that hybridization may decrease the plant's dependence on VAM and decrease VAM infection (Hetrick *et al.*, 1993)

To further complicate the issue, it would appear that VAM of the same species can have genetic variation dependent upon the environment from which the population was derived. This was shown in the varying effect that different populations of *Glomus mosseae* had on the growth of *Melilotus officinalis* plants when grown under the same conditions (Stahl and Christensen, 1990).

From the work so far completed, it would appear that there is great scope for co-selection of VAM/host combinations to improve the productivity of many plants and that plant breeding for better VAM colonization is possible.

The hypothesis that VAM represent a carbon cost to plant hosts which may be controlled by the plant genome was advanced by Graham and Eissenstat (1994). The VAM colonization control would be dependent on the benefit afforded by the VAM, compared to the carbon outlay required from the plant to sustain the association. If carbon outlay by the plant was too great then reduction of VAM colonization would be initiated by the genome. More work is needed.

Any breeding for increased VAM colonization would be dependent on the soil conditions and nutrient availability. Breeding for VAM colonization is only viable when the benefits of the VAM symbiosis outweigh the carbon cost to the plant.

An topical use for VAM is in the conservation of threatened plant species such as some of the indigenous Hawaiian plants. Cultivation of these species was found to be difficult and infection with VAM resulted in improved survival rates (Koske and Gemma, 1995).

A genetic manipulation of plants that could have far reaching implications on the study and production of VAM colonies is the introduction into plant roots of the root inducing plasmid genes from *Agrobacterium rhizogenes* (Becard and Fortin, 1988). These plasmid genes induce the roots to grow far more rapidly and abundantly than in the non-transformed plant. In this way cultures of root organs can be developed that can produce axenic VAM cultures when infected. These symbiont cultures can be maintained for long periods and axenic roots can be amplified using tissue culture techniques. This produces an ample supply of axenic roots for the study of VAM spore germination. In addition root attachment mechanisms have proved consistent and repeatable (Becard and Fortin, 1988)

The study of initial events in VAM infection and early plant colonization using transformed roots was made possible due to the stability of the system. Elucidation of the change in nutrient dependence of growing germ tubes, from spore to host dependence was made possible (Becard and Piche, 1989). The same authors also determined that the difference between the early events of infection of a mycotrophic and non mycotrophic plant root system occurred at the root attachment stage. Mycorrhizal plants allowed penetration of hyphae whereas the non-mycotrophic did not (Becard and Piche, 1990). A similar system has been used by Mugnier and Mosse (1987) to study nutrient exchange between the symbionts. These authors suggested that this system may be harnessed to provide large quantities of axenic VAM propagules for use in large scale inoculation of crops. Previously amplification was only possible on whole infected plants. This seems a good method of producing the inoculum for infecting micro propagated plants such as avocado (*Persea americana* Mill.), which benefit from VAM infection during transplantation to *ex-vitro* conditions. (Vidal *et al.*, 1992).

Very little work has been done on the DNA of mycorrhiza, again because of the inability to grow axenic cultures *in vitro*. Most work has been done on the DNA of spores, where a model by Burggraaf and Beringer (1989) assumes the nuclei form a single layer around the periphery of the cytoplasm. This model give an estimate of nuclei in *Gigaspora gigantea* of 27,000, much higher than the 2,600 estimated by Cooke *et al.* (1987) from direct observations of crushed spores. This model would appears to indicate excessively high numbers of nuclei, even from their own work with *Glomus caledonium*, visual estimates were over 1000, while calculated values would be around 9,000. More work is needed on the distribution of nuclei in spores.

The amount of DNA in spores is in the range of 10-60 ng , with *Glomus versiforme* having between 13-30ng and the larger *Scutellospora persica* having 43-49ng per spore (Burggraaf and Beringer, 1989). These authors, using their model for spore nuclei arrangement, estimated that *Scutellospora persica* would have about 1.7pg DNA per nuclei.

Genetic identification of VAM DNA would appear to be an advantageous method for distinguishing between VAM types and species. VAM spores contain large numbers of nuclei (Viera and Glenn, 1990) which could be used as a DNA source with which to attempt VAM identification. Identification of ectomycorrhizal fungi using restriction fragment length polymorphism (RFLP) of ribosomal RNA genes has shown that identification of fungal DNA is viable (Henrion *et al.*, 1992). Identification of VAM DNA in a similar manner would also be possible. Perhaps the use of the recently developed randomly amplified polymorphic DNA (RAPD) method (Newbury and Ford-Lloyd, 1993) would be quicker and possibly more definitive once perfected. By the correct choice of short PCR primers (which can have a wide variety of nucleotide base sequences), small differences in DNA makeup can often be distinguished .

This thesis is divided into three main projects :-

1. Evaluation of the effect that varied fertilizer regimes have on vesicular-arbuscular mycorrhizal spore counts with regards to quantity and diversity of spore populations. The spore population is taken as an indicator of the level of mycorrhizal infection in plants grown on the various fertilizer regimes.

2. Identification of VAM directly from spore DNA, employing the randomly amplify polymorphic DNA technique (RAPD) to amplify short lengths of the DNA. This is accomplished by completing a polymerase chain reaction (PCR) on the DNA using a single short primer (about nine bases long) to initiate chain formation.

3. A greenhouse trial to ascertain what effect a variety of different vesicular-arbuscular mycorrhizal floras have on the growth and nutrient uptake of the two grasses, *Themeda triandra* and *Trachypogon spicatus* (indigenous to South African grasslands), when grown on coal mine spoil topsoil.

CHAPTER 2

The effect of varied fertilizer levels on the mycorrhizal spore count of a long term fertility trial

2.1 Introduction

Since prehistoric times, endomycorrhizae or vesicular-arbuscular mycorrhiza (VAM) have been shown to form associations with plants. For example, Stubblefield (1985) suggested that fossil chlamydospores from the lower Devonian of the Palaeozoic Era are of VAM extraction. It is possible that VAM co-evolved with terrestrial plants as they first colonized the land, with the VAM symbiont being responsible for the absorption of the very low levels of certain nutrients (*eg:* phosphorous (P)) in the early soils. Some of these nutrients were then passed to the plant in exchange for fixed carbon products thus allowing for survival of both symbionts under the harsh conditions (Mallock, 1975; Pirozynski, 1981).

Nutrient uptake control, caused by VAM has been reported for numerous elements such as copper (Kothari *et al.*, 1991), iron (Cress *et al.*, 1986), manganese (McGee, 1987) and zinc (Faber *et al.*, 1990). However, the most important agricultural fertilizers are phosphorus, nitrogen, potassium and lime. Of these four nutrients, phosphorus (P) is probably the most important to the mutualistic association. One of the anomalies of the VAM plant association is that it may be adversely affected by the phosphorus that appears to give the association its main driving force. Mycorrhizal plants have been shown to take up P from low P soils significantly better than non mycorrhizal plants (Jakobsen, 1986; Ikombo *et al.*, 1991; Habte and Aziz, 1991; Bell *et al.*, 1989). Louis and Lim (1988) showed that two strains of *Glomus clarum* had different effects on soybean growth and nodulation at low P levels. At high P levels the effects were similar but VAM growth was decreased. This result implies that VAM have a preference to host and P level. The type of P (available or fixed) also has an effect on mycorrhizal growth (Young *et al.*, 1986).

Raju *et.al.* (1990a) ran a cost / benefit analysis on the sorghum / *Glomus fasciculatum* association. This showed that the low P mycorrhizal regime gave the most favourable results in this analysis. The added advantage was that the cost of production of the low P mycorrhizal plants was less than the non mycorrhizal high P plants. However, this research did not indicate how low the P should be for best growth. Waterer and Coltman (1988) showed that increasing the amount of inoculum caused increased infection, only at medium to low P levels. If the P level was either very low, or high, increase in the amount of inoculum did not increase infection. This result indicates that there is an optimum level of P for the greatest mycorrhizal growth. When comparing untreated fields and fields treated with high levels of P, there appears to be a change in the mycorrhizal flora over a 6-8 year timespan (Thomson *et.al.*, 1992). The authors assumed that this change indicated that some VAM are able to withstand high P levels better. However, no P tolerant strains were found as all VAM decreased with increasing P levels.

Most research suggests that P uptake is increased with VAM and that high levels of P inhibit the effect of VAM. De Miranda and Harris (1994) hypothesised that this was due to poor spore germination or poor mycorrhizal growth. They used the equivalent of 10 levels of phosphate from 0 - 250μgP/g of soil, to make up agar plates for germination and hyphal extension trials. Their results showed that both germination and hyphal extension are affected by P levels. An initial increase in germination occurred up to 12.5μgP/g soil beyond which germination steadily decreased. Similarly the hyphal extension of VAM increased with P increase up to a peak of 37.5μgP/g of soil after which the amount of extension gradually decreased. In field trials the effect of P on VAM has not shown the same consistency as in greenhouse trials. An interesting contradiction was shown by Still (1991). In two fields of similar soil type, a well tended field of high P had far higher VAM levels and diversity than did a low P field.

Little information is available on the effects of varying lime and potassium levels on the effect or level of VAM infection. Lack of nitrogen has been shown to negate the decrease in VAM infection at high P levels (Sylvia and Neal, 1990), but no reason is given for this effect. More work seems to have focused on the type of nitrogen assimilated by plants and mycorrhiza. Azcon *et.al.* (1992) and Johansen (1992) both suggest that VAM absorb nitrates while the roots prefer ammonium salts. The authors did not indicate if any advantages were gained from these

two methods of nitrogen assimilation.

Research to date has left the following questions unanswered:-

a. What is the effect of varied P levels on mycorrhizal colonization in a long term field trial ?
b. Do other normally used fertilizers such as potassium nitrogen and lime have any effect on VAM ?
c. As P affects VAM species differently, do high P levels cause selective pressures that result in a decrease in the diversity of VAM ?

In an attempt to answer some of these questions, a long running fertility trial at Kleinkopje Mine at Witbank, South Africa was visited to collect soil samples for mycorrhizal spore content analysis.

2.2 Materials and Methods

2.2.1 The Trial Site

The fertility trial plot is at Kleinkopje Mine and is run by the Amcoal Environmental Services research group. The trials main purpose is to determine the effect of various levels of fertilizer on the growth of four selected grasses [Lucerne (*Medicago sativa)*, *Eragrostis teff,* Rhodes *(Chloris gayana*) and Smuts (*Digitaria eriantha*) grasses]. This trial had been running for ten years prior to sampling and by this stage Smuts grass (*Digitaria eriantha*) had become the dominant species on all plots. The plot has a random complete block design with three replications, each containing 14 sub plots of 10m by 7m. Drainage was sufficient to stop permanent water from forming, which can adversely affect VAM. Interplot contamination was not a problem as the plot had only a very slight slope as seen in the photograph (Figure 2.1).

Figure 2.1 **The fertility trial plot at Klienkopje Mine Witbank.**
With from left to right, my supervisor Dr W. A. Cress, myself (Alan Lee), and Tracy, a member of the Amcoal Environmental Services team.

The layout of the plots fertilization regime for the three replications is shown in Figure 2.2

In Figure 2.2 the fertilizers not indicated in the block were applied at the 2 level of fertilizer indicated in Table 2.1. For instance, this means that plots marked K3 in Figure 2.2 have the regime K3, P2, N2, L2, only the fertilizer deviating from level 2 is indicated. The exceptions to this are the two unfertilized plots, marked as 0 in Figure 2.2, that act as controls. Plots marked N2 have the fertilizer regime N2, K2, P2, L2 and are used as the level 2 sample for each of the fertilizers.

72m

[1] N3	[12] 0	[13] L3	[24] L1	[25] K1	[36] P1	[37] K0	A
[2] P0	[11] P3	[14] 0	[23] N1	[26] N2	[35] L0	[38] K3	
[3] P1	[10] N3	[15] L0	[22] L3	[27] N1	[34] 0	[39] K1	B
[4] N2	[9] K3	[16] P0	[21] K0	[28] 0	[33] L1	[40] P3	
[5] 0	[8] L3	[17] L1	[20] 0	[29] P0	[32] K0	[41] P1	C
[6] L0	[7] K1	[18] K3	[19] N2	[30] N1	[31] P3	[42] N3	

42m

Figure 2.2 **Layout of the fertility trial plot at Kleinkopje Mine, Witbank.**

Letters A, B and C, on the right of the table, indicate the three replicates of the growth trial. The individual treatments are indicated by the symbols L = lime, K = potassium, N = nitrogen and P = phosphate, while 0-3 represent increasing fertilizer concentrations specified in Table 2.1.

Fertilizer levels are shown in Table 2.1.

Table 2.1 **Fertilizer levels used on the Klienkopje Mine fertility trial plots**

Phosphorus	P0 =	zero	Potassium	K0 =	zero
	P1 =	80 kgs/ha		K1 =	100 kgs/ha
	P2 =	160 kgs/ha		K2 =	200 kgs/ha
	P3 =	320 kgs/ha		K3 =	300 kgs/ha
Lime	L0 =	zero	Nitrogen	N1 = 100 kgs/ha	
	L1 =	1.1t/ha		N2 = 200 kgs/ha	
	L2 =	3.3t/ha		N3 = 300 kgs/ha	
	L3 =	5.5t/ha			

P was applied as superphosphate, K as potassium chloride, lime as agricultural lime and N as ammonium nitrate.

From this site, two samples were taken from each of the 42 plots. Samples were taken from the central region of the plot to minimize effects of fertilizer run over. To ensure uniformity of samples, all samples were taken from the rhizosphere soil of the dominant Smuts *(Digitaria eriantha)* grass. For the purpose of this trial, the samples were taken as being separate entities, therefore giving a total of six separate samples being taken for each of the fertilizer levels. Sampling was completed during the dry season (17-7-1993), when the plants had already died back and VAM sporulation was complete. The soil was sufficiently dry to allow for long term storage without noticeable degradation of the spores contained in the samples.

2.2.2 Spore separation

Spore separation was completed using wet sieving and then sucrose step gradient centrifugation.

2.2.2.1 Wet Sieving

A modified version of that described in Appendix 1 was used. For this spore separation, only 500g of soil was used and the 250μm filtration step was embodied. As all spore sizes were to be counted in this section, the 250 μm filtration step was considered superfluous. The filtered particles from the 70 μm filter were gently washed into a beaker. The contents of the beaker were then re-sieved through a small piece of mirror cloth, washed into a vial and made up to 20 ml. This coarse spore suspension was then stored in a refrigerator at ±4°C for two days to allow the spores to imbibe water. Most of the dry spores from the soil floated, so the imbibing of water helped in the next step, sucrose layer centrifugation.

2.2.2.2 Sucrose Layer centrifugation

Five millilitres of a 70% sucrose solution was pipetted into a glass centrifuge tube and then

5mls of the coarse spore solution was overlaid on top of the sucrose. The test tubes were then centrifuged at 4000 rpm for 5 min in a Hettich universal K2S. Most spores collect at the interface but a number still float and so the entire upper layer is collected. This is then filter washed to remove the sucrose and washed into a vial and made up to 20mls with 5% Triton X100 (to stop clumping of spores). The small amount of debris that is transferred in the process is mainly organic material and does not mask the spores during spore counting. Heavier soil particles sink through the sucrose during centrifugation.

2.2.2.3 Spore Counting

A 10cm diameter Petri dish lid was marked with 36 * 1cm^2 squares and 1ml of the spore solution was pipetted into the Petri dish. The solution was swirled to give even distribution of the spores and then 10 randomly selected squares were counted. This process was repeated 5 times for each of the samples. Five different types of spores were observed in the samples which were labelled A - E.

A = Small golden to light bronze(with age), 85 -120μm diameter. (*Glomus sp.*)

B = Small black spore, 85 - 120μm diameter. (possibly *Acaulospora sporocarpa*)

C = Clear to very light golden spore, 85 - 120μm diameter. (*Glomus sp.*)

D = Large golden spore, 180 -300μm diameter. (*Gigaspora sp.*)

E = Large brown - bronze, 180 -250μm diameter. (*Scutellispora sp.*)

These spores are shown in Figure 2.3.

One of the characteristics for identification of mycorrhiza is the way in which the spores are attached to the mycorrhizal hyphae. As the spores in these samples had already been released from the hyphae full identification was not possible.

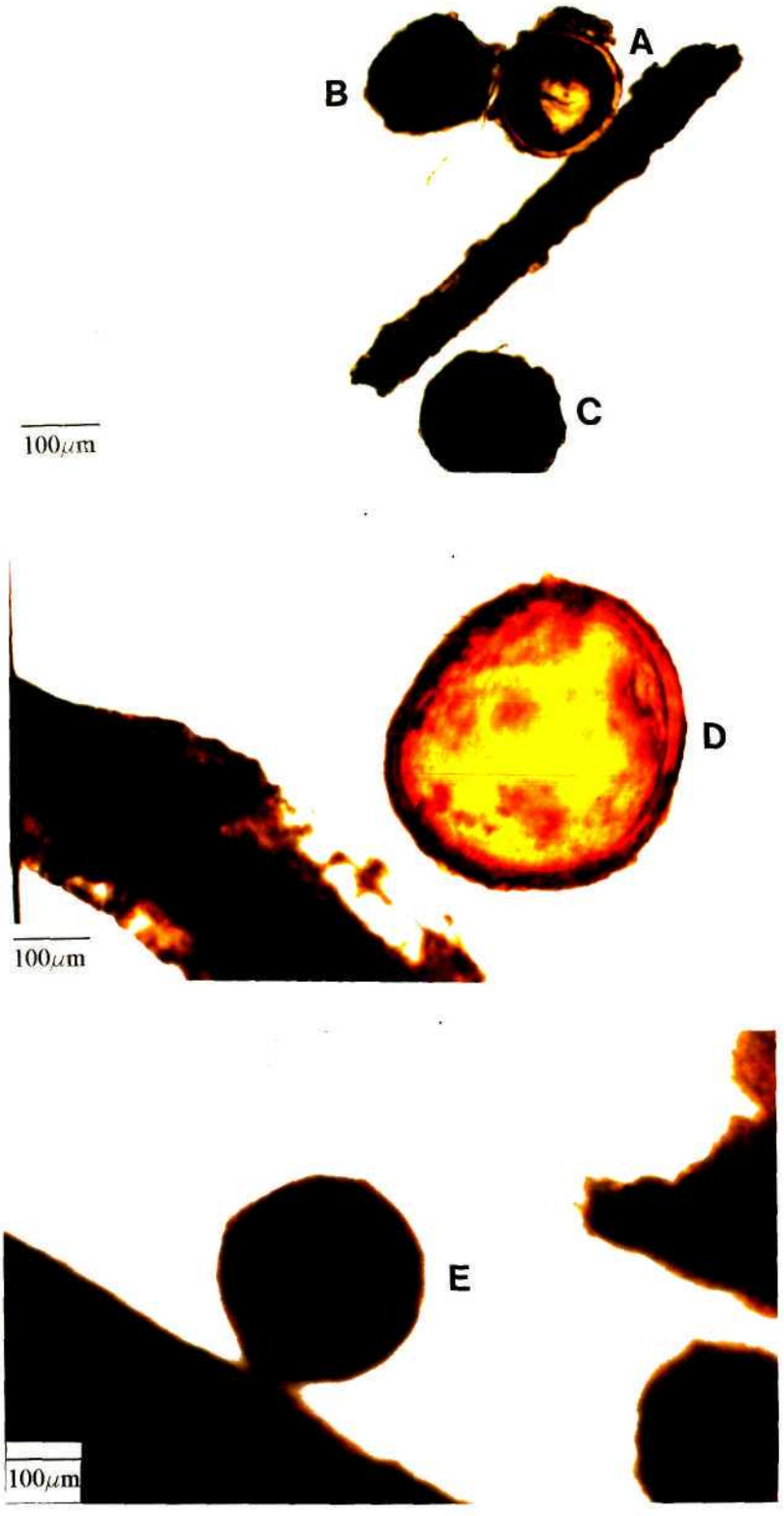

Figure 2.3 **The five types of Vesicular-arbuscular mycorrhizal spores found in the rhizosphere soils of the fertility trial plots**

A - E = the five spore types identified in the text. A = *Glomus sp.*

B = possibly *Acaulospora sporocarpa* C = *Glomus sp.* D = *Gigaspora sp.*

E = *Scutellispora sp.*

2.3 Results and Discussion

The ten random squares counted were tabulated and the total number of each spore type counted is indicated as a sub-total. Five separate counts were completed on each sample, giving five sets of sub-totals. The sub-totals for each spore type were added to give the final set of totals for each sample which was tabulated in Appendix 2.

From these totals we can acquire the following:-

a. The total of all spores for a sample
b. The total of high abundance propagules (usually less than 10 spores per sample)
c. The total of low abundance propagules (usually greater than 20 spores per sample)

High abundance propagules are those with normally greater than ten spores per sample (in this case types A and B), while low abundance propagules normally have less than ten spores per sample (types C-E).

The total spore counts are used to compare the effect of the different fertilizers on the mycorrhizal spore production. The assumption made is that the spore abundance will be proportional to the fungal growth.

All statistics were run on the Minitab Inc. (State College, P.A. 16801-3008, U.S.A.) statistical package. An ANOVA (F test) was run on spore count results to see if varying the level of the fertilizers had any significant effect on the spore abundance. The means and standard deviations from the analysis of the four fertilizer components are shown in Table 2.2.

In the phosphate study, Figure 2.4(A) shows the average spore abundances for the four fertilizer levels (P0-P3). There is a distinct increase from P0 to P1 and then a decrease from P1 to P3.

Utilizing the data in Appendix 2, ANOVA was run on the total spore counts of the zero

Table 2.2 **Means and standard deviations of spore counts for fertiliser components of the fertility trial**

FERTILISER	SPORE COUNT (Mean ± S.D.)
ZERO	50.7 ± 15.6^{b}
P0 (no P added)	49.3 ± 12.8^{b}
P1 (80 kgs P/ha)	146.7 ± 52.8^{a}
P2 (160 kgs P/ha)	$41.3 \pm 6.9b^{b}$
P3 (320 kgs P/ha)	46.5 ± 17.6^{b}
L0 (no L added)	67.3 ± 20.4^{ab}
L1 (1.1t L/ha)	59.2 ± 14.5^{ab}
L2 (3.3t L/ha)	41.3 ± 6.9^{b}
L3 (5.5t L/ha)	83.3 ± 22.7^{a}
K0 (no K added)	48.3 ± 24.3
K1 (100 kgs K/hA)	55.2 ± 12.6
K2 (200 kgs K/ha)	41.3 ± 6.9
K3 (300 kgs K/hA)	56.8 ± 21.4
N1 (100 kgs N/ha)	41.2 ± 14.1
N2 (200 kgs N/ha)	41.3 ± 6.9
N3 (300 kgs N/ha)	52.0 ± 13.9

P = phosphate, L = lime, K = potassium and N = nitrogen

For the separate fertiliser components, the superscript letters a and b represent groups that are significantly different from one another.

Where no superscripts are shown, there is no significant differences.

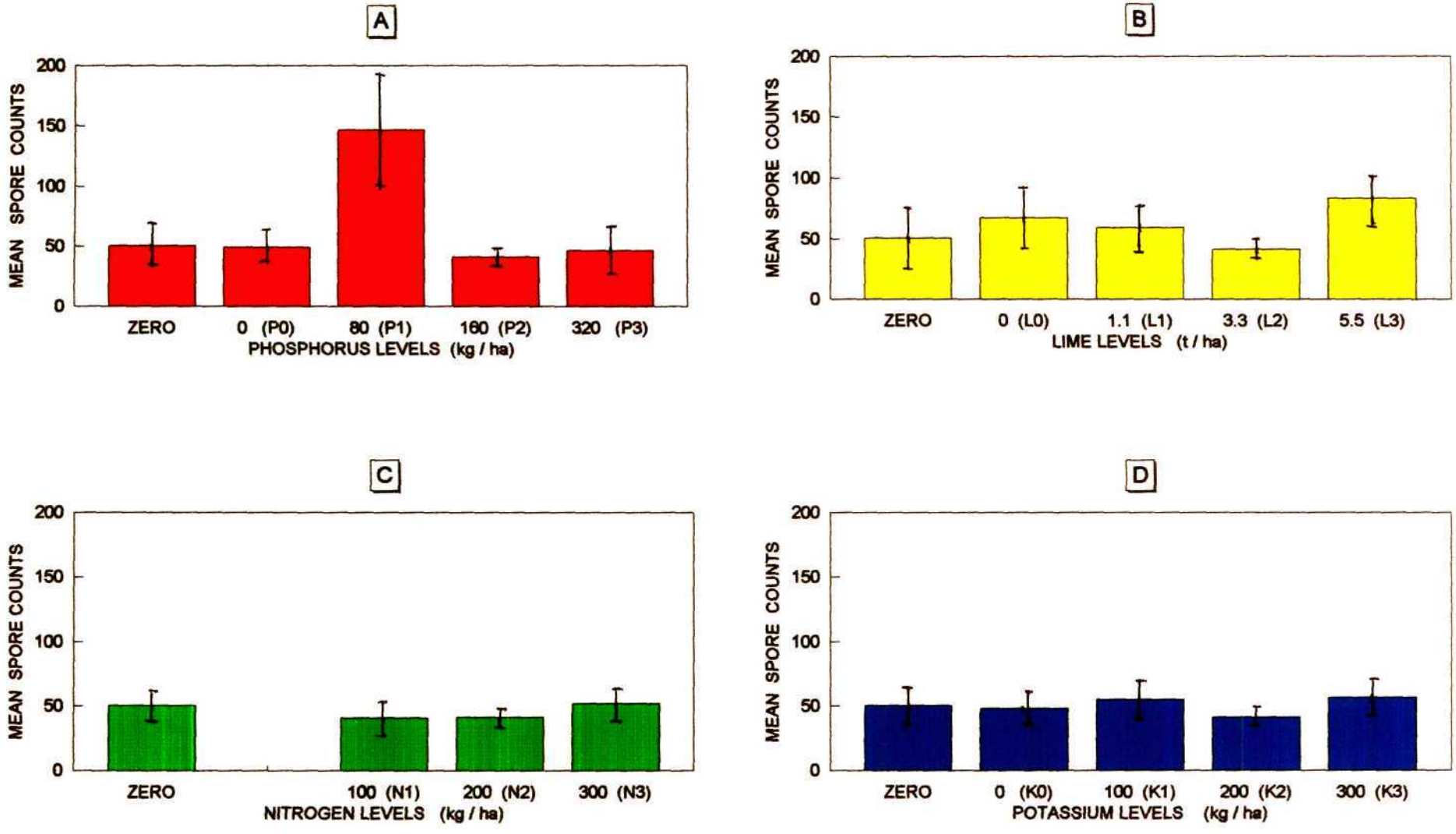

Figure 2.4 **Mean spore counts for the four fertiliser components used in the Kleinkopje Coal Mine Fertility trial**

Each graph shows the mean spore count for the differing concentrations of phosphorus, lime, nitrogen and potassium respectively

and phosphate treatments. As there was significant difference between some of the treatments, a Tukey's pairwise comparison was performed to see which of the samples were significantly different. In this test, if the 0 mean difference between the pair does not lie within the confidence interval then there is a significant difference between the treatments. In Table 2.3 the pair zero and P1 gives the confidence interval -34.7 to 37.3. As this contains the 0 difference there is no significant difference between the treatments at the 95% confidence interval.

Table 2.3 **Tukey's pairwise comparison of the zero and phosphate treatments.**

Treatment	Zero	P0	P1	P2
P0	-34.7			
	37.3			
P1	-132.0	-138.9		
	-60.0	-55.8		
P2	-26.7	-33.6	63.8	
	45.3	49.6	146.9	
P3	-31.8	-38.7	58.6	-46.7
	40.2	44.4	141.7	36.4

Zero = no fertilizer added. P0 = no P added, P1 = 80 kgs P/ha, P2 = 160 kgs P/ha and P3 = 320 kgs P/ha, from Table 2.1

Comparing the other samples in the same manner the P1 level of phosphate is significantly higher than the rest. This implies that there is an initial increase in spore count as P increases. Spore counts reach a peak around the P1 level of 80 kgs P / hectare (Table 2.1). Above this level the P causes a decrease in spores, which can be explained by the decreased germination of VAM

spores and the decreased hyphal growth noted by De Miranda and Harris (1994). It would appear that, in this field trial, the P effect corresponds to that found in greenhouse trials.

Figure 2.4(C) and 2.4(D) show the nitrogen and potassium average spore counts respectively. There appears to be no significant variation in the spore numbers with increase in fertilizer level. Completing an ANOVA and Tukey's multiple range test on the spore counts of the N and K treatments, no significant difference could be found.. It was concluded that K and N levels do not affect mycorrhizal spore formation. Reports by Sylvia and Neal (1990) that low N can negate the high P effect could not be validated as no plots contained very low N and high P. This was due to the initial top dressing of 50 kgs/hectare of N at the start of the trial and hence no N0 plots were available.

The final treatment, Figure 2.4(B) shows the effect of lime on the spore count. The comparison of the lime levels appeared more complex, as spore counts of L0 and L1 were higher than would have been expected and this could not be accounted for. As these plots contain the level P2 phosphate, it was thought that they would be similar to the K and N plots, whose spore counts are similar to L2. There was no significant difference between L3 and L0 or L1 but L3 was significantly different to both L2 and zero. This would then imply that high levels of lime cause an increase in the spore count. An increase in spore count would not be unexpected as by increasing the lime, more of the available P would be bound by the calcium in lime. This would decrease the P2 level of the L3 plots down towards P1 and so the high P inhibition of VAM is partially negated and the spore count increased. This implies that well limed plots can lock phosphate into the soil which reduces P run-off and enhances mycorrhizal growth, hence reducing the amount of P fertilizer needed in future years (Cress *et.al.* 1979)

The final question asked was, does an increase in phosphate fertilizer cause a decrease in the diversity of VAM symbionts. To try to show this it was decided that a comparison of the high and the low abundance propagules (HAP and LAP) at high and low P levels should be employed. What we needed to show was that the LAP spores decreased at a greater rate than the HAP spores. Looking at the ratio of HAP/LAP for the samples zero, P0 and P1, the ratios are 5.33, 4.92 and 4.40 respectively. These ratios are all of a similar level when compared to the

ratios of P2 and P3 which are 34.33 and 30.00 respectively. The great increase in ratio from low P (zero, P0 and P1) to high P (P1 and P2) implies that the LAP spores are decreasing at a greater rate than are the HAP spores, as the P level is increased. In the long term this decrease would lead to the loss of some of the low level spore types from the high P soils. Hence a decrease in diversity would occur as the P level was increased. The importance of this phenomenon is that more than one type of mycorrhiza may infect a plant at any point in time. As the different mycorrhiza may have a benefit to the plants at different periods of the year and may also help one plant species more than another, the maintenance of diversity of mycorrhizal species could have an important role in plant survival.

2.4 Conclusion

Work done on the fertility trial site at Kleinkopje Mine shows that an initial low level of phosphate is required for good mycorrhizal infection and growth. In this field trial the level of P needed was in the range 80 kgs P/hectare and using the hectare furrow slice this is equivalent to 35.7parts per million. Inhibition of mycorrhiza due to high P levels was demonstrated and corresponds to work done in greenhouse trials by Cress *et.al.*, (1979).

Neither nitrogen nor potassium showed any appreciable effect on mycorrhizal infection at the levels used in this trial. Although not conclusive, it would appear that high lime fertilization causes an increase of mycorrhizal infection when high levels of phosphate fertilizer have been used. This is possibly due to the binding of available P by the calcium in the lime.

Finally the greater rate of inhibition of LAPs compared to HAPs at high P levels, implies that eventually high P levels would result in a decrease in mycorrhizal diversity.

CHAPTER 3

Identification of Vesicular-Arbuscular Mycorrhizal spores using the Polymerase Chain Reaction and Randomly Amplified Polymorphic DNA techniques.

3.1 Introduction

Identification of Vesicular-arbuscular mycorrhizal (VAM) fungi is rendered difficult due to the fact that no method has yet been developed to grow VAM *in vitro*. Separation of VAM hyphae from inside root systems is not practical and no method has yet been perfected to identify VAM hyphae *in situ*. To amplify the VAM they must be inoculated onto host plants and grown to sporulation at which point the VAM can be identified. Using spore size, type, colour and type of attachment of the spore to the fungal hyphae, the VAM spore forms the chief characteristic by which VAM are identified (Schenck and Perez, 1990). This is time consuming and needs a great deal of experience to distinguish closely related species.

Henrion *et al.* (1992) developed a rapid method for identifying ectomycorrhizal fungi, using the polymerase chain reaction (PCR) to amplify their ribosomal DNA. PCR is a technique that amplifies DNA with a repetitive cyclic process consisting of three steps :-

I. Melting the DNA into separate strands at temperatures of around 95°C.

II. Cooling to 35-40°C to allow a short piece of "primer" DNA to anneal to a complimentary sequence on the DNA to be amplified.

III. Primer extension at 72°C, using a DNA polymerase to copy the template DNA.

These three steps of the first cycle are represented diagrammatically in Figure 3.1.

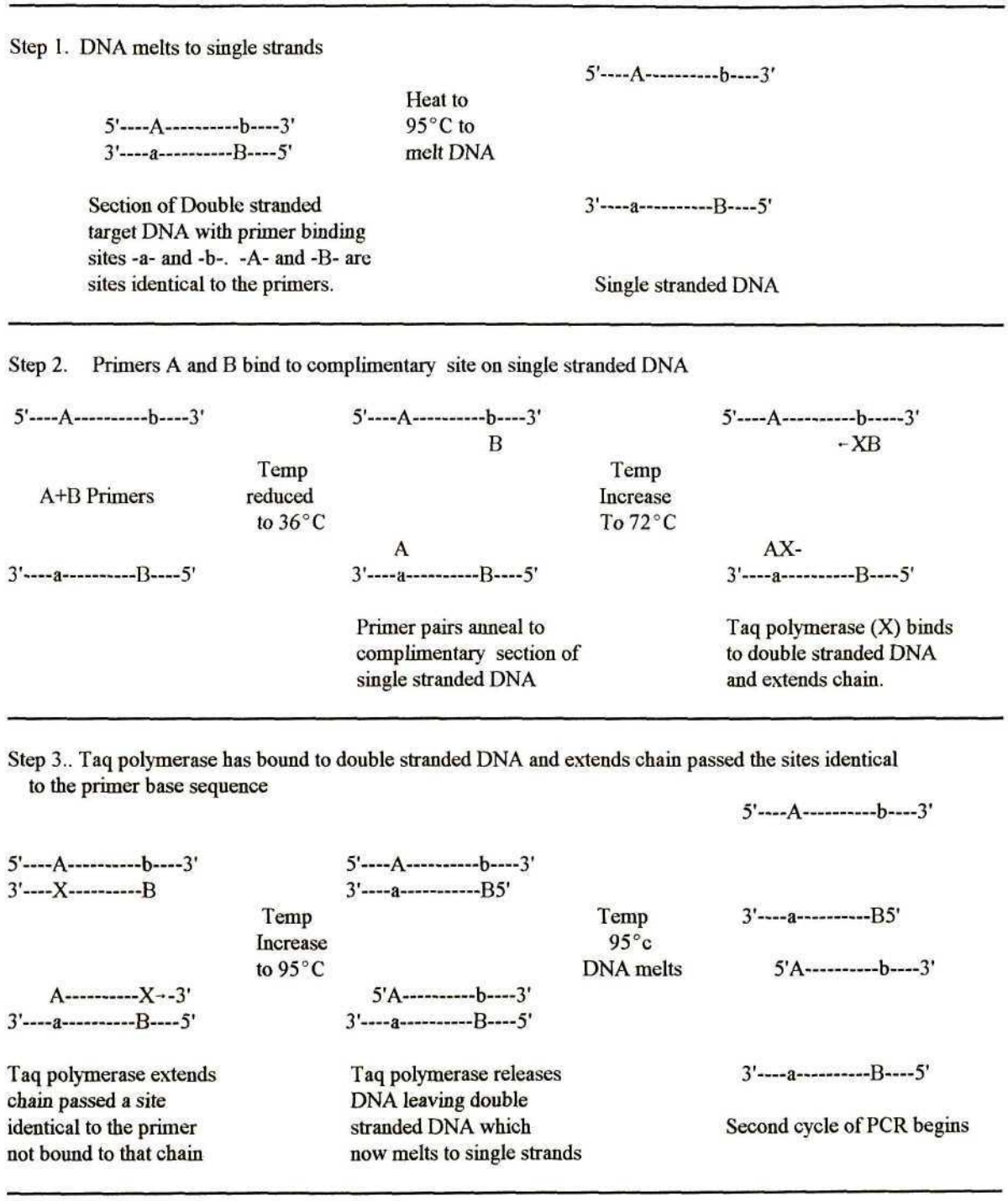

Figure 3.1 Diagrammatic representation of a polymerase chain reaction (PCR) cycle
A and B represent primers, while -a- and -b- represent sites on the DNA with base pair sequence complementary to the primer. -A- and -B- are sites on the DNA chain where the sequence is identical to that of the primers. X represents Taq polymerase.

Classical PCR uses two primers to exponentially amplify the DNA contained between the binding sites of the two primers (Figure 3.1). Henrions (1992) amplified ribosomal DNA in this manner, which was then subjected to a variety of restriction endonucleases, each of which cut the DNA at specific base sequences. This cutting forms restriction fragments which can be analysed for restriction fragment length polymorphism (RFLPs) using gel electrophoresis. The band patterns formed could be used to distinguish ectomycorrhizal species.

To eliminate the necessity of performing restriction enzyme fragmentation, a new technique was recently developed to aid in plant identification and philology (Newbury and Ford-Lloyd, 1993; Wolff and Peters-Van Rijn, 1993) using the same DNA variation employed in RFLP analysis. Using a single short primer, the Randomly Amplified Polymorphic DNA (RAPDs) technique relies on the likelihood that there will be a number of sites on the DNA chain that are complimentary to, and a number of sites that are identical in sequence to the primer (Ohan and Hiekkila, 1993). The technique only generates exponentially amplifying DNA fragments if , when generating a new strand in the first cycle, the Taq polymerase passes over a template site identical in sequence to the primer. This is easier to show diagrammatically in Figure 3.2

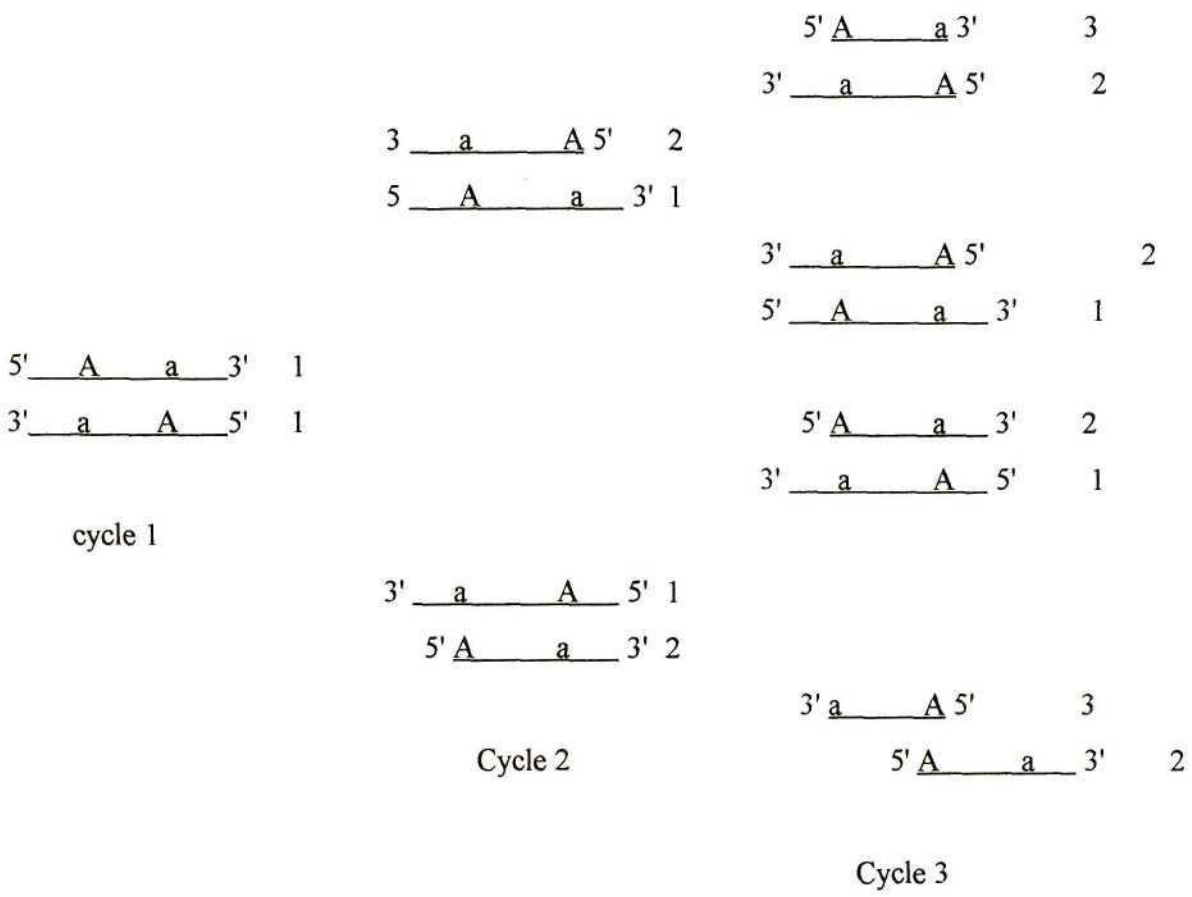

Figure 3.2 DNA strands generated in the first three cycles of RAPD PCR.

'A' represents a site where the DNA sequence is identical to the primer, 'a' represents a site where the DNA sequence is complementary to the primer

At the start of the first cycle of Figure 3.2 the full DNA chains are present with the primer binding site (-a-) and the site identical to the primer (-A-) being present. The DNA strand runs past the primer site and the site identical to the primer and is marked 1 indicating that it is the original DNA. In this diagrammatic system only two type 1 strands are ever present throughout the PCR. When the primer binds to site -a- in cycle 1, it generates a type 2 DNA strand where the 5' end terminates at -A-. At the start of cycle two the initial DNA has two type 1 and two type 2 DNA strands as the DNA has doubled. In cycle 2, when the type 2 strand is replicated, the new strand type formed has again been cut off at the 5' end at site -A-, generating a type 3 strand.

This type 3 strand terminates at the primer binding site -a- and the site identical to the primer -A-. It is this type 3 strand that causes an exponential amplification of this section of DNA in the rest of the PCR cycles. The type 2 strands only increase arithmetically by two strands per cycle and so are swamped by the exponential growth of the type 3 strands which double with each cycle. Only the type 3 strands will amplify the DNA sufficiently to be visible as bands when the PCR is subject to agarose gel electrophoresis. Hence after a RAPD PCR and agarose gel electrophoresis a band pattern is formed in the same manner as for RFLP band patterns, which can then be used in identification of the source of the DNA .

VAM spores are known to contain many nuclei (Burggraaf and Beringer, 1989) with reported numbers being variable and the exact range for any species still unclear. Cooke *et al.* (1987) estimated that *Gigaspora gigantea* and *Scutellospora erythropa* contained 2600 to 3850 nuclei per spore, while (Burggraaf and Beringer, 1989) estimated that *Glomus caledonium* had over 1000 nuclei per spore. This would mean that there is approximately 10 nanograms of DNA per spore (Viera and Glenn, 1990). It was considered feasible that this amount of DNA could be directly PCRed without first extracting the DNA. Identification of a single spore would then be possible using the RAPDs technique.

3.2 Materials and Methods

Rhizosphere soil was collected from grasses growing next to a highway offramp in Pietermaritzburg. Separation of spores was completed by wet sieving and sucrose gradient centrifugation (Section 2.1.2.1 and 2.1.2.2), followed by washing and physical spore separation using a narrow Pasteur pipette. The spores collected for the attempted PCR were *Glomus spp.* It was considered that if the DNA in the smaller *Glomus spp.* could be subjected to PCR, then the larger VAM spores with more nuclei (DNA) could also. A single spore was placed into a plastic PCR tube suitable for a Hybaid thermocycler PCR machine. To release the DNA from the spore, it was crushed using a Pasteur pipette that had the point melted into a ball the correct size to almost fill the bottom of the plastic PCR tube. Initially the spore was crushed when dry and then 5μl of water was added to allow the contents of the spore to be separated from the spore case by pressing a number of times with the modified pipette. The spore was now ready for PCR.

3.2.1 Polymerase Chain Reaction (PCR)

The following reagents were used for the PCR:-

1. PCR buffer. (Appendix 3)
2. Nucleotide tri-phosphates (dNTP) consisting of equal quantities of deoxyadenosine, deoxyguanosine, deoxycytidine and deoxythymidine tri-phosphates (from Boehringer Mannhiem) (Appendix 3)
3. *Thermophilus aquaticus* (Taq) polymerase (from Promega).
4. Primer (polynucleotide chains consisting of ten bases) a number of primers are available with differing base type and sequence. Operon Technologies Primers OPA-07 (base sequence GAAACGGGTG) and OPA-09 (base sequence GGGTAAACGCC) were used.

All water used in the PCR was ultra pure PCR water and all chemicals used were of molecular biology grade.

The constituents for a 25μl PCR reaction were as follows :-

10 x buffer	2.5μl	
1/100 x dNTPs	2.5μl	
Primer OPA-07	1μl	(= 5pM for 25μl PCR)
1/10 xTaq	1μl	(= 0.5units/μl)
DNA	1 spore	
PCR Water	18μl	(Make up to 25μl)
Total	25μl	

The PCR was run on a Hybaid thermocycler with the following cycle conditions :-

1. 95°C for 2min
2. 36°C for 1min
3. 72°C for 1min

35 of these cycles were completed to amplify the DNA $\pm 10^{10}$ times.

The complete PCR reaction volume was loaded onto a lane of a agarose gel.

3.2.2 Agarose Gel Electrophoresis.

The principle of separation behind agarose gel electrophoresis of DNA is that the charge to mass ratio of DNA is the same for all lengths. When a charge is applied across an agarose gel, the DNA strands will migrate towards the positive anode of the gel. The pore size of the agarose gel, controlled by the percentage of agarose, was such that the longer the DNA strands, the more resistance there is to the migration. Thus the longer the DNA the slower it migrates, so causing a separation of the DNA chains according to their length. This allows the strands of DNA of varying length, generated in the PCR, to be separated and visualized as bands on the gel. (Auaubel *et al.* 1989). The constituents of the agarose gel and loading buffer are recorded in Appendix 4.

3.2.2.1 Sample preparation

The sample was prepared for gel electrophoresis by adding 4μl of loading buffer

(Appendix 4) to the PCR product, heated to 65°C for 3 minutes, then placed in ice to cool. This linearizes the DNA, ensuring that any secondary structure (loops etc) of the DNA does not confuse the separation on length basis produced by electrophoresis. The complete reaction product was loaded into a gel lane well and the electrophoresis was run at 4 volts/cm until the front marker (Bromophenol blue) reached the end of the gel. The Bromophenol blue moves more rapidly than the DNA, so when it reaches the end of the gel, the DNA strands will have migrated a distance along the gel, dependent on the strand length. To visualize the DNA, the gel was submerged in a 0.5μg/ml solution of ethidium bromide until the gel appeared light pink when viewed under ultra violet (UV) light. The gel was then "cleared" of ethidium bromide by gently shaking in distilled water until the pink colour just disappears. At this point the ethidium bromide will still be bound to the DNA. Pink bands indicating where the DNA is present in the gel when viewed under UV light. The gel was then photographed with a Polaroid camera (fitted with an orange filter) to give a permanent record of the banding pattern.

3.2.3 Phenol/chloroform extraction of DNA

The principle behind this extraction is that the phenol/chloroform mixture dissolves phenolics and non polar constituents from the solubilized components of the spore. By shaking the phenol/chloroform with an equal volume of the solubilized spore constituents the non polar substances will partition into the phenol/chloroform. This leaves the partially purified DNA in the water fraction of the mixture, which is then microfuged ± 15 seconds to separate out the two phases. To remove water soluble impurities, the water phase is separated and subjected to an ethanol precipitation overnight (under refrigeration) and the DNA then pelleted and washed before dissolving in PCR water. (Auaubel *et al.* 1989)

As phenol is very acidic in nature it has to be buffered before use (Appendix 5), to prevent damage to the DNA.

Chloroform is water saturated by shaking it with an equal volume of distilled water and then allowed to settle upon which the two phases separate out and the required amount of

saturated chloroform can be extracted.

Both phenol and chloroform are left in bottles in this condition to maintain saturation and pH.

For the phenol/chloroform extraction mixture, phenol, chloroform and isoamyl alcohol are mixed in the following ratios:- 25/24/1.

3.2.3.1 Sample preparation

Glomus spp, spores were selected for extraction and 400 spores were placed into a 50μl microfuge tube and crushed with a modified Pasteur pipette (end melted into a ball). DNA and other spore contents were solubilized by adding 100μl of TE buffer pH 8.0 and the mixture crushed further to release the DNA. This preparation was then subjected to a phenol chloroform extraction..

A volume of phenol/chloroform/isoamyl alcohol (25/24/1) mixture equal to the volume of the DNA solution was added to the sample and the mixture shaken for 10 seconds in a vortex mixer. The microfuge tube was then transferred to a Hagar microfuge and spun for 15 seconds. This causes the aqueous phase and phenol/chloroform phase to separate out into two layers. The aqueous layer into which the DNA partitions was separated. Concentrated DNA at the interface between the two phases may precipitate out and adhere to the interface, so 100μl of TE buffer was added to the microfuge tube and the vortexing and centrifuging repeated. Again the aqueous layer was separated and the two aqueous fractions combined. Phenol will denature most proteins and so any phenol left in the aqueous fraction must be removed. This was done by chloroform isoamyl alcohol extraction of the aqueous fraction. Equal volumes of DNA solution and chloroform/ isoamyl alcohol (24/1) were vortexed for 10 seconds and then microfuged for 15 seconds and the water layer containing the DNA was separated. Any phenol that was present in the original water fractions, is partitioned into the chloroform layer and so the DNA water fraction is purified of phenol. Any chloroform left in the DNA solution can be removed in the

same manner as phenol, using ether to extract the chloroform. The purified DNA solution was now concentrated by an ethanol precipitation of the DNA from solution. At this point large quantities of DNA appear as a white candyfloss like precipitate, but small quantities are not normally visible.

3.2.4 Ethanol precipitation of extracted DNA

A volume of 3M NaAc (sodium acetate) equal to 1/10 of the volume of the DNA solution is added and the mixture vortexed to mix the NaAc. Ethanol at a volume of 2.5 times the volume of the DNA solution is now added to the sample and vortexed to mix. This addition is best done with refrigerated solutions as the precipitation works best with cold liquids. The solution was then placed in the freezer compartment of a refrigerator for at least 1 hour, preferably overnight to fully precipitate the DNA. After precipitation the precipitate was spun in a microfuge for 20 minutes to pellet the DNA. The liquid was decanted off and the DNA pellet was then washed with 70% ethanol to remove any remaining salt and solution. The DNA was then solubilized in 20μl of PCR water. (Auaubel *et al.* 1989)

3.3 Results and Discussion

The result of the first PCR using a single spore and the conditions in section 3.2.1 resulted in no bands being visualized. There are a number of possible reasons for this including

1. Insufficient amplification of the DNA to allow for visualization.
2. PCR conditions not suitable for this process.
3. Contaminants in the spore render the PCR ineffective.
4. DNA not released fully from the spore to become solubilized for the PCR.

Point 1 could indicate that there is insufficient DNA present in a single spore, to allow for the PCR to proceed correctly. To test this hypothesis, the number of spores used in the PCR was increased from one to ten. The PCR was run using the same conditions as above, but still no DNA bands could be visualized. To increase the amount of DNA effectively a ten fold increase in the starting material may not be sufficient to allow for visualization. Instead of increasing the starting material further, the exponential amplification of the PCR was used to greatly increase the final product.. This increase was facilitated by a double PCR, in which an initial amplification of the spore DNA was completed for 10 cycles with small amounts of dNTPs and Taq. After adding more Taq and dNTPs a further 35 cycles were completed amplifying the DNA an extra 1000-fold. The original PCR was not just increased in cycle number as the harsh conditions of PCR reduce the effectiveness of the Taq polymerase after 35 cycles which leads to reduced yield and erroneous replications. By using a double PCR most of these problems are averted.

The double PCR conditions are :-

	1st PCR		2nd PCR		
10x buffer	2.5 μl		-		
1/100dNTPs	1.0 μl		1.5μl		
Primer 7	0.5 μl		0.5μl		
1/10 Taq	1.0 μl		2.0μl		
DNA	1 spore		-		
Water*	20.0 μl		-		
Total	25.0μl	+	4.0μl	=	29μl

After agarose gel electrophoresis no bands could be detected on the gel.

Next the type of spore used was varied to see if a larger spore with more DNA in it would not give better results. The spore used was a large golden coloured spore ±150μm diameter *(Gigaspora spp.)*.

Conditions of the PCR were varied as follows from those of the first double PCR :-

	1st PCR		2nd PCR		
10x buffer	2.5 μl		-		
1/100dNTPs	1.5 μl		1.0μl		
Primer 7	0.5 μl		0.5μl		
1/10 Taq	2.0 μl		1.0μl		
DNA	1 spore		-		
Water*	20.0 μl		-		
Total	25.0μl	+	4.0μl	=	29μl

This also gave no bands after agarose gel electrophoresis.

To get more amplification of the DNA present, a double PCR was completed using 15 *Glomus spp.* spores, the volume of the first PCR was reduced to 10μl and the number of cycles increased to 15. The conditions were as follows :-

	1st PCR		2nd PCR		
10x Buffer	1.0 μl		1.5 μl		
!/100 dNTPs	1.0 μl		1.5 μl		
primer 7	1.0 μl		-		
1/10 Taq	3.0 μl		-		
spores	15		-		
PCR water	4.0μl		12.0 μl		
Total	10.0μl	+	15.0 μl	=	25μl

No bands were visualized on agarose gel electrophoresis.

The same conditions for the double PCR were used except the first PCR was increased

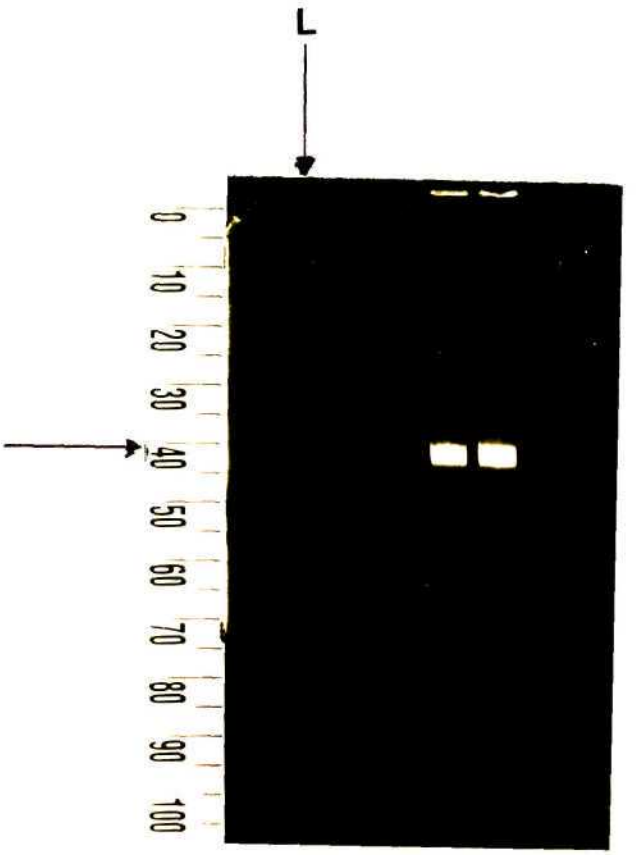

Figure 3.3 **Agarose gel from electrophoresis of RAPD PCR of 15 *Glomus sp.* spores.**

A very faint band is visible in the marked lane at position 42, which corresponds to a 3 Kilobase DNA length.

to 30 cycles and the PCR water in the second cycle decreased to 7μl. After agarose gel electrophoresis of the PCR product, a very faint band could be detected on the gel (Figure 3.3). The number of bases in the band was estimated to be 3Kb from a set of DNA markers run on the gel. The DNA marker from Boehringer Mannheim, was an ECO RI and HIND III restriction digestion of λ DNA, producing 13 DNA fragments ranging from 125 to 21226 bases long. The faintness of the bands indicated that amplification could be the problem with the method used and the result was not repeatable. To see if there was a problem with the release of the DNA from the spores, sonication of the crushed spores was attempted, as chemical methods (such as SDS addition) would adversely affect the PCR. As no results was obtained by electrophoresis after this additional mechanical disruption, release of the DNA from the spore was probably not the cause of the PCR failure.

As completing a PCR directly on the spore contents had not proved successful, it was decided that a phenol/chloroform DNA extraction (Auaubel *et al.* 1989) of a number of spores should be completed. The purpose of this extraction was two fold,

I. To determine how much DNA is present in the spores used and

II. To remove any contaminants present in the spore that may be detrimental to PCR of the spore DNA.

A number of selected spores (± 400) of the *Glomus spp.* were placed in a 50μl microfuge tube and crushed with the modified Pasteur pipette. The crushed spore contents were dissolved in 20 μl of distilled water and then the phenol/chloroform extraction plus ethanol precipitation was completed to extract the DNA. A 6μl portion of the 20μl of purified DNA was then used to determine the concentration of the DNA extracted. The 6μl of DNA solution was placed in a 1ml quartz cuvette and made up to 1ml. The diluted DNA was measured in a GeneQuant DNA calculator, which gave the following information:- absorbance at 260nm =0.021, at 280nm = 0.016, 260/280 ratio = 1.313, DNA purity = 73%, DNA in 6μl of sample = 1.05ug. The total amount of DNA in 400 spores was 3.5ug, from which the DNA content of a single spore was calculated to be 8.75ng.

The crushing stage of this extraction was not very successful as it was difficult to tell if

all spores were effectively broken open. This could cause low extraction level of DNA. To test this hypothesis two other spore preparation methods were tried:-

1. Sonication.
2. Nitrogen freezing and crushing, followed by addition of SDS to rupture nuclear membranes

The sonication was not effective as on extraction little DNA could be detected. The nitrogen freezing helped in the crushing of the spores which formed much smaller particles than did the spores crushed at room temperature. The SDS solution probably also added to the increased amount of DNA extracted. After preparation the spore DNA was extracted by Phenol/chloroform extraction and then ethanol precipitation. The DNA was dissolved in 20μl of PCR water and 4μl was added to a 1ml quarts cuvette for DNA analysis on GeneQuant, which gave the following results:- absorbance at wavelength 260nm = 0.024, at 240nm = 0.018, 260/280 ratio =1.384, DNA purity = 76%, protein present =0, DNA in 4.0μl of sample = 1.1μg. Total DNA in 20μl of sample = 5.5μg, which was the amount of DNA extracted from 300 spores, so the DNA concentration per spore = 18ng. This amount of DNA is higher than the 10ng per spore extracted by (Viera and Glenn, 1990) but is only 76% pure so is in the same range.

To see if the DNA could be PCRed to form RAPDs bands, a PCR using 3μl of the extracted DNA solution containing about 1.0μg DNA (assuming 76% purity) was attempted.

The PCR had the following ingredients :-

1/10 Taq	2.5μl	
1/100dNTPs	1.0μl	
10X buffer	2.5μl	
Primer OPA-07	1.0μl	
DNA	3.0μl	(1.0μg DNA)
PCR water	15.0μl	
Total	25.0μl	

After PCR and agarose gel electrophoresis, no bands could be detected. The DNA being only 76% pure possibly contained small amounts of phenol, left from the phenol/chloroform

extraction, that inhibits the Taq polymerase.

This work was being completed during a section of the growth trial, where plants were growing and little attention was needed to the trial. As positive results were not forthcoming and pressure of work from the growth trial and fertility trial was increasing, it was considered advisable by my supervisor to discontinue further work on this project.

CHAPTER 4

The effect of Vesicular-arbuscular mycorrhizal infection on the growth of grasses *Themeda triandra* and *Trachypogon spicatus* on coal mine spoil soils. A greenhouse trial.

4.1 Introduction

The main thrust of the current research is the hypothesis that mycorrhiza are able to benefit grass species that were, to some extent, dependent on the mycorrhizal symbiosis formed between the fungal and plant partners. This symbiosis was postulated to allow mycorrhizal plants to grow in low nutrient soils, where growth of high nutrient-requiring primary plant colonizers was no longer viable. Revegetation of disturbed land (such as coal mine spoils) would benefit from any system that could accelerate the formation of a natural sustainable ground cover.

Under natural conditions, badly disturbed land is first colonized by fast growing annual plants. These plants have extensive root systems that rapidly absorb the nutrients made available by the disturbance (Allen, 1991). As the nutrient supply of the soil becomes depleted, these annual plants are not as competitive as perennial secondary colonizers. Although the secondary colonizers do not grow as rapidly as the primaries, they are able to store nutrients from year to year. This allows the secondary colonizer to out-perform and take over from the annual primary colonizers as soil nutrient levels decrease. The successional process at this stage has advanced from non-mycorrhizal annual colonizers to facultatively-mycorrhizal perennial secondary colonizers. The next stage in the successional process is for the secondary colonizers to give way to tertiary colonizers. In natural South African grasslands, these tertiary colonizers are highly mycorrhizal grasses which become the climax community under the prevailing conditions (Roux, 1969). When land is badly disturbed, the successional process from primary to climax communities usually takes a long time, especially the secondary to tertiary progression. This

process may take many decades, as found by Roux (1969).

What drives this successional process ? The initial move from annual primary colonizers to perennial secondary colonizers could possibly be due to the perennial plants' ability to store nutrients from year to year. This allows the perennial plants to survive on lower soil nutrient levels than can the annual plants and hence they usurp the annuals. The next successional stage is not quite as easy to explain. Secondary colonizers often have more extensive root systems than do tertiary colonizers and therefore should be capable of absorbing more nutrients. Highly mycorrhizal tertiary colonizers however, have an extensive root-associated hyphal network that can penetrate into areas unavailable to the larger roots of grasses. This allows tertiary colonizers with their superior mycorrhizal symbiosis to absorb more nutrients than can secondary colonizers, driving the successional process towards tertiary colonisation (Allen, 1991).

Bound phosphates (notably calcium phosphate) may also play a role in this progression. Plant roots cannot absorb these phosphates at the extremely low levels of their solubility constants. Mycorrhiza are, in some manner, able to assimilate phosphates at these low levels and hence phosphate becomes less limiting in mycorrhizal associations (Cress *et al.*, 1979). This additionally drives the successional process towards highly mycorrhizal plants.

Revegetation of strip mine sites is notoriously difficult, as the re-established topsoil is usually very low in both nutrients and rhizosphere microorganisms (Jasper *et al.*, 1988). In particular, nitrogen (N), potassium (K) and phosphorous (P) are normally limiting. When fertilizer is added at adequate levels to support ground cover growth, annual plants normally thrive. Rapid and extensive root growth enables them to absorb the available nutrients, out-performing perennial plants such as grasses. High levels of phosphorus have been linked to the inhibition of VAM mycorrhizal infections (Bell *et al.*, 1989), which makes the perennial mycorrhizal dependent grasses even less competitive. As nutrient levels fall, facultative VAM-infected plants act as secondary colonizers and the annuals are reduced in number. Vegetation on older sites becomes more dependent on mycorrhiza for colonizing ability, a principle described by Kostkova and Cudlin (1989) using coalmine topsoil spoils of varying ages up to 18 years. A problem with allowing natural progression from annuals to perennials is that during the transition,

the ground cover is reduced and erosion can occur, slowing natural revegetation of the spoil. Lindsey *et al.* (1977) working with coal strip mine spoil material, showed that VAM infection helped the survival and growth of rabbitbrush (*Chrysothamnus nauseosus).* Two other plants, fourwing saltbrush (*Atriplex canescens*) and corn (*Zea mays*) did not show any advantage from *Glomus fasciculatus* infection . A high dependence on VAM colonization of the three grasses, *Andropogon gerardii, Panicum virgatum* and *Calamovilfa longifolia,* was demonstrated by Brejda *et al.* (1993). For survival on their native, poor sandy soil the three grasses had to be mycorrhizal. For reclamation on strip mine spoils it was emphasized that VAM colonization was essential for the promotion of plant succession on the spoil (Pflegor *et al.*, 1994).

Trachypogon spicatus and *Themeda triandra* are two naturally occurring grass species of the South African bushveld. They are mycorrhizal (personal observation), grow naturally on low nutrient soil and are palatable to both cattle and game species, and thus would make excellent plants for revegetating coal mine spoils. On disturbed land these two species would be late successional plants, only establishing after many years. Any method that could accelerate the rate of establishment of these species would be a great advantage in the spoil reclamation.

Before any reclamation can be attempted, the viability of the system must be examined. To this end a set of greenhouse trials was designed to demonstrate:-

I. That VAM have a positive effect on the growth and/or nutrient uptake of two naturally occurring grass species of the South African bushveld.

ii. That mycorrhizal flora from different habitats have varying effects on the growth of grasses.

4.1 Materials and Methods

In order to demonstrate these two points, a greenhouse trial was undertaken using five sets of VAM mycorrhizal flora. This consisting of VAM infection propagules and micro flora associated with the rhizosphere soil of the plants, from around which the soil was taken. These were to be inoculated on to two types of naturally occurring bushveld grasses. A control of

sterilized mycorrhizal flora was used to act as the inoculum for a set of control grass plants.

The mycorrhizal flora was extracted from rhizosphere soils collected from a number of regions where arid conditions and low nutrient soils prevail. Rhizosphere soils were sampled from the Cape Flats (CF), Beaufort West (BW), the Worcester (WOR) region of the Cape Province and from roadside grasses near a Pietermaritzburg south (PMB) highway offramp (disturbed low nutrient soil). The Pmb rhizosphere soil samples had two major mycorrhizal spore types indicated as Large (LRG) and Small (SML)(Figure 4.1). The two naturally occurring grasses chosen were *Themeda triandra* and *Trachypogon spicatus.*

The amount of inoculum gathered from these samples was insufficient to inoculate the two trials planned and so required amplification. This was done by inoculating two plant species, tomato (*Lycopersicon esculentum*) and maize (*Zea maize),* with the initial inoculum. After growing these inoculated plants the roots were harvested and used as inoculum for the trials.

Two separate greenhouse trials were planned, the first using the harvested inoculum to infect *Themeda triandra* and *Trachypogon spicatus* seedlings for growth on "topsoil" sampled from a coalmine reclamation site. The second trial was essentially the same except that potassium, nitrate and phosphate fertilizers were supplemented to the mine spoil soil to improve growth of the grasses. Phosphate fertilization was included at the equivalent of 50 kgs P / hectare, this level having been determined in Section 2.3 as the lowest required to give a high rate of mycorrhizal infection and growth on the grasses used in the trial.

4.1.1 Coarse separation of mycorrhizal spores by wet sieving

Coarse spore separation was completed by the wet sieving protocol (Appendix 1).

Plate 1

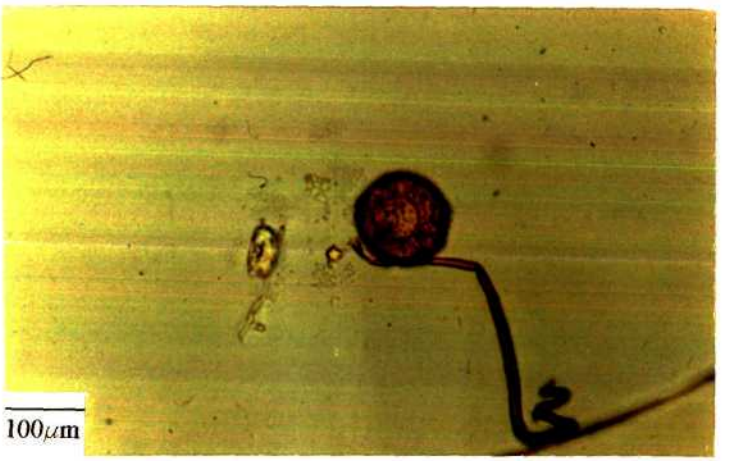

Plate 2

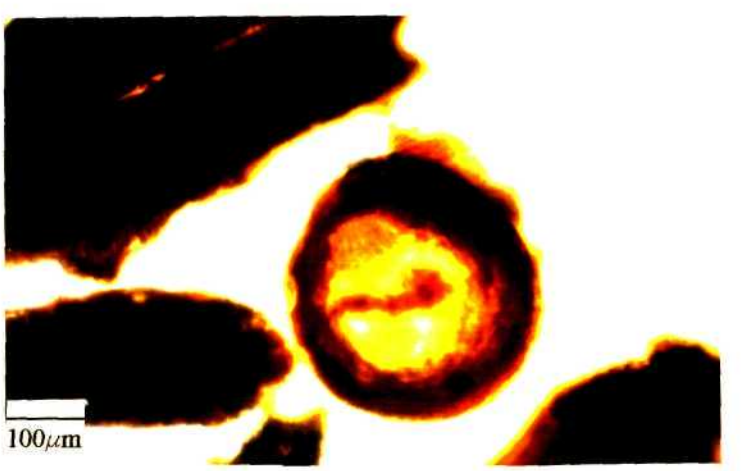

Figure 4.1 **The Large and Small spores from Pietermaritzburg used as inoculum cultures in the greenhouse trials**

Plate 1 Small golden spore (*Glomus* sp.)

Plate 2 Large golden spore (*Gigaspora* sp.)

4.1.2 Fine separation of mycorrhizal spores using sucrose gradient centrifugation

The spore separation achieved by wet sieving gives a very crude spore sample mixed with soil particles and small pieces of organic matter. To isolate the spores, sucrose gradient centrifugation is used. A number of gradient separations are listed in Shenck (1984). The method adopted was a modification of the 70% - 20% layered gradient using a gradient maker to form a continuous gradient from 70% up to 20%. A schematic of the gradient maker, which was manufactured in the local university workshops, is shown in Figure 4.2.

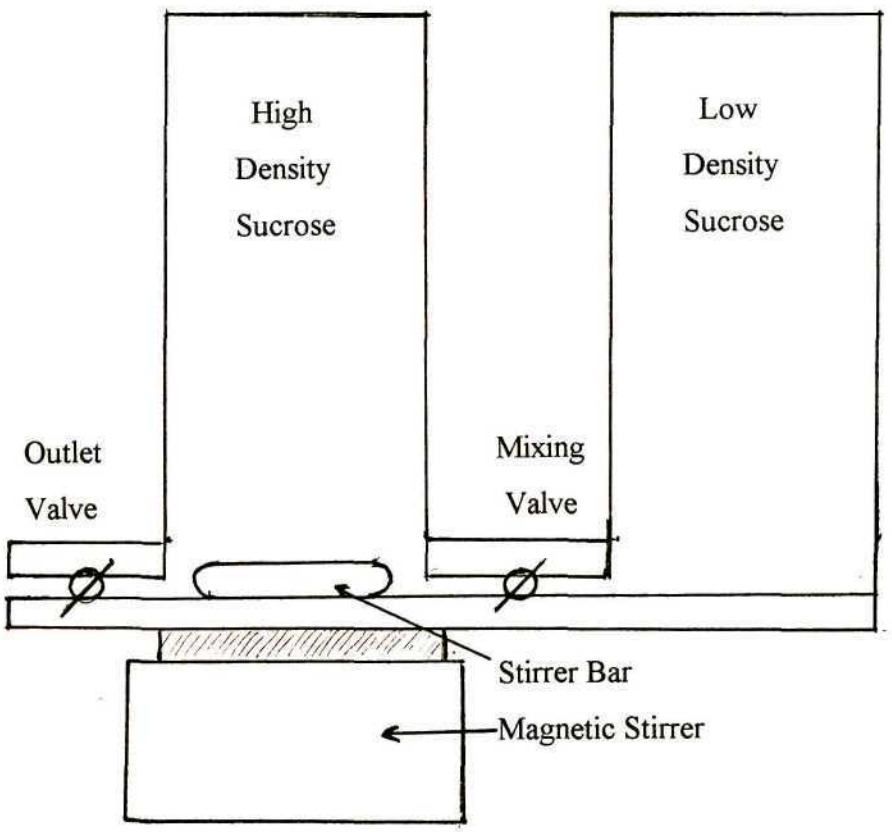

Figure 4.2 **Continuous gradient making equipment**

Gradient generation was completed as follows:-

1. The gradient making equipment was placed on a magnetic stirrer to allow free movement of the stirrer bar placed in vessel A, which had 15mls of 70% glucose solution added to it.
2. Vessel B had 15mls of 20% glucose solution added.
3. While the stirrer bar revolved, the output valve was opened to allow the peristaltic pump to remove solution at a constant rate into a 50ml centrifuge tube.
4. When about 0.5cm had been removed, valve B was slowly opened to allow the 20% solution to drain into the 70% solution. Thus the 70% solution slowly mixed with the 20% solution in vessel A to produce a continuous gradient. Stirring the contents of the 70% container allowed the two densities to mix uniformly.
5. 10 ml of the coarse spore separation from the 70μm wet sieving step was overlayed gently on top of the gradient. Some of the heavy soil particles immediately sank to the bottom. This would destroy the gradient if the overlaying were done too rapidly.
6. The tube was then centrifuged at 1000×g for 3 min at room temperature in a Hettich universal K2S centrifuge and three distinct areas formed. Organic material floated to the top of the gradient, soil particles and other heavy objects formed a pellet while the spores were usually seen as a band of varying width someway down the gradient which was removed with a Pasteur pipette. Additional layers within this band contained other material of similar density or other spores of a slightly different density.
7. The spores were then washed through a silk screen cloth filter (70um) to remove the sucrose which would otherwise cause reduction of spore germination due to osmotic shock.

4.1.3 Root staining to visualize vesicular-arbuscular mycorrhiza

Methods for clearing and staining mycorrhizal roots were assessed. The method which

gave best results with the least hazard was that taken from Koske and Gemma (1989) :

I. Clear with 2.5% KOH (90°C, 20 min)

ii. Bleach with 3.5% ammoniacal H_2O_2 (20 min)

iii. Acidify in 1% HCl (pH 0.65) overnight.

iv. Stain with 0.05% trypan blue dissolved in 70% acidified glycerol (90°C, 20 min)

v. Destain in 70% acidified glycerol

To show that the grasses to be used in the trial are naturally mycorrhizal, roots from *Trachypogon spicatus* and *Themeda triandra* plants collected from indigenous grasslands were stained and showed a high degree of VAM infection. Stained roots from *Trachypogon spicatus* are shown in Figure 4.3.

Figure 4.3 Stained *Trachypogon spicatus* roots showing internal VAM hyphae.

4.1.4 Growth trials of *Trachypogon spicatus* and *Themeda triandra* on minespoil soils

4.1.4.1 Inoculation and growth of plants used for the propagation of the mycorrhizal inoculum

As described, spore amplification using tomato and maize plants to amplify VAM inoculum was necessary. The mycorrhizal spores were extracted (wet sieving) from rhizosphere soils collected from the Cape Flats region, Beaufort West, and Worcester. The coarse wet sieving extract from the 70μm filter was used as inoculum and as the quantity of inoculum was small, it was deemed essential that all the potential inoculum, plus microflora be used for efficient inoculation. The Large and Small spores from Pietermaritzburg were manually separated, after wet sieving and sucrose gradient centrifugation, and the two spore types were used separately as inoculum.

The sand for planting was thoroughly washed and as much organic matter as possible removed before the sand was placed into pots of 25cm diameter. These pots were autoclaved at a temperature of 120°C for one hour to effect sterilization. A layer of coarse spore extract (CF, BW and WOR) or 50 spores, both LRG and SML were placed into the sand about one third of the way down the pots. To improve the chances of VAM infection and provide a better microflora for the CF,WOR and BW treatments, a thin layer (~ 2-3 mm) of each rhizosphere soil was placed half way down the pots. A soil wash filtered through a 70μm filter was used to give a natural microflora to the LRG and SML spore inoculum pots. Each group was used to infest 8 pots containing tomato seeds and 8 pots containing maize seeds giving a total of 80 propagule propagation plant pots. The tomato and maize seeds were planted three to a pot above the level of the inoculum. Pots were watered with 3/4 strength Hoaglands solution (Appendix 6) modified to contain a very low phosphate level of initially 20mgl^{-1}. The pots were then watered daily to prevent desiccation. Each week the pots were thoroughly watered to flush out residual Hoaglands solution and a further 50 mls was added. During the literature search it was discovered that VAM do not grow well in very moist conditions, so after two weeks the watering regime was reduced to twice weekly, plus flush out. After a month the phosphate level in the

Hoaglands solution was reduced to zero to encourage the growth of VAM in the pots.

All plants initially developed signs of phosphate depletion and was most pronounced in the tomato plants, as shown in Figure 4.4. The first sampling of propagule propagation plants for mycorrhizal infection commenced after a month of growth. A spare plant showing signs of recovery from phosphate depletion (Figure 4.4), from a LRG spore pot was removed and stained with trypan blue. Distinct blue hyphae (Figure 4.5) could be seen in the roots and signs of arbuscules were present, although these did not visualise well in the photographs. Plants from other treatments were sampled with similar results and the VAM infected plants were then left to grow and sporulate. The level of infection was however low, with a maximum of 20% of the total root length, while most plants tested were only in the 10% range. Some of the plants that did not become mycorrhizal died from phosphate deficiency. Root staining of these dead plants showed no signs of mycorrhizal activity.

The plants were allowed to mature for a further four months at which stage some of the plants were tested for sporulation. None could be detected in the plants tested and so it was decided to stimulate sporulation by simulating drought conditions. The plants were only watered when they showed signs of wilting. This drought stressing was completed three times by which stage the plants showed signs of sporulation, although on wet sieving, the level of spore collection was very low. To complete the first trial, it was decided that root fragments as well as spores would be utilized, to provide a greater inoculum base. It has been demonstrated that some types of mycorrhizal hyphae can act as infection propagules (Friese and Allen, 1991), which would supplement the low spore inoculum.

4.1.4.2 Trial 1 preparation and seed germination

The first grass growth trial, done on the mine reclamation "topsoil", commenced with a germination test of both *Trachypogon spicatus* and *Themeda triandra* seeds. Seeds from each species were placed on a circle of filter paper in a petri dish which was then moistened and the dish covered. The filter paper was re-moistened as necessary during the germination process.

Plate 1

Plate 2

Figure 4.4 **Comparison of a tomato plants showing signs of phosphorus depletion and plants recovering after becoming mycorrhizal**

Plate 1: Tomato plants showing signs of phosphorus depletion

Plate 2: Mycorrhizal tomato plants recovering from phosphorus depletion

Plate 1

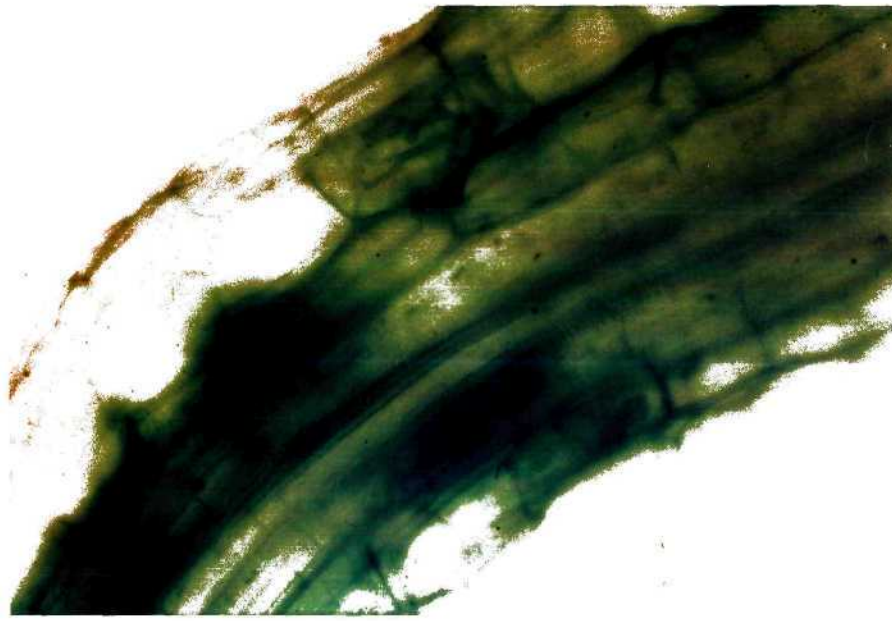

Plate 2

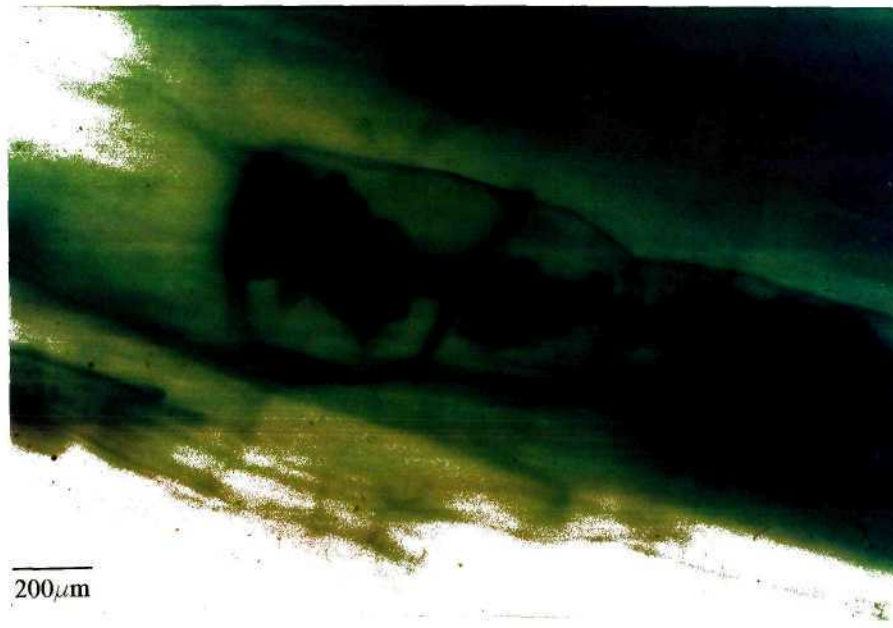

Figure 4.5 **Mycorrhizal tomato plants roots stained with trypan blue**

Plate 1: VAM hyphae can be seen growing along cell boundaries

Plate 2: Formation of arbuscules is clearly visible

Trachypogon spicatus took from 10 and 14 days to germinate with only 14 out of 500 seeds germinating, a 2.8% germination. *Themeda triandra* took four to five days to germinate and of 50 tested, 33 germinated giving a germination rate in of 66%. These tests were used to estimate the seed requirement for the trial.

Soil collected from a freshly topsoiled reclamation site at Klienkopje mine Witbank, was used as the minespoil topsoil for the trial. Thirteen 15cm diameter pots per group (ie: inoculum from samples from CF, WOR, BW, LRG, SML and CONtrol [autoclaved inoculum]) per grass species, were filled with mine spoil topsoil and autoclaved at 120°C for one hour. These pots were then covered for a week to allow any volatile compounds generated by the autoclaving process to dissipate. During this period *Themeda triandra* and *Trachypogon spicatus* seeds were germinated ready for the trial. For each of six days, 600 *Trachypogon spicatus* seeds and 50 *Themeda triandra* seeds were set in petri dishes to germinate. In this way the *Trachypogon spicatus* seeds would germinate a week after the *Themeda triandra* seeds. For *Trachypogon spicatus*, an extra 400 seeds were set on the first day to ensure that sufficient seedlings would germinate by planting time, since germination time varied.

4.1.4.3 Planting, inoculation and maintenance of grasses in Trial 1

When the *Themeda triandra* seedlings had germinated, they were planted and inoculation was commenced. Each day one of the inoculum groups (CF, WOR, BW, LRG , SML and CON) was used to inoculate 13 of the sterilized minespoil soil pots. As the procedures were the same for all six groups, only the initial Cape Flats inoculum planting is fully explained:

Two pots each of the eight tomato and maize plants were extracted for roots and spores. The sand around the roots was gently washed into a 10 litre bucket and the roots retained. All the remaining sand was emptied into the bucket and covered with water. This was swirled to release any root fragments not already separated and these were added to the rest of the roots. The remaining sand mixture was then subjected to wet sieving (Appendix 1) and the coarse spore separation from the 70μm sieve was retained. Separated roots were then cut into 1cm pieces

ready for use as inoculum. When all four pots were extracted, the root pieces were mixed together and this was used as the initial inoculum for the *Themeda triandra* seedlings. Coarse spore suspensions (taken directly from the filters, without being subjected to sucrose gradient centrifugation) from the four pots were also mixed and then stored at 4°C for secondary inoculation after two weeks.

The root pieces were divided into 20ml portions with the remaining roots used for inoculation of two tomato and two maize propagule propagation pots. This was to ensure that inoculum would be available for the second trial in case the present propagule propagation plants died before its completion. The root pieces were placed in the sterilized minespoil soil about half way down the pots. *Themeda triandra* seedlings were then planted above the inoculum and the pots watered. Ten pots were planted with only one seedling per pot, and three had three seedlings per pot. These were to be used to supplement for any seedlings that did not survive the initial planting. The other five groups were planted and inoculated at daily intervals in the same manner. For the control group, spare root fragments had been set aside and these were autoclaved and stored at 4°C for a week before use. This was to prevent any modification of the roots, due to autoclaving, from affecting the seedlings when planted.

The *Trachypogon spicatus* seeds were germinated and these seedlings were planted with the six groups of inoculum in the same manner as the *Themeda triandra* seedlings. All plants were watered twice weekly and after two weeks of growth, the seedlings were re-inoculated with the coarse spore suspension that had been made up to 100 ml and stored at 4°C after extraction. This was done by making four holes in the soil of each pot, angled in towards the root to a depth greater than half the pot. These holes were then injected with a total of 5 ml of the inoculum to allow the inoculum to settle below root depth. Once the inoculum had drained into the soil, the holes were filled in and the pots were watered to compact the soil and prevent the holes reopening and the spores desiccating. The grass seedlings were maintained through twice weekly watering for a total of four months before being harvested. New propagule propagation plants previously inoculated with root fragments were also reinoculated with the coarse spore suspensions and then maintained with the same regime as the original propagule propagation plants.

Any seedlings which died within the first three weeks were replaced from the spare seedlings. Most of the plants that died for reasons other than nutrient depletion did so within the first two weeks. Plants dying from nutrient depletion first showed signs of nutrient, especially phosphate, deficiency, (purple leaf colouration) but death generally took over a month to occur. These plants were not replaced.

4.1.4.4 Harvesting of Trial 1

Harvesting commenced four months after planting and the *Themeda triandra* plants inoculated with the Cape Flats preparation were harvested first. The soil was carefully removed from the pots in one piece and the root and soil gently separated by washing in a trickle of water. Separated plants were then stored in sealed plastic bags to prevent desiccation.

Roots and shoots were separated, their lengths measured and the results recorded (Appendix 7) . Measurement was completed manually as both roots and shoots were fairly short. Any roots longer than one centimetre were measured while both new and old shoots were measured, as some of the older shoots had died. This was more prevalent in plants that had shown extended periods of nutrient depletion, assumed to be plants that did not become mycorrhizal. Most of the CF plants had recovered from nutrient depletion fairly early and were considered to have become mycorrhizal. These mycorrhizal plants were in general larger and greener than the plants that had not become mycorrhizal (Fig. 4.6). Once measured, the roots and shoots were dried overnight in a drying oven at 60°C and then weighed to establish their dry weights (Appendix 7). Both roots and shoots were then stored in sealed plastic bags for chemical analysis. Some of the groups had spare plants that had not been used to replace dead plants and these root systems were stored at 4°C, for staining to determine if VAM were present. The same procedure was completed daily on the other five groups of plants.

Trachypogon spicatus trial plants were ready for harvesting two weeks after the *Themeda triandra*. The harvesting was completed in the same manner as for the *Themeda triandra* trial, and results recorded in Appendix 7.

Plate 1

Plate 2

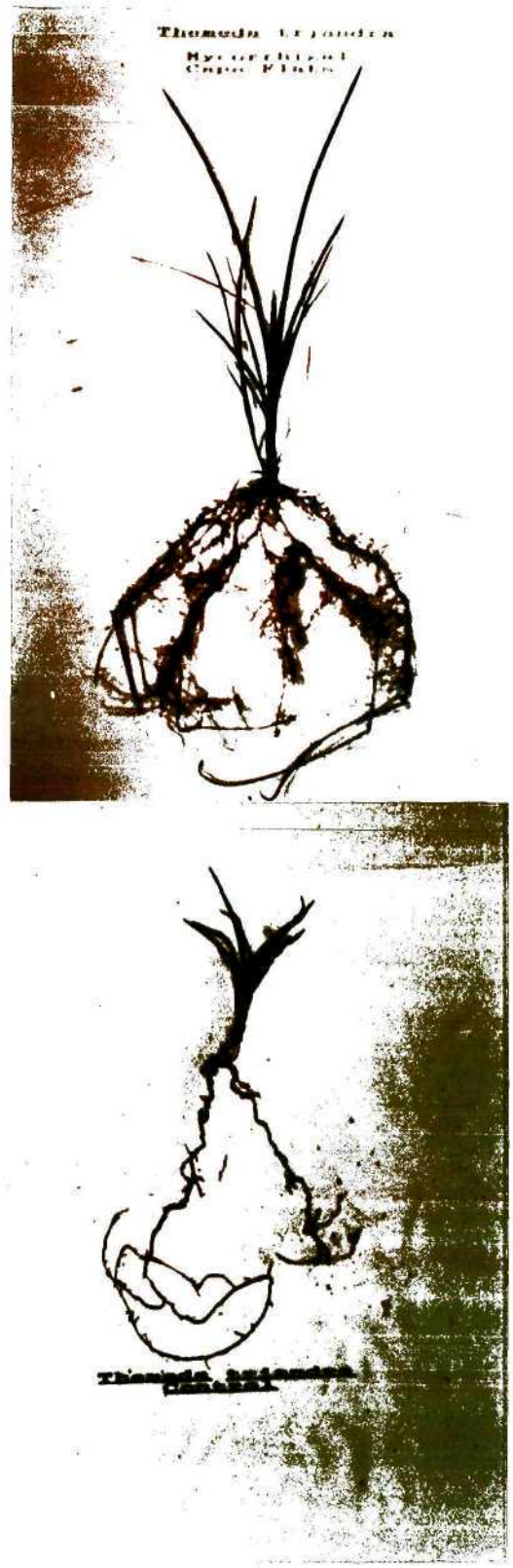

Figure 4.6 **Trial 1** ***Themeda triandra*** **mycorrhizal and control plants.**

Plate 1: Shows the longer and much more branched nature of the mycorrhizal roots and less stunted stems compared to the control plants

Plate 2: Control plant

In week two after harvesting *Themeda triandra*, the plants stored for root staining were analysed. Portions from plants assumed mycorrhizal showed low levels of mycorrhizal infection, while the more stunted plants that had remained nutrient depleted (purple), showed no sign of mycorrhizal activity.

The plant that appeared to be most mycorrhizal from the initial staining was subjected to further analysis. One centimetre root segments were stained for mycorrhiza and of fifty segments stained only five showed signs of blue stained mycorrhizal hyphae in the cortex. Some arbuscules could be seen penetrating beneath the cell walls of a few cells. The infection rate appeared to be low, in the range of 10%. As there was a limited quantity of root material available, a full statistical analysis of the percentage of roots becoming mycorrhizal could not be performed. From further tests on the roots available, the percentage of mycorrhizal roots in the grasses was between 0 and 10%.

4.1.4.5 Harvesting of Trial Two

Trial Two was planned as a repeat of Trial One, but the stunted nature of all the plants indicated that some fertilization was needed to improve the growth of the grasses. Section 2.3 showed that the optimum range of phosphate fertilization would be equivalent to adding 50-100 kgs P/ha. Maximum VAM spore production was seen at these levels, so good VAM infection could be anticipated. To minimise the possibility of VAM inhibition, caused by high phosphate levels, addition of the equivalent of 50 kgs P/ha was chosen. Potassium fertilizer was added at 200 kgs/ha and nitrate fertiliser at 200 kgs/ha. These were the middle level (level 2) used in the fertility trial plots (Section 2.1.1). The fertilizers used were potassium hydrogen phospate ($K_2H PO_4$), potassium nitrate, and ammonium nitrate, ammounts of which were calculated to give the equivalent of 50 kg/ha of P, 200kg/ha of K and 200kg/ha of N.

Planting, inoculation and maintenance of the second trial were all carried out in the same manner as in Trial One for both *Themeda triandra* and *Trachypogon spicatus*. To obviate having to transfer any plants from spare pots to trial pots, 15 identical pots were planted, and all

Plate 1

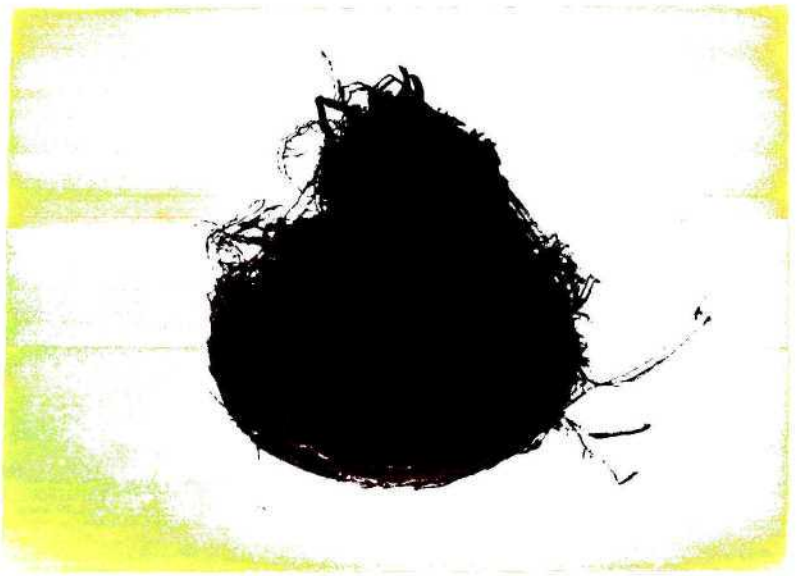

Plate 2

Figure 4.7 Trial 2 Comparison of *Themeda triandra* mycorrhizal and control root systems.

Plate 1: Shows the denser and more branched nature of the mycorrhizal root system compared to the control

Plate 2: Control plant roots

Fertilization has greatly increased the root lengths compared to *Themeda triandra* plants of Trial 1, shown in Figure 4.6.

growth was greatly improved by the addition of fertilizers as was expected. The root systems of mycorrhizal plants were much larger and more branched than the non-mycorrhizal control plants (Figure 4.7). After removing the roots from the soil, the roots and shoots were again separated and placed into plastic bags for measurement. As the root network was much more extensive, the measurements took several days to complete. Samples were stored at 4°C during this period to maintain freshness. The roots were then dried, weighed and the results recorded (Appendix 7).

4.1.4.6 Chemical analysis of harvested plants

Before chemical analysis, the dry roots and shoots were digested. This was done using a modified Kjeldahl digestion procedure (Appendix 9). Using aliquots from this digest, crude protein and phosphorus were analysed on a SCALAR segmented flow auto-analyser (Appendix 10) and copper, iron, manganese and zinc were analysed using a VARIAN model 20 atomic absorption spectrophotometer (Appendix 11). All methods are AOAC(Association of Official Analytical Chemists) approved and according to the instrument manufacturer's recommended procedures. The full results are presented in Appendix 7.

In Trial One, the Kjeldahl digest was completed using a mercury catalyst that contained potassium which prevented the measurement of plant potassium levels in Trial One. To eliminate this problem, a non-catalytic Kjeldahl digest was performed on the samples from Trial Two. The hydrogen peroxide used was changed from 100 volume to 200 volume to increase the oxidising potential and catalyst was omitted. A further modification to the analytical procedure was to eliminate the sample weight variable and use a standard root and shoot sample weight for all sample digests. Nitrogen, phosphorus, potassium, iron, manganese and zinc levels were assayed in the same manner as in Trial One and the results recorded in Appendix 7.

4.2 Results and Discussion

Initial values for the percentage VAM infection of the propagule propagation plants were very disappointing. Most researchers indicate that the level should be in the range of 50 - 70 percent for tomato and maize. However, the low infection level of 10-20% was perhaps not unexpected, as the initial watering regime was incorrect and VAM infection is reduced by excessive water (Stenlund and Charvat, 1994). This information was only accessed after commencement of the trials. Infection rates may also have been affected by the very low level of phosphorus supplementation of the sand. Recent reports (Diaz and Honrubia, 1993) indicate that very low levels of phosphorus reduce the infection rate of VAM, with 6mg P/ kgs (about 14 kgs P/ ha) soil, giving maximum infection rate for *Tetraclinis articulata*. Section 2.3 showed that, under local soil conditions, the optimum level of phosphorus for maximum VAM infection,, would be in the range of 50-80 kgs P/ ha for the grasses studied. The low level of infection would also have resulted in low levels of sporulation of the infective VAM, with the result that the inoculum did not have as many infection propagules as was expected. Hence root fragments as well as spore extracts were used to allow maximum numbers of infective propagules.

4.2.1 Trial 1 Results

In this trial, in order to examine the treatment effect on both of the grasses studied, no nutrient supplementation was provided. The low soil fertility was obvious from the stunted growth of the grasses, which all initially showed distinct signs of nutrient depletion.

4.2.1.1. *Themeda triandra* Trial 1

A number of visual observations of the *Themeda triandra* growth were made. The purpling of the leaves in these grasses showed that the main deficiency was lack of phosphorus. Plants grown on the Cape Flats plant inoculum were however much less affected by this deficiency and most of the plants recovered, becoming greener. Some of the grasses in this treatment did not become mycorrhizal and stayed purplish and stunted, resulting in a wide range

of root and shoot lengths. Plants proven to be VAM infected (Figure 4.8) had greener leaves and the root architecture differed from the non-VAM infected control plants in that the VAM infected roots were much more branched and the total root length was much greater than those of non-VAM infected plants (Fig 4.6). The number of plants assumed to have become mycorrhizal in the other treatments was much lower. Plants assumed not to have become mycorrhizal, resembled those from the control group which were nutrient depleted and had a single long undivided root.

Statistical analysis using an F test and Tukey's pairwise comparison was completed on the raw data tabulated in Appendix 7. The statistical summary of this data is presented in Appendix 8

Themeda triandra plants inoculated with the Cape Flats (CF) inoculum out-performed all the other treatments in physical parameters (root and shoot lengths and masses). Both root and shoot lengths and dry masses were all significantly higher than any of the other treatments. However, the root to shoot ratio was not significantly lower than the control as had been expected from literature reports (Allen, 1991).

Figure 4.8 ***Themeda triandra*** **roots showing Vesicular-arbuscular mycorrhizal infection**

Roots from Trial 1 *Themeda triandra plants* infected with Large spore inoculum, stained with trypan blue, show blue hyphae running along plant cell walls. The swelling of hyphae into the cells is possibly the start of arbuscule formation.

This may well be due to the low level of root colonization, as high levels of colonization are needed to cause a significant change in the root architecture. All treatments tended to have a higher root:shoot ratio than the control, although only the plants inoculated with Beaufort West (BW) inoculum were significantly greater. This increase in root:shoot ratio at low VAM colonization levels could possibly be due to root division instigated by VAM, causing greater root length.

Nitrogen levels in both roots and shoots of plants inoculated with either Cape Flats or Worcester (WOR) inoculum, were significantly greater than in the other four treatments. Beaufort West, Large (LRG) and Small (SML) spore inoculum treated plants showed no significant nitrogen difference from the control. This is probably due to the fact that mycorrhizal rhizosphere flora is usually rich in nitrogen fixing bacteria (Pacovsky, 1989), which allowed for greater nitrate absorption.

A problem arose, with respect to the phosphate levels measured. Only the plants inoculated with Worcester inoculum showed a significantly higher concentration of root phosphorus. None of the treatments were significantly different from the control with respect to shoot phosphate concentration. The phosphate concentration in mine spoil soil is extremely low. Hence any advantage that VAM association may have in phosphate absorption is offset by the low level of phosphate that was absorbed. Both the mean dry weight and phosphate content of the plants inoculated with the Cape Flats inoculum were nearly twice that of the control plants. Hence the total amount of phosphorus contained in a Cape Flats plant root system would have been nearly four times the amount of phosphorus contained in the control plant root systems. As seedling growth is a rapid growth stage, the phosphate absorbed was immediately used for growth. Phosphate probably limited plant growth even in the VAM-associated plants, due to the low soil phosphate level. Hence the level of phosphate per gram of dry root would not be much greater in the VAM plants than in the control. This is probably why the plants inoculated with Cape Flats inoculum grew larger than the control, but did not have significantly more phosphate per gram of dry weight than did the control.

Trends in plant iron content were similar to those of phosphorus, with plants inoculated

with either Cape Flats and Worcester inoculum containing significantly more root and shoot iron than the control. Root iron levels of these two treatments were significantly higher than the rest of the treatments. Shoot iron levels of plants inoculated with Beaufort West or Large spore inoculum were not significantly lower, but the iron level was only half that of the plants inoculated with Cape Flats and Worcester inoculum. VAM have been linked to enhanced iron uptake (Cress *et al.*, 1986).

Manganese showed almost the same distribution patterns as iron, with the roots of plants inoculated with Cape Flats or Worcester inoculum having significantly greater concentrations than other treatments, while in shoots, only the Cape Flats plants were significantly higher. Uptake of manganese in manganese deficient soils, has been shown to be increased by VAM infection, while in soils with toxic levels of manganese, uptake can be reduced (Pacovsky, 1986; Raju *et al.*, 1990b).

There were no significant differences in zinc levels between any of the treatment's shoots and those of the control. In roots, the small spore inoculum had a significantly higher level of zinc than the control, although the reason for this difference is unknown.

4.2.1.2. *Trachypogon spicatus* Trial 1

Trachypogon spicatus responded similarly to *Themeda triandra* with respect to VAM colonization. However, the number of plants becoming mycorrhizal , and the level of infection in *Trachypogon spicatus* were less than in *Themeda.* The Cape Flats inoculum again appeared to give the highest infection rate which was however less than 10%. The small root systems made it impossible to take sufficient roots for statistical analysis of infection rates without affecting other parameter measurements. Probably only eight of the ten plants inoculated with Cape Flats inoculum had become mycorrhizal at all and most of these had very low infection levels. In three of the treatments, Worcester, Small spore and the Control, only five of the plants survived, while in the other two treatments up to 50% were either non-VAM, or had very low VAM infection.

Grasses supplemented with Cape Flats inoculum were the only treatment to exhibit

significantly longer root lengths and overall dry masses than the control, but shoot lengths were not significantly greater. Although plants inoculated with Cape flats inoculum tended to have greater physical parameters (longer root and shoot lengths and greater root and shoot masses), there were no significant differences between them and any of the treatments other than the control.

Of the nutrients analysed, only the shoot phosphorus and shoot iron levels showed any significant differences in any of the treatments. Surprisingly, the shoots of plants inoculated with Cape Flats inoculum were significantly lower in phosphorus than the control or plants inoculated with Beaufort West or Large spore inoculum. Reasons for this can only be speculated upon. Possibly the phosphorus from the dead leaves, common in the non-VAM plants, was translocated to growing shoots. This would increase the unit dry weight level of phosphorus in the lighter non-VAM shoots, so when expressed in this form, non-VAM shoots show high phosphorus levels. Iron showed a similar but less marked trend, and the only significant difference was between the Cape Flats and the Large spore plants.

In those mycorrhizal grasses, the low levels of VAM infection, did not permit clear expression of a number of mycorrhizal effects. It was expected that the root to shoot ratios would indicate that VAM plants had shorter, more divided root systems than the non-VAM plants. This was postulated on the theory that VAM hyphae absorb large amounts of nutrients, some of which are transferred to the plant roots in exchange for fixed sugar compounds. Hence an extensive root system becomes less critical. With the low level of VAM infection and the nutrient limiting growth conditions, this did not occur. No significant shortening of root systems due to VAM infection could be detected.

The mycorrhizal plants grown on the Cape Flats inoculum were visually distinctly healthier than the control plants. However, the expected significant increase in phosphate level between VAM and non-VAM control plants, did not arise in either grass. This could be attributed to low VAM colonization and low soil nutrient levels. As VAM colonization was low, widespread utilization of the soil phosphorus by VAM was not possible. Additionally, the low level of soil phosphorus was probably limiting, even with VAM infection and microflora.

The death of so many non-VAM *Trachypogon spicatus* plants would indicate that VAM infection in *Trachypogon spicatus* is more essential for growth than in *Themeda triandra*.

4.2.2 Trial 2 Results

In Trial One, the plants were extremely stunted and the three major inorganic nutrients (nitrogen, potassium and phosphorus) were at levels limiting to growth. Addition of nitrogen and potassium at levels sufficient to give good plant growth and low levels of phosphorus would obviate this problem, as now only phosphorus would be limiting. The supplementation of these fertilizers had a major effect on growth patterns of the grasses and VAM colonization of the treatments.

4.2.2.1 *Themeda triandra* Trial 2

Fertilization reduced the number of plants that died and there were few signs of nutrient depletion in any of the treatments, including the control. The visual difference between the shoots of VAM infected plants and non-VAM plants was not as obvious as in Trial One. Plants that had become mycorrhizal were generally taller and had a greater number of shoots than the control. It was noted however that the number of dying (browning) leaves on the control and non-mycorrhizal plants was higher than on the plants that had become VAM infected. Root differences were more pronounced, with the mycorrhizal roots being denser, heavier and much more divided than Control plant roots (Figure 4.7). This within treatment difference in root size between VAM and non-VAM plants, caused high standard deviations in the VAM treatment.

Statistical analysis of the data collected from the trial was completed in the same manner as for Trial One. The statistical summary of the data is presented in Appendix 8.

Plants inoculated with Large spore inoculum, had significantly longer root systems than any other treatment, while Worcester and Beaufort West inoculated roots were significantly

longer than the control. Root dry mass figures showed the same trends of significance. Both shoot length and dry mass of the plants inoculated with Cape Flats inoculum were significantly lower than the other treatments, while the shoot lengths of large spore grasses was significantly higher than the control. All treatments had significantly higher root:shoot ratios than the control.

This is a clear change from Trial One, where plants inoculated with Cape Flats inoculum had significantly superior physical parameters to the other treatments. In Trial Two, the plants inoculated with large spore inoculum demonstrated the best physical results. This was not totally unexpected, as Thomson *et al.* (1992) demonstrated that some VAM species are more susceptible to phosphorus inhibition than others. The low level of infection of the plants inoculated with Cape Flats inoculum (less than 5%) and the number of plants apparently not becoming mycorrhizal indicated such an inhibition.

The results of the elemental analysis was somewhat confusing, as the levels of a number of elements was not higher in the more mycorrhizal plants, as would have been expected.

Nitrogen was not limiting, so the fact that the root nitrogen levels of the large spore inoculum roots was significantly lower than the control was perhaps not unexpected. The larger mycorrhizal plants use more of the nitrogen for growth and so there is less excess. This could manifest itself as a lower level of nitrogen per gram of dry weight of root. The shoots showed little significant differences in nitrogen levels except that the Small spore inoculum analysed significantly higher than the controls. This could not be explained. It is confusing that the Cape Flats treatment yielded plants with significantly higher nitrogen levels than any of the other treatments, including the control. A possible explanation could be that the Cape Flats microflora contains nitrogen fixing bacteria, which are common in normal VAM associated microflora. This would increase the amount of absorbable nitrogen that these plants could absorb, although there is no proof to sustain this hypothesis.

Even more difficult to explain is that the root phosphorus and potassium levels where higher in the Cape Flats and Control groups than in the other treatments. The Cape Flats shoots were significantly higher in both phosphorus and potassium than the other treatments. As the

most mycorrhizal Large spore treatment had lowest unit levels of nitrogen, phosphorus and potassium, there may be a correlation between VAM infection and the levels of these elements. Similarly, potassium would also not be limiting and so the potassium would be more widely distributed in the larger mycorrhizal plants, so appearing to have relatively lower unit levels. However, it was assumed that the low level of phosphorus added would be limiting, so VAM infected plants should show significantly higher phosphorus levels (or at worst, no different) to non-VAM plants. As the VAM infection levels were relatively low, the enhanced uptake of phosphorus associated with high levels of VAM infection may not become evident. This however would indicate that phosphorus levels should be approximately equal in all plants with the VAM infected plants being larger as they absorb more phosphorus. Reasons why VAM plants have a lower unit dry mass level of phosphorus when VAM infection is low, can only be speculation. The only reason that this author can postulate, is that the low level VAM infected plants have a heavier basic structure, hence the mass per unit volume of plant material is higher in VAM infected plants. In dry plants therefore, the ratio of elements to structural material would be lower in VAM plants, which is not offset by their enhanced phosphorus uptake.

Plants inoculated with Cape Flats inoculum had significantly higher levels of root iron than did the control grasses, although in the shoots, there were no inter-treatment differences. This mirrored the *Themeda triandra* trend of Trial One. As Cape Flats inoculated plants had much less VAM infection in Trial Two, this would suggest that, in this case, the enhanced iron uptake may be due more to the microflora of the treatment rather than the VAM.

Manganese levels were significantly lower in the mycorrhizal plants' roots than in the control's, while the Cape Flats inoculum plants had similar levels to the control. In the case of the shoots, there were no differences in manganese levels between the Control and the more mycorrhizal plants although the Cape Flats inoculated plants analysed significantly higher than the others. This could not be explained. Results from zinc analyses were very similar to those of manganese, although only the large spore inoculum plants showed significantly lower root zinc levels than the control.

4.2.2.2 ***Trachypogon spicatus* Trial 2**

Again, *Trachypogon spicatus grasses* showed lower levels of VAM infection than did *Themeda triandra* plants. None of the plant roots tested showed VAM infection greater than 10%, while most were 5% or less. With this low level of VAM infection it was not surprising that there were few significant differences between the treatments and the control. Root and shoot lengths, dry masses and ratio showed no inter-treatment differences. The only physical difference between treatments was shown in the survival rate of the grass plants. Of the 15 planted per treatment, the non-VAM Control and the Small spore inoculated plants only had seven survivors. The other treatments fared better, with Large spore inoculum showing nine plants surviving, while the other three treatments had eleven to twelve survivors. This may have been due to the mycorrhizal infection, but is as likely due to the rhizosphere flora associated with VAM infected plants. Plants with more mycorrhizal infection, were in general slightly larger than control plants, but the difference was not as marked as in the *Themeda triandra* grasses.

Likewise, nitrogen, potassium and phosphorus analyses detected no significant differences in root or shoot levels of these elements between any of the treatments and the control. Moreover, root phosphorus levels showed no differences between any treatment. The phosphorus level in shoots of the Cape Flats inoculum treatment was significantly higher than in either the Beaufort West or the Large spore inoculum treatments. In the case of unit mass potassium levels though, the reverse held true, with the Cape Flats roots having a higher level than Beaufort West and Large spore inoculum treatments, while shoot levels showed no significant differences.

Iron levels in both roots and shoots tended to be higher in the control than in the mycorrhizal treatments, although only Beaufort west was significantly lower in both cases. The reason for this is unclear. Zinc levels followed the same trend with the more VAM infected treatments (Worcester, Beaufort West and Large spore) being significantly lower in zinc than the control. Manganese levels in the roots were not significantly different between any of the treatments or the control, while in the shoots, manganese levels were only significantly higher in the Cape Flats than the Beaufort West inoculum treatments, with none showing any differences

from the control.

As the levels of VAM infection of *Trachypogon spicatus* were low in this trial and as fertilisers had been supplemented, little difference were seen between the control and any of the treatments.

4.3 Summary

A major limitation with these two sets of trials was caused by the initial watering and phosphorus fertilization regimes imposed on the plants used to amplify the initial inoculum. This resulted in low infection rates in the inoculum amplification plants and hence propagule levels were low in the trial inocula. The eventual effect was to produce infection level ranges of 0-10%, with some of the plants from the five VAM inoculum treatments showing no signs of mycorrhizal activity. Not only did this influence results, but also caused high within-treatment standard deviations because non-VAM plants were often vastly different in size from the infected plants within a treatment. In Trial One, the fact that a large number of *Trachypogon spicatus* plants that had not become VAM infected died, indicated that VAM do play a part in plant survival. The analysis of variance incorporated a missing plot system, any dead plants did not contribute to the analysis. The small size of the plants and probable low nutrient levels, are not shown in the results.

In extremely poor soils such as the minespoil soil used in the trials, VAM infection resulted in a significant increase in the physical size and weight of both species of grasses. This was most marked with the inoculum from the Cape Flats rhizosphere soil, even though the VAM infection level was 10% or less. The low infection levels did not allow the demonstration of the enhanced uptake of a number of the nutrients analysed, in particular phosphorus. Total phosphorus uptake was much greater in the Cape Flats inoculum plants than in the controls. However, the low VAM infection levels did not cause sufficient uptake to increase the unit dry mass phosphorus levels above those of the control. This indicates that despite the enhanced uptake, phosphorus is used as rapidly as it is absorbed, and is therefore a limiting nutrient.

The two trials showed a marked change in the dominance of treatments with respect to the physical parameters of the *Themeda triandra* plants. There was a distinct change in the dominant treatment, from the Cape Flats treatment in the first trial, to the Large spore treatment in the second trial. Thomson *et al.* (1992) demonstrated that some VAM infection and colonization are suppressed more than others by higher phosphate levels. Indications are that the VAM in the Cape Flats inoculum was adversely affected by the increase in phosphate level from Trial One to Trial Two. As the region from which this inoculum was extracted is very sandy and extremely low in nutrients, it is quite likely that higher phosphate levels could inhibit those VAM present. Further investigation in to the level of phosphate at which Cape Flats VAM are most effective and if lime supplementation allows for higher phosphate levels before inhibition occurs, could be considered. The two grasses displayed very similar reactions to VAM infection in Trial One, where the Cape Flats inoculum gave the greatest increase in growth of both of the grasses. Because of the low levels of infection, this increase in growth was not reflected in the uptake of nutrients. In the second trial the benefit of VAM infection was only noticeable in the *Themeda triandra*.

Mortality of *Trachypogon spicatus* was much greater than in *Themeda triandra* and in both cases the plants that died were all non-VAM infected. From these results it would appear that VAM do confer a survival advantage, especially in low nutrient soils, where plant sizes were significantly higher in the VAM infected plants than in controls. Of the two grasses, *Themeda triandra* was much hardier than *Trachypogon spicatus*. The *Themeda* seeds had a much higher germination rate, less seedling mortality after planting and appeared to respond better to VAM infection. The best combination of symbionts in the low fertility soils of Trial One, proved to be *Themeda triandra* infected with the Cape Flats inoculum. This was modified by the higher fertility levels in Trial Two, where Cape Flats inoculum seemed to be inhibited, but the Large spore inoculum was not.

Further study is required to assess the mycorrhizal potential of the grasses in the presence of an inoculum with more infection propagules. Higher VAM infection might also demonstrate whether the grasses used show enhanced or controlled uptake of some nutrients as noted in Sections 1.6 and 1.7. The inhibitory effect of elevated nutrient levels on the Cape Flats inoculum

indicates that more work may determine if it is in fact the increase in phosphorus level that caused the inhibition. If so, then a study to assess at what level the Cape Flats inoculum is most effective and if it is more effective than the Large spore inoculum is needed. The effect that lime addition might have on VAM effectivity under the conditions of Trial Two, could also be appraised.

Finally, these trials indicate that VAM infection of grasses is necessary for their survival on low nutrient soils. This implies that in order to rehabilitate minespoil soils with a natural grassland ecosystem, the grasses may need to be mycorrhizal. As there are no VAM infection propagules in the minespoil soils, some form of inoculation would be required.

CHAPTER 5

5.1 General Conclusion

A number of points have been partially resolved by work done during this research, but even more interesting questions have been raised.

The fertility trial results indicated that, under trial conditions, a concentration of phosphate in the region of 80 kgs / ha will cause an increase in the colonization of grasses by VAM. Further increase in phosphate lead to inhibition of the VAM. By adding lime the inhibition of VAM was reduced, so higher phosphate concentrations could be added to the soil without adversely affecting VAM. As VAM appear to be able to assimilate the low concentrations of phosphate in tricalcium phosphate (Cress *et al.*, 1979), the growth enhancement proffered by VAM infection can be made effective with higher soil concentrations of P. Although the links between these two pieces of research seem tenuous, results of the two projects combine well in demonstrating effective use of VAM and fertilizer. The greenhouse trial indicated that VAM infection has a positive effect on grass plant growth and that different VAM cultures are effective at different P concentrations. It was also purported that high P concentrations may cause a decrease in VAM diversity.

So what is the link ? The first greenhouse trial showed that, on unamended soil, the Cape Flats VAM inoculum gave best plant growth which was inhibited by P fertilization in the second greenhouse trial. The Large spore inoculum was more effective at aiding plant growth in the amended soil than was the Cape Flats inoculum. By adding lime to the amended soil, the effective concentration of available phosphate would be reduced. This would reduce the inhibition of VAM infection and also help to maintain the diversity of VAM which this research indicated was also affected by high P concentrations.

The practical implication of this was somewhat inadvertently alluded to in work done by Still (1991). This study indicated that a 'well tended' field with high P concentrations had a

higher VAM colonization and greater VAM diversity than did a 'badly tended' field with low P concentrations. The 'well tended' field had a high level of humus and had been well limed, while the 'badly tended' field was low in both lime and humus. Available P in the 'well tended' field was probably not much greater than in the 'badly tended' field, however the tricalcium phosphate caused by lime addition is not easily leached from the soil and so available P concentrations remain constant. This allows for high infection by VAM and also aids in generating a diverse VAM population. For subsistence farmers, this means that only low levels of P fertilizer need to be added if the soil is well limed and composted, as then P leaching does not occur. Mining companies attempting to re-establish ground cover on strip-mined land may also benefit from VAM colonization. Study of VAM effects on the growth of the grasses, that they wish to introduce, may make revegetation more rapid and allow for lower fertilization than present methods of revegetation. This could save both time and money.

This outlook now implies further research projects. A primary goal would be for trials to determine what levels of lime and P are optimum for maximum VAM colonization, and to determine if at these levels, growth of plants similar to high P fertilized plants could be attained. As indicated in the greenhouse trial, different VAM cultures have different growth effects on the same plant. For optimum growth using VAM therefore, trials must be completed to determine the most effective VAM culture for the plant to be grown.

If future research demonstrates that plants grown with VAM colonization and low fertilization can be competitive to plants grown on highly fertilized fields, the necessity for an convenient method of identification of VAM will become essential. RAPD PCR has worked well with identification of plant species and would be a great asset in the identification of the otherwise problematic VAM fungi. Suggestions for future work would be to first separate pure DNA from the spores of an axenic strain of VAM. This DNA could then be used to develop a working RAPD PCR system. Once a good banding pattern has been obtained, work could commence on the extraction and amplification of the DNA in a single VAM spore.

Appendix 1

Coarse Spore Separation by Wet Sieving

A modified version of that described by Danials and Skipper (Schenck, 1984) was used. Wet sieving is the process whereby soil samples are suspended in water and particles can be separated from the soil into sieves of decreasing pore size. The required particles (in this case mycorrhizal spores) are thus separated from the soil, along with objects of a similar size range.

A quantitative evaluation of spore number can be made in the following manner:-

1. A mass of rhizosphere soil (e.g. 1 kg) is suspended in about two litres of water in a ten litre bucket (volume is not critical as it is sieved off). Once suspended the mixture is swirled by hand and the vortex formed is then stopped by placing the flat of the hand against the flow. The larger and heavier particles will sink rapidly and the rest of the material is filtered through the first sieve (in this case a 2.5 mm mesh size) into a second bucket. The soil left in the first bucket is then washed with about 500ml more water and stirred again, stopped and then the liquid poured through the filter. This step is repeated again to remove all the spores from the soil. Large stones and pieces of organic matter left in the sieve are quickly washed under a stream of water (under pressure, to remove any spores adhering to them) while the strained liquid containing the spores, is collected in the second bucket. The sieved liquid is retained for the next stage.

2. The same process of swirling, stopping and sieving is repeated using a 710 μm sieve. This will remove smaller soil particles and organic matter which would otherwise block the finer sieves. The collected strained liquid is then used for the next sieving.

3. The process in 2. is repeated using a 250 μm filter and the sieving collected, are particles the size expected for sporocarps and some of the larger mycorrhizal spores,. Sporocarps are not common and difficult to identify compare to spores, but this extract can be used for inoculum. This sieving also removes smaller particles that would otherwise block the final fine sieving.

4. The final sieving is done in the same manner as 2. with a 70 μm mirror cloth (silk screen fabric) filter. This filters out all but the very small or immature mycorrhizal spores. It is important at this stage to make sure that the flow of the liquid through the filter is kept at a rate that allows rapid drainage. If it is too slow or fast the filter clogs up and greatly reduces the speed of filtration. This is also the reason why filtration through all three sieves simultaneously is not effective. Most of the endomycorrhizal spores are found in this size range and the filtration gives a coarse spore separation that can be used for an inoculum if the spores are not from a single culture.

Appendix 2

Spore count totals for each sample from the Kleinkopje mine fertility trial, listed in fertilizer groups

FERTILIZER	SAMPLE	SPORE TYPE							
		A	B	C	D	E	TOT	HAP	LAP
ZERO	5A	19	17	4	2	2	44	36	8
	5B	21	20	5	4	3	53	41	12
	12A	36	36	4	1	5	82	72	10
	12B	32	32	3	0	4	71	64	7
	14A	25	32	2	2	0	61	57	4
	14B	20	10	1	1	1	33	30	3
	20A	15	11	1	0	1	28	26	2
	20B	21	19	2	4	3	49	40	9
	28A	15	15	3	2	3	38	30	8
	28B	18	16	3	2	1	40	34	6
	34A	18	26	7	2	2	55	44	11
	34B	18	20	7	4	5	54	38	16
P0	2A	22	15	2	2	0	41	37	4
	2B	23	22	3	3	2	53	45	8
	16A	17	14	2	5	7	45	31	14
	16B	12	16	3	0	3	34	28	6
	29A	27	19	5	0	1	52	48	6
	29B	29	30	2	4	6	71	59	12

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Appendix 2 continued

FERTILIZER	SAMPLE	SPORE TYPE							
		A	B	C	D	E	TOT	HAP	LAP
P1	3A	44	35	8	3	9	99	79	20
	3B	43	37	7	3	7	97	80	17
	36A	72	67	7	0	7	153	139	14
	36B	59	96	13	61	3	232	155	77
	41A	69	37	2	5	5	119	106	13
	41B	79	79	11	6	5	180	158	22
P2	4A	13	17	0	0	0	30	30	0
	4B	23	21	1	0	0	45	44	1
	19A	17	19	0	0	0	36	36	0
	19B	20	20	1	0	2	43	40	3
	26A	25	23	0	0	0	48	48	0
	26B	21	22	3	0	0	46	43	3
P3	11A	20	11	0	0	0	31	31	0
	11B	24	25	0	0	1	50	49	1
	31A	14	11	0	0	1	26	25	1
	31B	17	23	1	0	1	42	40	2
	40A	23	31	2	0	0	56	54	2
	40B	26	45	1	0	2	74	71	3

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Appendix 2 continued

FERTILIZER	SAMPLE	SPORE TYPE							
		A	B	C	D	E	TOT	HAP	LAP
L0	6A	49	45	1	1	5	101	94	7
	6B	26	23	2	1	3	55	49	6
	15A	31	43	6	0	1	81	74	7
	15B	20	20	3	0	1	44	40	4
	35A	27	31	0	3	0	61	58	3
	35B	31	29	2	0	0	62	60	2
L1	17A	33	24	2	0	5	64	57	7
	17B	35	44	3	0	1	83	79	4
	24A	20	27	1	0	0	48	47	1
	24B	22	42	1	0	0	65	64	1
	33A	22	20	0	0	2	44	42	2
	33B	22	25	0	0	4	51	47	4
L2	4A	13	17	0	0	0	30	30	0
	4B	23	21	1	0	0	45	44	1
	19A	17	19	0	0	0	36	36	0
	19B	20	20	1	0	2	43	40	3
	26A	25	23	0	0	0	48	48	0
	26B	21	22	3	0	0	46	43	3

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Appendix 2 continued

FERTILIZER	SAMPLE	SPORE TYPE							
		A	**B**	**C**	**D**	**E**	**TOT**	**HAP**	**LAP**
L3	8A	52	45	4	5	4	110	97	13
	8B	40	34	4	5	3	86	74	12
	13A	35	50	4	2	0	91	85	6
	13B	20	20	0	1	0	41	40	1
	22A	42	39	3	0	3	87	81	6
	22B	39	36	5	2	3	85	75	10
K0	21A	15	7	1	0	0	23	22	1
	21B	15	11	1	1	0	28	26	2
	32A	22	12	1	0	0	35	34	1
	32B	24	22	0	2	2	50	46	4
	37A	25	43	1	0	2	71	68	3
	37B	39	42	2	0	0	83	81	2
K1	7A	26	22	2	0	3	53	48	5
	7B	23	22	1	0	0	46	45	1
	25A	25	18	1	0	0	44	43	1
	25B	24	25	2	0	1	52	49	3
	39A	32	42	2	0	3	79	74	5
	39B	32	22	0	0	3	57	54	3

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Appendix 2 continued

FERTILIZER	SAMPLE	SPORE TYPE							
		A	B	C	D	E	TOT	HAP	LAP
K2	4A	13	17	0	0	0	30	30	0
	4B	23	21	1	0	0	45	44	1
	19A	17	19	0	0	0	36	36	0
	19B	20	20	1	0	2	43	40	3
	26A	25	23	0	0	0	48	48	0
	26B	21	22	3	0	0	46	43	3
K3	9A	21	22	1	0	1	45	43	2
	9B	20	18	1	0	0	39	38	1
	18A	24	28	3	0	0	55	52	3
	18B	19	15	1	1	0	36	43	2
	36A	42	39	2	2	1	86	81	5
	36B	34	41	2	3	0	80	75	5
N1	23A	21	13	1	1	0	36	34	2
	23B	29	22	1	0	1	53	51	2
	27A	16	14	1	0	1	32	30	2
	27B	28	32	2	0	2	64	60	4
	30A	19	9	0	0	1	29	28	1
	30B	16	13	2	1	1	33	29	4

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Appendix 2 continued

FERTILIZER	SAMPLE	SPORE TYPE							
		A	B	C	D	E	TOT	HAP	LAP
N2	4A	13	17	0	0	0	30	30	0
	4B	23	21	1	0	0	45	44	1
	19A	17	19	0	0	0	36	36	0
	19B	20	20	1	0	2	43	40	3
	26A	25	23	0	0	0	48	48	0
	26B	21	22	3	0	0	46	43	3
N3	1A	30	39	0	0	3	72	69	3
	1B	24	23	0	0	1	48	47	1
	10A	20	22	0	0	1	43	42	1
	10B	18	23	0	0	0	41	41	0
	42A	22	18	0	0	1	41	40	1
	42B	22	42	2	0	1	67	64	3

A-E are spore types identified in the text. Sample number corresponds to the plot number indicated in the trial site description. TOT is total of all spores for the sample, HAP total of high amplitude propagules and LAP total of low amplitude propagules.

Appendix 3

PCR Buffer solution preparation

PCR buffer is made up as follows :-

			For 10ml
10x Buffer	=	100mM Tris-HCl	0.121 g
		500mM KCl	0.323 g
		20mM MgCl	0.041 g
		0.01% Gelatin	0.001 g
		0.1% triton X100	0.01 g

made up to 10ml with PCR water

dNTP solution preparation

50mls dNTPs at a concentration of 200μM/reaction (2.5μl in 25μl reaction volume) :-

each dNTP	0.5μl
1x PCR buffer.	48.0μl

Appendix 4

Agarose gel preparation

Reagents

Agarose
Bromophenol blue
Distilled water
Ethylenediaminetetraacetic acid (EDTA)
Glacial acetic acid
Sodium hydroxide (NaOH)
Glycerol
Tris-Acetate buffer (50x)
Trizma base

1.2% agarose gel for a mini gel (gel size 10cm^2) is prepared as follows:-

Agarose.		0.33 g
Distilled water.	29.34 ml	
50 x Tris-Acetate buffer.		0.66 ml
Total volume		30.00 ml

Melt the agarose in the buffer at 100°C, cool to 55°C before pouring into gel making tray. Allow to cool and set before use.

To prepare 200ml of 50 x Tris-Acetate buffer for gel:-

Trizma base	48.4 g
Glacial acetic acid	11.4 ml
EDTA	3.72 g
Distilled water	188.4 ml
Total volume	200.0 ml

Dissolve the solid ingredients in the distilled water.

Preparation of running buffer for agarose gel electrophoresis.

1x Tris-Acetate final concentration:-

50 x Tris-Acetate buffer	12.24 ml
Distilled water	600.0 ml
Total volume	612.24 ml

Preparation of 10x loading buffer

NaOH	50.0 mM
EDTA	1.0 mM
Glycerol	2.5%
Bromophenol blue	0.025%

Mix all ingredients by stirring well, store in light protected bottle in refrigerator.

When the gel is ready for use it is placed in the electrophoresis equipment in its carrying tray. The equipment is then filled with running buffer to above the height of the gel, ready for sample application.

Appendix 5

Buffering of phenol

Buffering of phenol is with a Tris-EDTA (TE) buffer, prepared as follows :-

For 100mls buffer phenol

10mM EDTA	0.37g
25mM Tris	0.30g
Distilled water	Make up to 100ml

Dissolve EDTA and Tris in 100ml volumetric flask, then adjust the TE buffer to pH 8.0 and make up to 100ml.

Buffered phenol is made by shaking equal volumes of phenol (melted at 65°C and cooled to about room temperature) with TE buffer pH 8.0. Cooling of the phenol is essential, or the two solutions do not separate out and the phenol cannot be separated from the TE buffer solution. The anti oxidant 8 - hydroxy quinoline is added to the melted phenol before it is mixed with the TE buffer. After settling, the mixture is shaken again and the process repeated three or four times to make sure the phenol is well buffered. The mixture is then left to separate until two clear distinct layers are formed. Buffered phenol can be stored under the buffer and extracted when needed.

Appendix 6

Hoaglands solution

The working Hoaglands solution was made up from stock solutions as follows:-

	Stock conc.	Volume used / litre
KNO_3	0.5M	9ml
$Ca(NO_3)_2$	0.5M	6ml
$MgSO_4$	0.5M	1ml
Micronutrients	As below	0.5ml
Chelated Fe	20mM	1ml
$Na_2H_2PO_4$	80mM	1ml

Micronutrient Stock Solution :

	Concentration
KCl	50mM
H_2BO_3	25mM
$MnSO_4$	2.0mM
$ZnSO_4$	2.0mM
$CuSO_4$	0.5mM
H_2MoO_4	0.5mM

When the stock solutions are mixed, the phosphate is often precipitated by the calcium, giving a faint white haze. Shake solution well just before use to ensure that the phosphate is evenly distributed.

Appendix 7

Legend for the Four Trial sets of Appendix 7

The Six inoculum types are labelled CF = Cape Flats, WOR = Worcester, BW = Beaufort West, LRG = Large spore, SML = Small spore and CON = control (sterile inoculum), indicating the origin of the inoculum.

rt = root, sh = shoot, rt : sh = root to shoot ratio, dms = dry mass, N =nitrogen, P = phosphorus, K = potassium, Fe = iron, Mn - manganese and Zn = zinc

APPENDIX 7

Raw data for Greenhouse Trial I - *Themeda triandra*

TRT	rt lngth	rt : sh	rt dms	sh lngth	sh dms	rt N	rt P	st Fe	rt Mn	rt Zn	sh N	sh P	sh Fe	sh Mn	sh Zn
	cm		g	cm	g	mg/g sample	mg/g sample	mg/g sample	mg/g sample	mg/g sample	mg/g sample	mg/g sample	mg/g sample	mg/g sample	mg/g sample
CF 1	85	1.809	0.0398	47	0.0298	59.221	75.402	24.296	1.736	0.339	74.765	105.906	8.356	0.695	0.386
CF 2	255	2.965	0.3454	86	0.0984	45.698	20.307	10.452	0.903	0.056	95.112	49.360	8.770	0.813	0.163
CF 3	326	2.117	0.2021	154	0.0902	71.554	18.590	19.263	1.215	0.092	121.763	37.727	4.124	1.279	0.171
CF 4	335	4.136	0.2474	81	0.0719	44.394	14.074	15.263	1.218	0.078	123.908	45.591	7.344	1.281	0.231
CF 5	333	4.012	0.2237	83	0.0838	51.582	18.198	17.251	1.279	0.095	118.425	41.313	4.189	1.369	0.212
CF 6	44	1.189	0.0245	37	0.0138	68.612	114.857	75.592	1.404	0.469	136.522	203.333	27.826	1.949	0.391
CF 7	102	2.684	0.0855	38	0.0276	46.608	38.292	31.813	1.049	0.184	83.297	90.435	11.630	0.830	0.391
CF 8	255	1.977	0.0871	129	0.0735	98.037	35.786	25.936	1.563	0.188	123.619	44.653	4.925	2.597	0.186
CF 9	39	1.083	0.0198	36	0.0106	117.424	144.293	72.727	0.667	0.480	200.189	262.170	25.377	4.415	0.406
CF10	333	4.012	0.2241	83	0.0835	51.990	18.050	17.135	1.228	0.091	118.383	41.377	4.132	1.323	0.202
WOR1	101	2.971	0.0361	34	0.0261	71.496	98.947	48.366	1.507	0.424	77.241	88.851	10.345	1.257	0.475
WOR2	88	2.444	0.0182	36	0.0244	89.945	181.154	60.165	2.000	0.522	106.148	133.033	14.795	2.258	0.434
WOR3	88	2.200	0.0465	40	0.0284	58.559	70.989	34.925	1.060	0.258	75.035	83.028	10.845	0.746	0.415
WOR4	57	2.036	0.0166	28	0.019	92.771	197.470	58.554	1.819	0.536	101.947	168.579	15.316	1.300	0.500
WOR5	143	2.424	0.0561	59	0.0425	82.264	59.733	37.861	0.929	0.262	96.659	78.235	6.776	0.760	0.336
WOR6	100	2.174	0.0288	46	0.0349	92.708	104.410	40.278	1.403	0.438	87.364	92.006	7.163	0.656	0.352
WOR7	122	2.773	0.0526	44	0.0295	50.456	63.194	37.319	0.956	0.289	92.881	95.661	8.373	0.929	0.468
WOR8	56	1.400	0.0501	40	0.0267	58.224	64.331	42.216	0.980	0.234	100.637	110.712	17.678	0.880	0.367
WOR9	86	2.389	0.0319	36	0.0217	70.408	94.138	40.533	1.285	0.433	101.889	131.843	10.046	1.157	0.484
WOR10	81	2.382	0.0481	34	0.0197	57.339	58.170	32.141	1.010	0.277	99.239	164.162	12.792	1.091	0.614
BW 1	161	5.031	0.0625	32	0.0201	16.336	11.952	10.272	0.018	0.213	29.453	34.428	1.443	0.463	0.642
BW 2	137	5.074	0.0612	27	0.0204	11.912	13.235	14.346	0.314	0.208	23.824	36.324	2.402	0.466	0.735
BW 3	127	3.432	0.0805	37	0.0282	12.584	9.242	10.311	0.271	0.184	18.404	23.262	1.241	0.511	0.422
BW 4	77	2.406	0.0573	32	0.0197	10.035	12.269	10.838	0.344	0.265	20.152	36.447	3.046	0.052	0.284
BW 5	117	3.250	0.0412	36	0.0205	17.694	20.704	21.602	0.408	0.289	25.317	35.902	2.146	1.005	1.400
BW 6	116	2.367	0.0862	49	0.0301	10.998	8.968	12.146	0.216	0.202	24.751	23.621	12.326	1.189	0.512
BW 7	110	3.548	0.0425	31	0.0201	9.553	17.765	17.176	0.367	0.221	20.199	37.910	3.234	0.612	0.920
BW 8	160	4.444	0.0591	36	0.0279	15.770	13.452	15.635	0.316	0.310	22.079	26.774	2.043	0.513	0.573
BW 9	75	2.500	0.036	30	0.0192	15.083	19.611	13.250	0.378	0.450	23.646	37.396	26.771	0.969	0.604
BW 10	105	2.917	0.0524	36	0.0257	13.912	14.122	12.672	0.315	0.363	26.187	27.938	0.156	0.545	0.339
LRG 1	82	1.745	0.0467	47	0.0216	16.660	15.525	13.555	0.274	0.441	26.620	31.389	1.435	0.407	0.500
LRG 2	169	2.770	0.0911	61	0.0289	15.741	8.200	11.405	0.292	0.153	18.512	16.747	1.453	0.779	0.270
LRG 3	122	3.389	0.089	36	0.0229	14.090	8.573	10.596	0.291	0.158	21.441	49.258	4.498	0.620	0.581
LRG 4	56	1.647	0.1212	34	0.0254	8.556	6.287	8.094	0.215	0.116	24.882	27.402	1.142	0.531	0.441
LRG 5	83	2.862	0.0487	29	0.0161	12.156	14.209	10.308	0.312	0.251	17.640	43.478	13.292	0.503	1.118
LRG 6	90	2.432	0.0481	37	0.0246	14.990	15.468	10.811	0.439	0.177	20.447	29.024	1.585	0.378	0.496
LRG 7	112	2.545	0.0443	44	0.0258	13.183	17.043	13.183	0.363	0.260	26.395	29.380	3.023	0.558	0.236
LRG 8	95	2.568	0.0403	37	0.0258	17.891	18.189	23.995	0.467	0.266	19.186	28.256	1.783	0.554	0.899
LRG 9	87	3.107	0.0384	28	0.018	17.083	15.938	9.583	0.430	0.174	76.944	45.611	2.611	0.744	0.667
SML 1	67	2.393	0.0345	28	0.0184	19.797	54.725	8.464	0.307	0.278	56.087	112.174	7.283	0.592	0.429
SML 2	114	3.455	0.051	33	0.0252	9.471	22.392	8.353	0.296	0.876	23.571	45.595	2.937	0.968	0.647
SML 3	66	1.784	0.0527	37	0.0279	12.941	22.903	9.355	0.306	0.996	21.577	40.789	3.333	0.559	0.846
SML 4	147	3.500	0.0644	42	0.0353	19.193	35.994	6.025	0.360	0.144	38.697	54.929	3.371	0.453	0.320
SML 5	99	2.829	0.0336	35	0.0308	12.262	29.583	8.810	0.405	1.271	39.058	59.481	1.883	0.425	0.377
SML 6	72	0.017	0.0245	42	0.0271	30.857	120.000	10.327	0.820	0.612	34.502	60.295	2.325	0.483	0.406
SML 7	64	1.778	0.0327	36	0.022	20.642	79.786	10.092	0.398	0.187	35.864	95.318	2.909	0.450	0.245
SML 8	76	2.815	0.0388	27	0.014	26.598	80.593	9.149	0.291	0.229	47.643	154.286	3.571	0.564	0.364
SML 9	91	2.022	0.0495	45	0.0303	26.444	44.444	9.212	0.509	0.277	37.558	59.373	2.739	0.393	0.211
SML10	75	2.027	0.0415	37	0.0236	21.542	59.759	6.940	0.349	0.267	35.847	77.797	3.305	0.521	0.263
CON 1	82	2.103	0.1124	39	0.0218	11.717	17.215	2.580	0.171	0.088	39.541	73.578	3.716	0.427	0.665
CON 2	87	1.977	0.1101	44	0.0374	13.070	15.577	5.413	0.243	0.070	31.310	46.925	2.112	0.364	0.457
CON 3	105	2.333	0.0794	45	0.0315	11.776	23.224	6.045	0.248	0.147	35.873	54.317	1.651	0.197	0.273
CON 4	70	2.000	0.0625	35	0.0232	18.992	26.608	5.776	0.306	0.198	38.879	71.681	3.750	0.319	0.440
CON 5	93	2.214	0.0639	42	0.0209	16.150	27.637	4.742	0.291	0.327	41.722	80.622	2.105	0.182	0.321
CON 6	64	1.829	0.0446	35	0.0198	19.507	40.762	7.691	0.388	0.276	42.727	97.929	3.889	0.449	0.480
CON 7	50	1.563	0.0284	32	0.0153	20.915	65.176	8.873	0.324	0.285	45.686	114.967	4.248	0.725	0.784
CON 8	87	1.261	0.0977	69	0.03	10.481	20.297	4.176	0.164	0.162	35.500	60.333	1.367	0.787	0.400
CON 9	97	1.940	0.0702	50	0.0247	13.775	24.744	3.860	0.272	0.157	38.178	88.057	2.024	0.308	0.308

APPENDIX 7

Raw data for Greenhouse Trial I - *Trachypogon spicatus*

TRT	rt lngth cm	rt : sh	rt dms g	sh lngth cm	sh dms g	rt N mg/g sample	rt P mg/g sample	rt Fe mg/g sample	rt Mn mg/g sample	rt Zn mg/g sample	sh N mg/g sample	sh P mg/g sample	sh Fe mg/g sample	sh Mn mg/g sample	sh Zn mg/g sample
CF 1	11	0.379	0.0057	29	0.0147	65.088	129.825	18.596	0.789	2.298	40.952	45.850	1.769	0.803	1.095
CF 2	333	5.842	0.1686	57	0.0567	19.128	4.389	6.246	0.187	0.142	33.263	13.580	0.635	0.956	0.317
CF 3	365	7.157	0.1305	51	0.0441	24.115	5.747	7.839	0.164	0.269	41.678	17.460	0.794	1.594	0.474
CF 4	188	3.418	0.1329	55	0.0489	14.966	6.155	6.388	0.150	0.139	32.372	17.628	2.127	0.785	0.413
CF 5	205	6.029	0.1264	34	0.0250	10.127	6.646	7.207	0.163	0.131	33.000	32.560	0.600	1.292	0.892
CF 6	283	5.340	0.1586	53	0.0381	13.247	5.624	7.465	0.132	0.232	35.276	21.759	0.052	1.520	0.625
CF 7	443	5.829	0.1777	76	0.0942	22.729	4.682	7.175	0.201	0.195	28.907	9.193	0.605	0.988	0.242
CF 8	77	1.791	0.0598	43	0.0423	19.264	13.796	3.963	0.236	0.468	33.452	19.338	2.222	0.908	0.499
CF 9	215	3.839	0.0833	56	0.0483	18.523	9.952	9.268	0.210	0.157	27.164	17.391	0.849	0.826	0.275
CF 10	157	3.925	0.0953	40	0.0457	20.042	9.003	8.447	0.189	0.154	25.033	18.621	1.050	0.621	0.379
WOR 1	54	2.000	0.0213	27	0.0160	25.634	40.141	15.493	0.333	0.690	34.625	44.625	4.000	0.906	2.163
WOR 2	42	2.333	0.0233	18	0.0166	17.983	30.644	8.884	0.270	1.197	36.747	47.048	0.422	1.006	1.000
WOR 3	224	2.732	0.1791	82	0.0671	10.307	4.461	6.287	0.138	0.102	33.219	12.027	1.237	1.115	0.276
WOR 4	136	2.833	0.0702	48	0.0270	10.513	10.812	6.724	0.144	0.808	31.444	43.556	0.333	0.948	0.844
WOR 5	77	2.265	0.0377	34	0.0235	16.393	20.716	9.894	0.438	0.316	41.915	31.319	1.404	1.060	0.536
BW 1	234	5.707	0.0946	41	0.0293	19.683	8.562	8.932	0.158	0.181	41.502	26.894	0.614	1.348	0.618
BW 2	70	3.182	0.0608	22	0.0113	11.349	12.895	7.615	0.153	0.952	46.195	64.867	1.416	1.177	2.681
BW 3	83	2.441	0.0410	34	0.0250	20.317	18.585	9.927	0.180	0.356	37.800	32.720	4.560	0.900	1.016
BW 4	37	1.609	0.0291	23	0.0142	25.498	44.089	13.471	0.192	0.832	51.620	90.915	9.225	0.958	1.014
BW 5	58	1.813	0.0347	32	0.0180	27.176	47.089	15.504	0.193	1.161	51.944	66.667	5.500	1.100	5.533
BW 6	209	4.750	0.0748	44	0.0303	28.971	18.837	10.722	0.104	0.778	34.158	115.017	3.564	1.205	0.944
BW 7	198	4.213	0.2068	47	0.0266	11.576	9.241	4.231	0.110	0.213	46.165	73.910	4.812	1.850	0.632
BW 8	92	3.172	0.1171	29	0.0323	12.425	12.579	5.790	0.092	0.304	43.467	47.059	4.644	0.659	0.802
BW 9	120	3.158	0.0664	38	0.0280	23.554	23.313	10.181	0.125	0.297	45.357	54.000	3.893	1.268	0.911
BW10	140	5.385	0.0753	26	0.0218	16.972	16.773	8.499	0.104	0.776	29.404	59.037	5.505	0.917	0.936
LRG1	108	2.769	0.0305	39	0.0288	24.033	47.246	15.967	0.174	0.934	24.028	87.708	3.958	0.608	0.757
LRG 2	163	3.705	0.0766	44	0.0288	15.261	15.822	10.052	0.111	0.336	33.021	41.806	3.264	0.788	0.882
LRG 3	115	2.347	0.0503	49	0.0410	22.584	25.825	11.491	0.141	0.614	29.951	31.683	2.561	0.812	1.249
LRG 4	58	2.231	0.0630	26	0.0201	18.556	23.127	9.079	0.162	0.717	49.851	61.493	5.522	1.080	2.905
LRG 5	144	4.000	0.0675	36	0.0284	18.563	23.926	9.185	0.179	0.342	45.000	46.972	3.873	1.021	0.796
LRG 6	74	1.721	0.0996	43	0.0362	14.608	18.032	7.319	0.147	0.255	36.022	35.000	3.039	0.677	0.677
LRG 7	58	3.053	0.0302	19	0.0107	20.662	41.987	14.040	0.318	0.904	92.804	114.019	16.075	1.243	2.299
LRG 8	119	4.577	0.0461	26	0.0248	27.874	35.163	13.579	0.282	0.527	44.113	42.661	4.032	1.395	1.173
LRG 9	128	3.879	0.0515	33	0.0253	25.146	22.621	12.233	0.283	0.654	43.241	74.585	4.190	1.830	1.296
LRG10	414	9.628	0.1649	43	0.0443	13.038	10.703	9.794	0.108	0.494	30.948	62.912	2.912	0.797	0.894
SML 1	194	4.409	0.0743	44	0.0291	20.592	22.746	10.781	0.234	1.016	39.897	53.849	3.918	0.983	0.845
SML 2	47	1.424	0.0285	33	0.0184	28.667	81.860	14.035	0.277	1.014	50.326	78.098	5.978	1.364	1.630
SML 3	87	2.023	0.0443	43	0.0237	25.643	29.774	10.858	0.246	0.517	40.844	62.152	4.051	0.785	1.945
SML 4	177	4.116	0.0146	43	0.0269	71.761	93.557	62.234	1.364	1.926	22.379	40.297	4.498	1.357	1.071
SML 5	75	1.829	0.0523	41	0.0214	12.428	23.212	7.763	0.212	0.432	29.252	53.178	5.187	1.481	0.981
CON 1	40	1.290	0.0175	31	0.0202	22.171	63.429	16.343	0.549	1.057	29.010	55.446	0.495	1.139	1.817
CON 2	66	1.610	0.0494	41	0.0260	12.024	24.130	9.332	0.154	0.457	32.308	45.577	3.269	0.935	1.200
CON 3	46	1.438	0.0172	32	0.0212	26.221	66.802	13.547	0.378	1.936	38.160	59.623	3.632	0.835	1.703
C0N 4	98	2.130	0.0329	46	0.0293	30.608	42.492	13.769	0.316	0.687	28.396	39.829	3.311	1.130	1.239
CON 5	33	1.571	0.0240	21	0.0093	17.167	71.875	6.833	0.375	1.329	33.226	129.355	8.172	1.473	3.247

APPENDIX 7

Raw data for Greenhouse Trial II - *Themeda triandra*

TRT	rt lngth cm	rt : sh	rt dms g	sh lngth cm	sh dms g	rt N mg/g sample	rt P mg/g sample	rt K mg/g sample	rt Cu mg/g sample	rt Fe mg/g sample	rt Mn mg/g sample	rt Zn mg/g sample	sh N mg/g sample	sh P mg/g sample	sh K mg/g sample	sh Cu mg/g sample	sh Fe mg/g sample	sh Mn mg/g sample	sh Zn mg/g sample
CF 1	1902	3.3021	1.46	576	0.96	4.837	2.134	2.411	0.007	0.621	0.067	0.012	7.223	2.897	5.792	0.007	0.189	0.099	0.015
CF 2	4289	3.0658	2.35	1399	2.06	2.100	1.011	1.532	0.004	0.416	0.047	0.005	2.879	1.288	2.354	0.003	0.029	0.047	0.007
CF 3	1716	2.3443	1.69	732	1.28	3.741	1.552	1.568	0.005	0.418	0.057	0.008	5.406	1.853	3.672	0.004	0.020	0.079	0.010
CF 4	2655	3.1835	2.39	834	1.20	2.222	1.108	1.146	0.003	0.307	0.036	0.006	6.123	1.951	3.550	0.004	0.043	0.140	0.013
CF 5	1319	2.0261	2.08	651	0.87	3.402	1.296	1.692	0.003	0.280	0.055	0.007	8.306	2.655	5.103	0.006	0.049	0.128	0.017
CF 6	1997	3.2738	3.10	610	0.89	1.936	0.863	0.648	0.002	0.291	0.029	0.004	8.279	3.048	4.067	0.006	0.064	0.131	0.016
CF 7	1394	2.9723	1.84	469	0.88	2.310	1.488	0.875	0.004	1.429	0.126	0.005	7.443	2.703	4.807	0.005	0.169	0.166	0.017
CF 8	1518	2.765	1.70	549	0.72	4.049	1.394	1.365	0.005	0.298	0.054	0.007	10.421	3.271	5.500	0.005	0.051	0.213	0.018
CF 9	2283	3.0851	3.95	740	1.21	1.454	0.581	0.575	0.001	0.179	0.031	0.002	6.142	1.978	3.777	0.003	0.037	0.112	0.011
CF 10	995	3.1094	1.54	320	0.39	3.981	1.438	1.357	0.004	0.937	0.055	0.007	15.590	5.528	7.051	0.010	0.092	0.294	0.025
CF 11	1507	2.6815	3.45	562	0.89	0.943	0.710	0.301	0.001	0.193	0.011	0.002	8.519	3.060	4.921	0.004	0.047	0.113	0.016
CF 12	1851	1.7076	4.13	1084	1.95	2.220	0.956	1.189	0.002	0.360	0.035	0.004	2.848	1.216	3.092	0.002	0.013	0.038	0.006
WOR 1	2915	1.7167	7.24	1698	3.10	0.770	0.287	0.510	0.001	0.137	0.011	0.002	1.785	0.705	2.177	0.001	0.020	0.031	0.004
WOR 2	3187	3.3547	6.99	950	1.54	0.628	0.301	0.300	0.001	0.248	0.007	0.001	4.983	1.437	2.942	0.003	0.060	0.103	0.009
WOR 3	5005	3.5396	11.32	1414	2.41	0.492	0.184	0.226	0.000	0.142	0.005	0.001	2.264	0.866	2.432	0.001	0.034	0.050	0.004
WOR 4	4090	2.5263	8.29	1619	2.62	0.635	0.260	0.277	0.001	0.260	0.011	0.001	2.133	0.853	1.958	0.001	0.076	0.049	0.005
WOR 5	3621	1.5541	3.77	2330	2.79	1.408	0.571	0.687	0.002	0.359	0.026	0.002	2.444	0.782	1.885	0.001	0.021	0.043	0.004
WOR 6	3456	3.2119	5.04	1076	1.62	1.142	0.567	0.437	0.002	0.525	0.028	0.003	4.048	1.537	4.074	0.003	0.064	0.132	0.011
WOR 7	3856	2.827	4.54	1364	2.14	0.970	0.464	0.419	0.001	0.447	0.016	0.002	3.685	1.039	2.033	0.002	0.042	0.062	0.006
WOR 8	4312	3.9451	5.20	1093	1.93	1.121	0.410	0.640	0.001	0.189	0.016	0.001	3.489	1.145	2.663	0.002	0.033	0.057	0.005
WOR 9	3375	1.7924	2.59	1883	2.09	2.014	0.824	1.409	0.003	0.593	0.034	0.003	3.047	1.253	3.124	0.002	0.052	0.057	0.006
WOR 10	5411	3.6685	11.44	1475	2.53	0.252	0.182	0.151	0.001	0.285	0.003	0.001	2.192	0.859	1.783	0.002	0.030	0.050	0.004
WOR 11	5341	2.7963	8.98	1910	2.34	0.464	0.194	0.223	0.001	0.122	0.004	0.006	2.617	0.847	2.068	0.002	0.043	0.051	0.005
WOR 12	4499	2.9521	6.98	1524	2.66	0.601	0.359	0.360	0.001	0.317	0.009	0.001	1.798	0.926	2.342	0.002	0.024	0.020	0.003
WOR 13	4768	4.0717	3.22	1171	1.48	2.128	0.763	1.314	0.002	0.366	0.027	0.003	3.938	1.613	3.399	0.004	0.024	0.111	0.007
WOR 14	4208	2.5612	5.62	1643	2.41	0.771	0.405	0.423	0.001	0.506	0.014	0.001	2.328	0.920	1.776	0.002	0.024	0.050	0.003
BW 1	3789	2.7942	3.64	1356	2.14	1.680	0.649	1.176	0.002	0.070	0.022	0.004	2.227	1.145	3.051	0.002	0.006	0.050	0.007
BW 2	5181	2.981	4.07	1738	2.22	1.156	0.574	0.803	0.001	0.193	0.020	0.002	2.450	1.075	2.635	0.002	0.009	0.081	0.009
BW 3	3126	2.1485	3.42	1455	2.44	1.746	0.740	1.173	0.002	0.121	0.025	0.005	2.107	1.110	3.135	0.002	0.006	0.040	0.006
BW 4	3691	2.3243	6.94	1588	2.68	0.713	0.445	0.432	0.001	0.161	0.014	0.002	1.849	0.921	2.683	0.001	0.004	0.028	0.005
BW 5	4513	2.0952	5.69	2154	2.97	0.741	0.418	0.353	0.001	0.133	0.014	0.001	1.664	0.841	2.428	0.001	0.007	0.003	0.004
BW 6	3151	2.348	5.07	1342	1.89	1.119	0.484	0.834	0.001	0.106	0.018	0.002	2.829	1.288	2.735	0.002	0.024	0.057	0.007
BW 7	3719	2.8281	4.14	1315	1.40	1.524	0.565	0.575	0.001	0.181	0.024	0.002	4.638	1.778	3.793	0.002	0.013	0.124	0.013
BW 8	4035	2.1989	4.59	1835	2.96	1.224	0.495	0.708	0.002	0.279	0.025	0.002	1.813	0.744	2.399	0.002	0.019	0.030	0.003
BW 9	5447	3.5006	7.80	1556	3.00	0.593	0.264	0.317	0.001	0.190	0.016	0.001	1.447	0.618	2.107	0.002	0.013	0.021	0.002
BW 10	4269	2.7019	6.82	1580	2.41	0.643	0.278	0.443	0.001	0.162	0.013	0.001	2.561	0.858	2.290	0.003	0.042	0.033	0.004
BW 11	3748	2.3722	12.33	1580	3.01	0.385	0.168	0.160	0.001	0.205	0.005	0.000	1.577	0.734	2.312	0.002	0.017	0.030	0.004
LRG 1	4960	2.8637	5.73	1732	2.82	0.673	0.360	0.396	0.001	0.508	0.013	0.001	1.927	0.752	2.348	0.002	0.027	0.040	0.002
LRG 2	5321	2.6251	9.85	2027	3.26	0.326	0.211	0.281	0.001	0.159	0.015	0.001	1.549	0.526	2.080	0.002	0.028	0.036	0.003
LRG 3	6239	4.1483	6.51	1504	2.34	0.582	0.327	0.445	0.001	0.053	0.019	0.002	2.456	0.959	2.962	0.003	0.033	0.054	0.007
LRG 4	4103	2.9141	17.27	1408	3.44	0.208	0.119	0.141	0.000	0.019	0.005	0.001	1.419	0.622	1.628	0.002	0.037	0.035	0.005
LRG 5	6761	3.5886	10.62	1884	3.05	0.326	0.197	0.266	0.001	0.071	0.008	0.001	1.474	0.714	1.941	0.002	0.016	0.032	0.010
LRG 6	5637	2.8045	18.33	2010	3.47	0.177	0.115	0.115	0.000	0.024	0.004	0.001	1.474	0.636	1.810	0.002	0.012	0.035	0.009
LRG 7	7509	3.8449	5.88	1953	2.02	0.748	0.422	0.549	0.001	0.081	0.018	0.003	2.487	1.073	2.594	0.003	0.061	0.081	0.007
LRG 8	5303	3.3457	17.08	1585	3.00	0.182	0.118	0.092	0.000	0.056	0.004	0.001	1.652	0.731	2.083	0.002	0.011	0.027	0.007
LRG 9	4627	3.0642	9.61	1510	2.45	0.553	0.224	0.247	0.001	0.125	0.011	0.001	2.233	0.889	1.939	0.003	0.018	0.054	0.008
LRG 10	5113	3.3794	13.11	1513	2.67	0.427	0.162	0.178	0.001	0.029	0.005	0.001	1.932	0.815	1.914	0.003	0.024	0.044	0.006
LRG 11	5038	3.1725	13.61	1588	2.94	0.332	0.160	0.198	0.001	0.042	0.010	0.001	1.838	0.753	1.782	0.002	0.016	0.035	0.005
LRG 12	5207	3.2402	18.14	1607	2.74	0.166	0.116	0.067	0.000	0.056	0.005	0.000	1.994	0.806	1.788	0.003	0.013	0.046	0.005
SML 1	2985	4.1172	2.15	725	0.76	3.537	0.776	1.019	0.002	0.102	0.056	0.009	9.642	1.875	6.237	0.005	0.171	0.227	0.038
SML 2	3923	2.7037	3.76	1451	1.94	1.256	0.551	0.636	0.002	0.071	0.029	0.006	3.651	0.749	2.165	0.002	0.057	0.101	0.012
SML 3	4230	3.384	3.14	1250	1.63	1.273	0.539	0.389	0.002	0.137	0.038	0.005	4.621	0.866	1.669	0.003	0.114	0.136	0.021
SML 4	3178	2.7707	3.91	1147	1.85	1.497	0.438	0.721	0.001	0.059	0.022	0.006	3.510	0.740	2.124	0.003	0.055	0.083	0.022
SML 5	3513	2.6414	5.14	1330	2.45	1.135	0.338	0.527	0.001	0.025	0.015	0.003	2.573	0.569	1.665	0.003	0.027	0.056	0.009
SML 6	2317	3.0852	2.21	751	1.05	3.129	0.750	0.986	0.004	0.146	0.037	0.005	7.456	1.257	3.457	0.004	0.060	0.131	0.014
SML 7	2944	2.036	3.03	1446	1.60	1.475	0.721	0.703	0.004	0.156	0.036	0.013	3.733	0.878	2.694	0.033	0.042	0.113	0.017
SML 8	3032	2.6903	3.19	1127	1.50	2.254	0.813	1.357	0.005	0.338	0.046	0.004	4.561	1.035	3.947	0.004	0.016	0.112	0.018
SML 9	3724	2.6374	4.18	1412	2.28	0.983	0.491	0.380	0.002	0.427	0.017	0.004	2.791	0.600	1.895	0.003	0.016	0.059	0.006
SML 10	3138	2.6259	2.37	1195	1.77	2.440	0.726	0.747	0.004	0.034	0.036	0.022	4.714	0.819	2.305	0.003	0.018	0.104	0.014
SML 11	3353	1.9472	2.84	1722	2.65	1.904	0.712	0.926	0.002	0.056	0.030	0.006	2.136	0.535	1.838	0.002	0.053	0.049	0.004
SML 12	2641	2.4613	3.89	1073	1.68	1.414	0.515	0.576	0.001	0.089	0.029	0.006	3.801	0.857	3.440	0.002	0.027	0.085	0.009
SML 13	2927	2.7509	2.98	1064	1.66	0.993	0.548	0.768	0.002	0.156	0.036	0.002	1.884	0.795	2.536	0.004	0.042	0.062	0.005
CON 1	3366	2.4551	1.90	1371	1.73	1.358	1.071	1.689	0.005	0.104	0.057	0.006	1.940	1.340	3.890	0.004	0.017	0.080	0.009
CON 2	2124	1.2673	1.46	1676	2.15	3.467	1.533	1.781	0.006	0.195	0.057	0.007	2.143	1.032	2.121	0.004	0.009	0.050	0.006
CON 3	2762	1.9261	2.07	1434	1.74	1.646	1.043	1.053	0.004	0.252	0.036	0.004	2.541	1.269	3.075	0.004	0.013	0.069	0.006
CON 4	2495	1.4273	1.84	1748	2.42	1.330	1.212	1.587	0.005	0.174	0.028	0.005	0.999	0.917	2.029	0.003	0.012	0.050	0.004
CON 5	2538	2.0095	2.65	1263	2.07	1.157	0.828	0.868	0.004	0.167	0.050	0.003	1.934	1.057	2.797	0.004	0.006	0.061	0.005
CON 6	2912	2.5212	1.95	1155	1.70	1.430	1.132	1.518	0.006	0.302	0.072	0.006	1.470	1.253	2.812	0.005	0.005	0.078	0.005
CON 7	2370	1.8647	2.26	1271	1.64	1.069	0.959	1.239	0.005	0.154	0.088	0.004	1.583	1.371	3.305	0.005	0.004	0.118	0.007
CON 8	2564	1.6394	2.26	1564	2.30	1.211	1.385	0.845	0.005	0.247	0.054	0.003	1.537	1.392	2.265	0.004	0.002	0.042	0.004
CON 9	2962	1.8352	2.09	1614	2.25	1.228	1.055	1.129	0.004	0.138	0.033	0.003	1.203	0.960	2.778	0.004	0.003	0.043	0.004
CON 10	2927	2.3047	2.10	1270	1.72	1.119	1.016	1.100	0.004	0.160	0.048	0.011	2.063	1.202	3.052	0.005	0.008	0.079	0.010
CON 11	2676	2.1668	1.89	1235	1.73	3.532	0.772	0.878	0.004	0.154	0.056	0.002	4.152	0.747	2.538	0.002	0.006	0.080	0.003
CON 12	2316	1.6218	2.04	1428	1.98	1.234	0.619	1.206	0.003	0.167	0.086	0.002	1.616	0.668	2.737	0.002	0.002	0.079	0.003
CON 13	2163	1.6587	1.56	1304	2.01	1.726	0.781	1.449	0.005	0.080	0.044	0.003	1.978	0.641	2.547	0.006	0.413	0.053	0.036

APPENDIX 7

Raw data for Greenhouse Trial - *Trachypogon spicatus*

TRT	rt lngth cm	rt : sh	rt dms g	sh lngth cm	sh dms g	rt N mg/g sample	rt P mg/g sample	rt K mg/g sample	rt Cu mg/g sample	rt Fe mg/g sample	rt Mn mg/g sample	rt Zn mg/g sample	sh N mg/g sample	sh P mg/g sample	sh K mg/g sample	sh Cu mg/g sample	sh Fe mg/g sample	sh Mn mg/g sample	sh Zn mg/g sample
CF1	706	1.512	1.54	467	0.63	3.302	1.673	2.052	0.004	0.493	0.044	0.017	9.889	4.354	7.333	0.007	0.176	0.312	0.016
CF2	2830	3.920	1.62	722	1.37	4.898	1.614	3.469	0.004	0.573	0.073	0.005	5.642	2.258	3.051	0.002	0.078	0.212	0.009
CF3	995	1.501	0.52	663	0.76	19.369	4.967	5.808	0.006	3.356	0.093	0.001	9.003	3.500	6.382	0.005	0.161	0.336	0.014
CF4	830	1.718	1.04	483	0.76	7.604	2.497	4.808	0.006	2.059	0.077	0.006	9.236	3.395	9.316	0.006	0.137	0.273	0.013
CF5	5044	2.765	3.52	1824	4.32	0.734	0.732	0.761	0.002	0.228	0.031	0.002	0.776	0.616	0.850	0.001	0.011	0.045	0.001
CF6	3146	2.721	3.05	1156	2.47	2.017	0.827	1.315	0.002	0.248	0.037	0.019	1.449	1.062	2.368	0.001	0.024	0.090	0.004
CF7	2250	2.451	2.07	918	1.47	3.906	1.365	2.304	0.003	0.599	0.065	0.003	4.431	1.670	1.884	0.002	0.054	0.162	0.005
CF8	1992	2.292	2.22	869	1.41	3.600	1.243	2.784	0.002	0.537	0.046	0.001	4.129	1.815	3.184	0.003	0.053	0.121	0.005
CF9	956	1.266	1.35	755	0.86	6.179	2.032	2.326	0.005	1.171	0.092	0.003	7.420	3.577	5.558	0.005	0.106	0.304	0.005
CF10	820	3.216	0.61	255	0.51	13.136	4.098	5.410	0.011	1.793	0.191	0.007	11.867	4.908	11.765	0.009	0.278	0.458	0.024
CF11	1326	0.964	1.06	1375	1.61	5.983	2.345	4.566	0.007	0.938	0.162	0.006	3.886	1.497	3.646	0.003	0.084	0.085	0.003
WOR1	6391	5.150	3.01	1241	2.46	2.129	0.812	1.153	0.003	0.145	0.022	0.001	1.947	0.992	1.614	0.002	0.019	0.070	0.002
WOR2	1387	1.659	1.89	836	1.59	4.675	1.420	2.016	0.006	0.503	0.052	0.003	2.598	1.474	3.868	0.003	0.056	0.121	0.003
WOR3	1772	1.797	1.72	986	1.88	5.007	1.421	2.843	0.005	0.431	0.062	0.002	3.317	1.341	2.670	0.003	0.032	0.078	0.002
WOR4	5228	4.886	2.97	1070	1.83	2.029	0.809	2.293	0.003	0.168	0.031	0.001	2.387	1.404	2.945	0.003	0.041	0.113	0.003
WOR5	1236	2.457	0.51	503	0.72	20.369	4.767	2.353	0.017	2.492	0.182	0.007	7.464	3.250	6.306	0.008	0.124	0.269	0.007
WOR6	2706	2.214	1.91	1222	2.40	5.707	1.242	1.524	0.004	0.393	0.072	0.003	1.691	0.977	1.996	0.002	0.035	0.083	0.002
WOR7	2147	1.771	1.51	1212	2.68	7.189	1.603	3.026	0.006	0.675	0.132	0.003	1.745	0.869	1.877	0.002	0.027	0.058	0.002
WOR8	3395	3.296	2.41	1030	1.71	2.858	1.030	2.162	0.004	0.359	0.065	0.003	3.012	1.432	3.538	0.004	0.047	0.133	0.006
WOR9	3562	2.774	2.70	1284	2.04	3.143	0.891	2.030	0.003	0.267	0.042	0.001	2.260	1.192	2.681	0.003	0.040	0.089	0.003
WOR10	1765	2.052	1.97	860	1.52	5.213	1.232	2.020	0.002	0.423	0.051	0.003	4.829	1.529	4.296	0.002	0.043	0.110	0.005
WOR11	1783	2.140	1.84	833	1.25	3.429	1.211	1.609	0.003	0.360	0.074	0.003	4.664	1.876	5.400	0.003	0.080	0.184	0.005
WOR12	5079	5.954	2.48	853	1.96	2.676	0.854	1.306	0.005	0.166	0.049	0.003	2.795	1.138	2.944	0.002	0.034	0.108	0.004
BW1	2289	2.616	1.68	875	1.50	5.260	1.258	2.643	0.003	0.243	0.050	0.002	4.070	1.451	3.687	0.002	0.057	0.119	0.005
BW2	3897	3.787	3.21	1029	1.98	2.326	0.653	0.944	0.002	0.144	0.042	0.002	1.945	1.069	2.768	0.002	0.040	0.073	0.003
BW3	1828	1.749	3.00	1045	1.55	2.922	0.670	1.143	0.002	0.183	0.028	0.001	4.897	1.408	4.116	0.002	0.055	0.111	0.006
BW4	4394	3.250	1.37	1352	2.76	3.751	1.490	2.679	0.006	0.345	0.089	0.003	1.713	0.733	1.895	0.001	0.036	0.071	0.003
BW5	4921	3.754	6.57	1311	2.58	0.777	0.303	0.508	0.001	0.080	0.023	0.000	1.781	0.814	1.671	0.002	0.045	0.091	0.002
BW6	2633	2.431	3.04	1083	2.08	2.570	0.666	1.905	0.002	0.107	0.037	0.002	2.458	0.998	2.659	0.002	0.055	0.074	0.002
BW7	3407	3.330	3.65	1023	1.93	2.796	0.592	1.227	0.002	0.130	0.035	0.001	3.223	1.015	2.881	0.002	0.069	0.078	0.001
BW8	4357	2.658	4.58	1639	2.86	1.734	0.426	0.801	0.001	0.132	0.023	0.001	1.801	0.722	2.098	0.002	0.036	0.081	0.002
BW9	4031	2.759	4.36	1461	2.27	1.955	0.440	0.906	0.002	0.124	0.023	0.001	2.379	0.884	2.454	0.002	0.051	0.095	0.004
BW10	2184	2.597	3.03	841	1.47	3.177	0.652	1.667	0.002	0.227	0.034	0.001	3.609	1.369	4.088	0.003	0.099	0.125	0.002
BW11	1912	1.093	1.44	1749	2.13	3.564	1.376	1.222	0.006	0.901	0.098	0.003	2.353	0.955	2.911	0.002	0.061	0.078	0.003
BW12	2784	2.513	2.87	1108	1.60	2.740	0.750	1.930	0.002	0.318	0.073	0.002	4.996	1.189	1.463	0.005	0.123	0.050	0.002
LRG1	2460	3.710	4.87	663	1.57	1.225	0.413	0.649	0.002	0.432	0.024	0.001	3.644	1.276	4.656	0.003	0.082	0.122	0.002
LRG2	4649	3.672	7.83	1266	1.93	0.746	0.233	0.488	0.001	0.123	0.012	0.000	2.616	1.027	2.591	0.003	0.078	0.170	0.003
LRG3	3141	2.837	3.74	1107	1.93	2.514	0.530	1.160	0.002	0.149	0.028	0.000	2.576	1.043	1.943	0.003	0.072	0.153	0.003
LRG4	3031	2.948	2.65	1028	1.96	3.320	0.680	1.615	0.002	0.113	0.033	0.000	2.818	0.972	2.500	0.003	0.073	0.100	0.001
LRG5	3576	2.337	3.08	1530	2.54	2.942	0.631	1.487	0.003	0.247	0.032	0.001	1.542	0.763	1.531	0.002	0.053	0.077	0.001
LRG6	2660	2.521	3.19	1055	2.05	3.190	0.588	1.524	0.001	0.175	0.020	0.005	2.600	1.002	2.600	0.002	0.072	0.114	0.008
LRG7	1890	1.267	1.65	1492	1.43	7.209	1.214	2.982	0.004	0.282	0.104	0.011	4.993	1.499	3.874	0.003	0.155	0.205	0.018
LRG8	1785	1.721	3.34	1037	1.80	3.003	0.599	0.874	0.002	0.283	0.024	0.005	4.098	1.186	2.961	0.002	0.105	0.088	0.009
LRG9	1865	2.228	1.88	837	1.30	6.906	1.088	3.218	0.003	0.311	0.060	0.009	5.352	1.557	3.623	0.003	0.137	0.135	0.015
SML1	3379	2.531	2.20	1335	2.37	2.702	0.932	0.927	0.006	0.256	0.093	0.008	2.833	0.852	1.751	0.001	0.092	0.062	0.007
SML2	1535	2.372	1.22	647	0.87	9.843	1.732	4.361	0.005	0.773	0.107	0.014	9.239	2.686	8.874	0.005	0.251	0.199	0.022
SML3	2090	2.606	2.48	802	1.19	4.165	0.825	1.544	0.004	0.612	0.071	0.008	6.799	1.673	6.487	0.003	0.197	0.185	0.017
SML4	1478	2.419	1.02	611	0.80	11.893	2.005	3.980	0.007	1.070	0.106	0.016	10.523	2.741	7.288	0.006	0.271	0.262	0.026
SML5	2174	1.904	2.15	1142	1.39	3.678	0.955	1.712	0.005	0.607	0.059	0.008	5.229	1.471	3.324	0.004	0.147	0.207	0.016
SML6	2857	3.079	2.55	928	1.59	3.829	0.796	2.647	0.003	0.236	0.037	0.007	3.113	1.172	2.069	0.003	0.086	0.121	0.011
SML7	2212	2.353	2.15	940	1.43	4.319	0.953	1.363	0.004	0.836	0.054	0.008	5.099	1.392	3.063	0.003	0.103	0.112	0.012
CON1	3286	2.077	3.84	1582	2.41	1.071	0.569	1.193	0.003	0.255	0.014	0.004	1.706	0.870	1.892	0.002	0.063	0.107	0.008
CON2	798	2.036	0.89	392	0.61	8.587	2.398	5.562	0.007	2.239	0.073	0.017	14.074	3.915	10.279	0.012	0.352	0.529	0.043
CON3	1485	1.578	2.03	941	1.82	1.486	1.003	1.315	0.003	0.372	0.032	0.008	1.708	1.117	2.775	0.002	0.080	0.103	0.011
CON4	2550	2.632	2.85	969	1.82	1.045	0.716	1.351	0.003	0.392	0.040	0.006	2.247	1.161	3.253	0.003	0.099	0.177	0.011
CON5	625	1.517	0.84	412	0.52	15.283	2.314	2.179	0.007	2.707	0.053	0.017	24.777	4.008	9.731	0.010	0.396	0.315	0.035
CON6	3309	2.567	2.31	1289	1.95	1.266	0.862	1.554	0.004	0.648	0.028	0.007	2.203	1.053	3.328	0.003	0.097	0.101	0.009
CON7	1976	2.179	0.86	907	1.46	5.666	2.384	2.465	0.011	1.586	0.065	0.018	4.692	1.554	4.438	0.004	0.136	0.129	0.126

Appendix 8

Results from the Statistical Analysis of the Greenhouse Trials Raw Data

Trial 1. Mean values and standard deviations from statistical analysis of *Themeda triandra* roots

Inoculum	Root:Shoot	Root length cm	Rt dry mass g	Root N mg/g sample	Root P mg/g sample	Root Fe mg/g sample	Root Mn mg/g sample	Root Zn mg/g sample
Cape Flats	2.60 ±1.16^{ab}	210.7 ±128.0^{a}	0.150 ±0.113^{a}	65.5 ±24.5^{a}	49.8 ±46.3^{b}	31.0 ±23.6^{a}	1.23 ±0.31^{a}	0.21 ±0.16^{b}
Worcester	2.32 ±0.43^{ab}	92.2 ±26.6^{b}	0.039 ±0.014^{b}	72.4 ±16.1^{a}	99.3 ±50.5^{a}	43.2 ±9.6^{a}	1.30 ±0.38^{a}	0.37 ±0.12^{ab}
B. West	3.50 ±1.03^{a}	118.5 ±29.5^{b}	0.059 ±0.016^{b}	13.4 ±2.8^{b}	14.1 ±4.0^{a}	13.8 ±3.6^{b}	0.29 ±0.11^{b}	0.27 ±0.08^{ab}
Large	2.56 ±0.57^{ab}	99.6 ±32.7^{b}	0.063 ±0.030^{b}	14.5 ±2.9^{b}	13.3 ±4.4^{a}	12.4 ±4.7^{b}	0.34 ±0.09^{b}	0.22 ±0.10^{b}
Small	2.26 ±1.01^{b}	87.7 ±26.6^{b}	0.042 ±0.012^{b}	20.0 ±6.9 ^{b}	55.0 ±31.1^{a}	8.7 ±1.3^{b}	0.40 ±0.16^{b}	0.51 ±0.40^{a}
Control	1.91 ±0.33^{b}	81.7 ±17.4^{b}	0.074 ±0.029^{b}	15.2 ±3.9^{b}	29.3 ±15.4^{a}	5.5 ±1.9^{b}	0.27 ±0.07^{b}	0.19 ±0.09^{b}

Root:Shoot is the root to shoot ratio. N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 8 continued.

Trial 1. Mean values and standard deviations from statistical analysis of *Themeda triandra* shoots

Inoculum	Shoot length cm	Sh dry mass g	Shoot N mg/g sample	Shoot P mg/g sample	Shoot Fe mg/g sample	Shoot Mn mg/g sample	Shoot Zn mg/g sample
Cape Flats	77.4 ±40.2^{a}	0.05 ±0.034^{a}	119.6 ±34.6^{a}	92.2 ±78.8^{a}	10.6 ±8.7^{ab}	1.66 ±1.12^{a}	0.27 ±0.10^{b}
Worcester	39.7 ±8.6^{b}	0.027±0.007^{b}	93.9 ±10.7^{a}	114.6 ±33.1^{a}	11.4 ±3.7^{a}	1.10 ±0.46^{ab}	0.45 ±0.08^{ab}
B. West	34.6 ±6.0^{b}	0.023±0.004^{b}	23.4 ±3.3^{b}	32.0 ±5.9^{b}	5.5 ±8.2^{abc}	0.63 ±0.33^{b}	0.64 ±0.33^{a}
Large	39.2 ±10.2^{b}	0.023±0.004^{b}	28.0 ±18.7^{b}	33.4 ±10.5^{b}	3.4 ±3.8^{bc}	0.56 ±0.13^{b}	0.58 ±0.28^{a}
Small	36.2 ±5.9^{b}	0.025±0.006^{b}	37.0 ±10.1^{b}	76.0 ±35.3a^{b}	3.4 ±1.5^{c}	0.54 ±0.16^{b}	0.41 ±0.20^{ab}
Control	43.4 ±11.15^{b}	0.024±0.007^{b}	38.8 ±4.3^{b}	76.5 ±21.7a^{b}	2.8 ±1.1^{c}	0.41 ±0.21^{b}	0.45 ±0.17^{ab}

N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 8 continued

Trial 1. Mean values and standard deviations from statistical analysis of *Trachypogon spicatus* roots

	Root : Shoot	Root length	Rt Dry Mass	Root N	Root P	Root Fe	Root Mn	Root Zn
Cape Flats	4.36 ±2.10	227.7 ±131.8^{a}	0.114 ±0.054^{a}	22.7 ±15.5	19.6 ±38.8^{b}	8.3 ±3.9^{b}	0.24 ±0.19	0.41 ±0.67
Worcester	2.43 ±0.35	106.6 ±74.94^{ab}	0.066 ±0.066^{ab}	16.2 ±6.3	21.4 ±14.5^{ab}	9.5 ±3.7^{ab}	0.26 ±0.13	0.62 ±0.42
B. West	3.54 ±1.43	124.1 ±68.8^{ab}	0.080 ±0.052^{ab}	19.8 ±6.6	21.2 ±13.7^{a}	9.5 ±3.3^{ab}	0.14 ±0.04	0.58 ±0.35
Large	3.79 ±2.24	138.1 ±103.1^{ab}	0.068 ±0.040^{ab}	20.0 ±4.9	26.5 ±11.6^{a}	11.3 ±2.7^{a}	0.19 ±0.08	0.58 ±0.23
Small	2.76 ±1.39	116.0 ±65.36^{ab}	0.042 ±0.023^{ab}	31.8 ±23.2	50.2±34.6^{ab}	21.1 ±23.1^{ab}	0.47 ±0.50	0.98 ±0.59
Control	1.61 ±0.32	56.6 ±26.2^{b}	0.028 ±0.013^{b}	21.6 ±7.3	53.75 ±20.0^{a}	12.0 ±3.8^{ab}	0.35 ±0.14	1.09 ±0.58

Root:Shoot is the root to shoot ratio. N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 8 continued.

Trial 1. Mean values and standard deviations from statistical analysis of *Trachypogon spicatus* shoots

Inoculum	Shoot length cm	St Dry Mass g	Shoot N mg/g sample	Shoot P mg/g sample	Shoot Fe mg/g sample	Shoot Mn mg/g sample	Shoot Zn mg/g sample
Cape Flats	49.4 ±13.5	0.046 ±0.021^{a}	33.1 ±5.4	21.3 ±10.5^{b}	1.07 ±0.72^{b}	1.03 ±0.33	0.52 ±0.28
Worcester	41.8 ±25.0	0.030 ±0.021^{ab}	35.6 ±4.0	35.7 ±14.6^{ab}	1.48 ±1.49^{ab}	1.01 ±0.08	0.96 ±0.73
B. West	33.6 ±8.8	0.024 ±0.007^{b}	42.8 ±7.2	63.1 ±26.2^{a}	4.37 ±2.36^{a}	1.14 ±0.32	1.51 ±1.53
Large	35.8 ±9.7	0.029 ±0.010^{ab}	42.9 ±19.3	59.9 ±26.1^{ab}	4.94 ±4.00^{a}	1.03 ±0.38	1.29 ±0.74
Small	40.8 ±4.5	0.024 ±0.004^{ab}	36.5 ±10.9	57.5 ±13.9^{ab}	4.73 ±0.86^{ab}	1.19 ±0.30	1.29 ±0.47
Control	34.2 ±9.7	0.021 ±0.008^{b}	32.2 ±3.9	66.0 ±36.3^{b}	3.78 ±2.77^{ab}	1.10 ±0.24	1.84 ±0.83

N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 8 continued.

Trial 2. Mean values and standard deviations from statistical analysis of Themeda triandra roots

Inoculum	Root:Shoot	Root length cm	Rt Dry Mass g	Root N mg/g sample	Root P mg/g sample	Root K mg/g sample	Root Fe mg/g sample	Root Mn mg/g sample	Root Zn mg/g sample
Cape Flats	2.79 ±0.51^{ab}	1952.2 ±860.8^{e}	2.47 ±0.95^{d}	2.77 ±1.20^{a}	1.21 ±0.43^{a}	1.22 ±0.57^{a}	0.48 ±0.36^{a}	0.050 ±0.028^{ab}	0.0057 ±0.0025^{a}
Worcester	2.89 ±0.80^{ab}	4146.0 ±793.1^{b}	6.52 ±2.77^{b}	0.96 ±0.56^{bc}	0.41 ±0.21b^{c}	0.53 ±0.39^{bc}	0.32 ±0.15^{ab}	0.015 ±0.010^{d}	0.0019 ±0.0014^{bc}
B. West	2.57 ±0.43^{b}	4060.8 ±745.1^{bc}	5.87 ±2.59^{bc}	1.05 ±0.47^{bc}	0.46 ±0.17^{bc}	0.63 ±0.34^{bc}	0.16 ±0.06^{bc}	0.017 ±0.006^{cd}	0.0020 ±0.0013^{bc}
Large	3.25 ±0.45^{a}	5484.8 ±939.7^{a}	12.15 ±4.8^{a}	0.39 ±0.20^{c}	0.21 ±0.11^{c}	0.25 ±0.15^{c}	0.10 ±0.13^{c}	0.010 ±0.005^{d}	0.0010 ±0.0006^{c}
Small	2.76 ±0.55^{ab}	3223.5 ±523.1^{cd}	3.29 ±0.86^{cd}	1.79 ±0.82^{b}	0.61 ±0.15^{b}	0.74 ±0.27^{b}	0.13 ±0.12^{bc}	0.033 ±0.011^{bc}	0.0071 ±0.0052^{a}
Control	1.90 ±0.38^{c}	2628.8 ±354.8d^{e}	2.01 ±0.31^{d}	1.65 ±0.84^{b}	1.03 ±0.25^{a}	1.26 ±0.32^{a}	0.18 ±0.06^{bc}	0.055 ±0.019^{a}	0.0045 ±0.0025^{ab}

Root:Shoot is the root to shoot ratio. N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 8 continued.

Trial 2. Mean values and standard deviations from statistical analysis of *Themeda triandra* shoots

Inoculum	Shoot length cm	St Dry Mass g	Shoot N mg/g sample	Shoot P mg/g sample	Shoot K mg/g sample	Shoot Fe mg/g sample	Shoot Mn mg/g sample	Shoot Zn mg/g sample
Cape Flats	710.5 ±289.3^{c}	1.11 ±0.48^{c}	7.43 ±3.40^{a}	2.62 ±1.15^{a}	4.47 ±1.30^{a}	0.067 ±0.056	0.130 ±0.071^{a}	0.0142 ±0.0052^{a}
Worcester	1510.7 ±378.2^{ab}	2.26 ±0.49^{b}	2.91 ±0.97^{bc}	1.06 ±0.30^{b}	2.48 ±0.69^{b}	0.039 ±0.018	0.062 ±0.032^{bc}	0.0054 ±0.0023^{b}
B. West	1590.8 ±247.3^{a}	2.47 ±0.53^{ab}	2.29 ±0.89^{bc}	1.01 ±0.33^{b}	2.69 ±0.48^{b}	0.015 ±0.011	0.045 ±0.033^{c}	0.0057 ±0.0032^{b}
Large	1693.4 ±219.6^{a}	2.85 ±0.44^{a}	1.87 ±0.38^{c}	0.77 ±0.15^{b}	2.07 ±0.39^{b}	0.025 ±0.014	0.043 ±0.015^{c}	0.0062 ±0.0024^{b}
Small	1207.2 ±278.3^{b}	1.76 ±0.52^{b}	4.24 ±2.16^{b}	0.89 ±0.35^{b}	2.77 ±1.27^{b}	0.054 ±0.044	0.101 ±0.047^{ab}	0.0145 ±0.0091^{a}
Control	1410.2 ±187.1^{ab}	1.96 ±0.27^{b}	1.94 ±0.78^{c}	1.07 ±0.27^{b}	2.77 ±0.50^{b}	0.039 ±0.112	0.068 ±0.021^{bc}	0.0077 ±0.0087^{ab}

N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 8 continued

Trial 2 Mean values and standard deviations from statistical analysis of *Trachypogon spicatus* Roots

Inoculum	Root:Shoot	Root length cm	Rt Dry Mass g	Root N mg/g sample	Root P mg/g sample	Root K mg/g sample	Root Fe mg/g sample	Root Mn mg/g sample	Root Zn mg/g sample
Cape Flats	2.21 ±0.91	1900.0 ±1349.0	1.69 ±0.96^{b}	6.43 ±5.41	2.13 ±1.33	3.24 ±1.70^{a}	1.09 ±0.96^{a}	0.089 ±0.051	0.0063 ±0.0060^{ab}
Worcester	3.01 ±1.49	3038.0 ±1713.0	2.08 ±0.70^{ab}	5.37 ±4.98	1.44 ±1.08	2.03 ±0.57^{ab}	0.53 ±0.64^{ab}	0.070 ±0.045	0.0028 ±0.0015^{b}
B. West	2.71 ±0.78	3220.0 ±1080.0	3.23 ±1.47^{a}	2.80 ±1.13	0.77 ±0.39	1.47 ±0.71^{b}	0.25 ±0.22^{b}	0.046 ±0.026	0.0016 ±0.0009^{b}
Large	2.58 ±0.82	2784.0 ±939.0	3.58 ±1.86^{a}	3.45 ±2.23	**26.22 ±76.50**	1.56 ±0.96^{b}	0.24 ±0.11^{b}	0.037 ±0.028	0.0035 ±0.0041^{b}
Small	2.47 ±0.35	2246.0 ±681.0	1.97 ±0.60^{ab}	5.78 ±3.57	1.17 ±0.49	2.36 ±1.34^{ab}	0.63 ±0.30^{ab}	0.075 ±0.027	0.0097 ±0.0037^{a}
Control	2.08 ±0.43	2004.0 ±1101.0	1.95 ±1.16^{ab}	4.92 ±5.42	1.46 ±0.85	2.23 ±1.54^{ab}	1.17 ±1.00^{a}	0.044 ±0.021	0.0108 ±0.0059^{a}

Root:Shoot is the root to shoot ratio. N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

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Appendix 10

Nitrogen Determination

Principle :

The AOAC (Association of Official Analytical Chemists) approved determination of nitrogen is based on the measurement of an emerald green colour formed by the reaction of ammonia, sodium salicylate, sodium nitroprusside and sodium hypochlorite in an alkaline medium (pH 12.8-13.0). The absorbance of the ammonia-salicylate complex is measured at 660 nm. The nitrogen test according to Kjeldahl encompasses all organically bound nitrogen. Before the samples can be analysed, they must be digested to convert the nitrogenous organic compounds to ammonia using a Kjeldahl digestion (Appendix 9).

Reagents :

Ammonium sulphate $(NH_4)_2SO_4$
Di-sodium hydrogen phosphate (Na_2HPO_4)
Potassium Sulphate (K_2SO_4)
Sodium chloride (NaCl)
Sodium hydroxide (NaOH)
Sodium hypochlorite (NaOCl)
Sodium nitroprusside
Sodium potassium tartrate $(NaKC_4H_4O_6)$
Sodium salicylate (HOC_6H_4COONa)
conc. Sulphuric acid (H_2SO_4)
Brij (buffer solution)
Distilled water

Solutions :

1. Acid / salt solution

Sulphuric acid (conc)	7.5 ml
Sodium chloride	100 g
Distilled water	1000 ml
Brij (buffer solution)	2 ml

Dissolve 100g of NaCl in ± 600 ml distilled water. Add 7.5ml sulphuric acid. Mix and dilute to 1000 ml with distilled water. Add 2 ml Brij solution and mix thoroughly.

2. Sodium hypochlorite solution

Sodium hypochlorite 15%	0.3 ml
Distilled water	1000 ml

Dilute 0.3 ml of 15% sodium hypochlorite solution to 100 ml with distilled water and mix thoroughly. Prepare fresh daily.

3. Sodium salicylate - Sodium nitroprusside solution

Sodium salicylate	150 g
Sodium nitroprusside	0.3 g
Distilled water	1000 ml

Dissolve 150 g of sodium salicylate and 0.3 g of sodium nitroprusside in about 700 ml of distilled water and mix thoroughly. Vacuum filter the reagent and store in a light resistant container.

4. Buffer solution

Di-sodium hydrogen phosphate ($12H_2O$)	35.81 g
Sodium potassium tartrate	50 g
Sodium hydroxide 50% w/w	108 g
Distilled water (make up to)	1000 ml

Dissolve 35.81 g of $Na_2HPO_4.12H_2O$ and 50g of sodium potassium tartrate in about 800ml s of distilled water. Add 108 g sodium hydroxide solution (50% w/w), mix and cool to room temp. Dilute to 1000 ml and mix thoroughly.

5. Standard diluent solution

Kjeldahl catalyst	2.5 g
Hydrogen peroxide (100 vol)	3.0 ml
Sulphuric acid (conc)	5.0 ml

Add 2.5 g Kjeldahl catalyst, 3 ml hydrogen peroxide and 5 ml sulphuric acid to digestion tubes. Digest at 410° for 45 minutes. Make up to 100 ml with distilled water.

6. Nitrogen standard solution (stock = 3.000 mg N / l)

Ammonium sulphate	1.4151 g
Standard diluent soln.	100 ml

Dry ammonium sulphate in oven overnight and then dissolve 1.4151 g in about 80 ml of standard diluent solution. Make up to 100 ml and mix thoroughly.

Working Standards :

Pipette the required amount of nitrogen standard solution into a 100 ml volumetric flask. Dilute to volume with standard diluent solution and mix thoroughly.

Volume of N standard sol (ml)	Sol nitrogen conc (mg/l)
1	30
2	60
4	120
6	180
8	240

Start-up procedure :

Check the level of all reagents to ensure an adequate supply for the run. Place all solution supply lines into their relevant containers with the exception of the Na salicylate-Na nitroprusside solution line which should be allowed to pump water-brij solution for five minutes. After this time place the Na salicylate-nitroprusside line into its own container and allow ten minutes equilibration time before attempting to set the zero nitrogen base line. Samples are automatically run and the results stored in computer memory ready for printing at the end of the run. At the end of the run place the Na salicylate-nitroprusside line into the water-brij (wash) container for five minutes. When this time has elapsed the rest of the reagent lines can be placed into the wash solution container. This process cleans out all the lines and prevents sodium salicylate-nitroprusside residues from building up and affecting the next run.

Appendix 11

Atomic Absorption spectrophotometry

Atomic Absorption spectrophotometry relies on the fact that when solutions containing metal ions are vaporized in a flame they absorbe light at specific wavelengths. By setting up standard curves from the absorbance of a series of standard concentrations of the metal ions to be analysed, the concentration of that metal ion in an unknown sample can be determined.

Concentrations of the ions of the metals potassium, iron, manganese and zinc were determined. The wavlengths at which the atomic absorbtion was completed were as follows

Metal	**Wavelength** (nm)
Potassium	404.4
Iron	386.0
Manganese	279.5
Zinc	213.9

Appendix 8 continued

Mean values and standard deviations from statistical analysis of *Trachypogon spicatus* Trial 2 Shoots

Inoculum	Shoot length cm	St Dry Mass g	Shoot N mg/g sample	Shoot P mg/g sample	Shoot K mg/g sample	Shoot Fe mg/g sample	Shoot Mn mg/g sample	Shoot Zn mg/g sample
Cape Flats	862.3 ±449.1	1.47 ±1.10	6.16 ±3.60	2.61 ±1.41^{a}	5.03 ±3.39	0.105 ±0.078^{ab}	0.218 ±0.129^{a}	0.0090 ±0.0071^{b}
Worcester	994.2 ±229.9	1.84 ±0.54	3.23 ±1.68	1.46 ±0.63^{b}	3.35 ±1.43	0.048 ±0.028^{b}	0.117 ±0.058^{ab}	0.0036 ±0.0018^{b}
B. West	1209.7 ±292.0	2.06 ±0.49	2.94 ±1.20	1.05 ±0.26^{b}	2.72 ±0.89	0.061 ±0.026^{b}	0.087 ±0.022^{b}	0.0030 ±0.0012^{b}
Large	1112.8 ±282.0	1.83 ±0.37	3.36 ±1.26	1.15 ±0.26^{b}	2.92 ±0.98	0.092 ±0.034^{ab}	0.129 ±0.041^{ab}	0.0067 ±0.0063^{b}
Small	915.0 ±260.0	1.38 ±0.53	6.12 ±2.92	1.71 ±0.73a^{b}	4.69 ±2.81	0.164 ±0.077^{a}	0.164 ±0.068^{ab}	0.0158 ±0.0066^{ab}
Control	927.4 ±430.9	1.51 ±0.71	7.34 ±8.87	1.95 ±1.39a^{b}	5.10 ±3.44	0.175 ±0.139^{a}	0.209 ±0.160^{ab}	0.0348 ±0.0427^{b}

N = nitrogen, P = phosphorus, Fe = iron Mn = manganese and Zn =zinc.

The superscripts a, b, c *etc.* represent groups that are significantly different from one another.

Appendix 9

Kjeldahl Digestion

Before any chemical analyses on the grass samples could be completed, they had first to be digested to release the analytes required. This is performed with a modified Kjeldahl procedure.

Reagents :

conc. Sulphuric acid (H_2SO_4)

Hydrogen peroxide (*100* volume or *200* volume as indicated)

Mercury based Kjeldahl catalyst (Merck)

Method :

Weighed (4 decimal places) samples were placed in calibrated 100ml glass digestion tubes together with 5ml of conc. sulphuric acid, a Kjeldahl catalyst tablet and approximately 3 ml of 100 volume hydrogen peroxide. The tubes were then heated to 410°C in a closed fume cabinet and maintained at this temperature for 45 minutes. Splashes of digestate were washed back down the tubes with hydrogen peroxide every 15 minutes, during the process, to ensure complete digestion of the samples. Once digested, the samples were allowed to cool to room temperature and then made up to 100ml with distilled water. Aliquots of the each sample were then analysed on a SCALAR segmented flow auto-analyser, from which nitrogen and phosphorus content were determined.