Effects of Arbuscular Mycorrhiza on plant growth of four ornamental annuals (*Dianthus chinensis x barbatus, Impatiens wallerana, Petunia x hybrida* and *Viola x wittrockiana*) commonly grown in South Africa

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DECLARATION

Student number: 36855103

I declare that **Effects of Arbuscular Mycorrhiza on plant growth of four ornamental annuals (*Dianthus chinensis x barbatus, Impatiens wallerana, Petunia x hybrida* and *Viola x wittrockiana*) commonly grown in South Africa** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of references.

12/07/2016

Signature

Date

ABSTRACT

Commercial AM fungi isolates, *Rhizophagus clarus*, *Gigaspora gigantea*, *Funneliformis mosseae*, *Claroideoglomus etunicatum* and *Paraglomus occulum* were tested on four seasonal ornamentals, *Dianthus chinensis* x *barbatus*, *Impatiens wallerana*, *Petunia* x *hybrid* and *Viola* x *wittrockiana* planted in peat-base medium. The experiment was conducted in a glasshouse with three replicates in a completely randomised design. Various vegetative (height, width, length, number of leaves, leaf area and dry biomass) and reproductive (number of flowers and buds) plant parts were measured in the course of three months. AM fungi was found to increase seedling growth and reduced seedling mortality rate of all the plants studied. Inoculated plants produced more leaves (16-33%) and grew taller (12-28%). Dry biomass of inoculated *Dianthus*, *Impatiens* and *Viola* plants were significantly increased by 25-53%. All plants under low colonisation rates displayed mycotrophic qualities and net growth output thereof were found to be similar to plants with equal or higher colonisation rate. Mortality were less frequent in inoculated plants and they were also less susceptible to transplant shock.

Key words: arbuscular mycorrhiza, annuals, rhizosphere, inoculation, symbiosis, extraradical mycelium, hyphae, mycorrhizal dependency, root cortices, interface.

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AM arbuscular mycorrhiza

ARB arbuscules

ANOVA analysis of variance

CMN common mycorrhizal network

DA discriminant analysis

DM mycorrhizal dependency

ECM ectomycorrhiza

ERM extraradical mycelium

FB fine branching

GRSP glomalin-related soil proteins

IH intercellular hyphae

LMWOAs molecular weight organic acids

LSD least significant difference

MCP mycorrhizal colonisation percentage

µm micrometer

MHB mycorrhization helper bacteria

MWD mean weight diameter

Myc $^{-}$ / AM $^{-}$ uninoculated control

Myc $^{+}$/AM $^{+}$ inoculated with mycorrhiza fungi

MNR mycorrhizal N responsive

MPR mycorrhizal P responsive

NFB N-fixing bacteria

PGPR plant-growth-promoting-rhizobacteria

ppm parts per million

PSB phosphate-solubilizing bacteria

RH runner hyphae

RMD root mycorrhizal dependency

S1/S2 sample 1/sample 2

SMD shoot mycorrhizal dependency

SOM soil organic mass

sp. species (singular)

SRR shoot root ratio

TDB total dry biomass

VA vesicular-mycorrhiza

v/v volume/volume

CHAPTER 1
LITERATURE REVIEW

CHAPTER 1

1. LITERATURE REVIEW

1.1 Arbuscular Mycorrhiza

Paleobotanical fossils indicate that mycorrhizal fungi were present 450 million years ago at which point they were found to initiate the transition of plants from water to land-based environments (Redecker, Kodner & Graham 2000). Various other forms of lineage dating suggest that Arbuscular Mycorrhiza (AM) fungi may have been present before terrestrial-based plants (Schubler 2012; Smith *et al*., 2010). Mycorrhizal fungi are non-pathogenic and form various close relationships with terrestrial plant roots. These Mycorrhizal relationships are responsible for the acquisition and uptake of nutrients through their specially modified hyphal structures supplying them to the plants they colonise (Allen 1992; BassiriRad 2005). Mycorrhizal relationships are classified broadly into two main groups (Figure 1.1). Endomycorrhiza, of which Arbuscular mycorrhizas are the largest group, and ectomycanhizas.

Arbuscular mycorrhizal relationships were discovered in the late 1800s by Gallaude who first named the characteristic tree-like structures ‘arbuscules’ from the Latin word *arbusculum* (Genre & Bonfante 2010b). However, it was only in the 1950s that Mosse proved that symbiosis between plants and fungi existed. AM fungi, being biotrophic obligates, require a host plant to complete their life cycle. They form mutualistic symbiotic associations with between 70-90% of plant species (Avio *et al*., 2010; Hause & Fester 2005). They colonise mostly Bryophytes, Pteridophytes, Gymnosperms and Angiosperms (Schubler 2012). Colonisation by AM fungi is commonly found in forest biomes (Aggangan & Moon 2013; Hawley & Dames 2004), agricultural crops (Cavagnaro *et al*., 2011; Miranda

et al., 2011; Nzanza *et al*., 2011; Yaseen *et al*., 2011) and ornamental bedding plants (Gaur & Adholeya 2005; Koide *et al*., 1999; Linderman & Davis 2003).

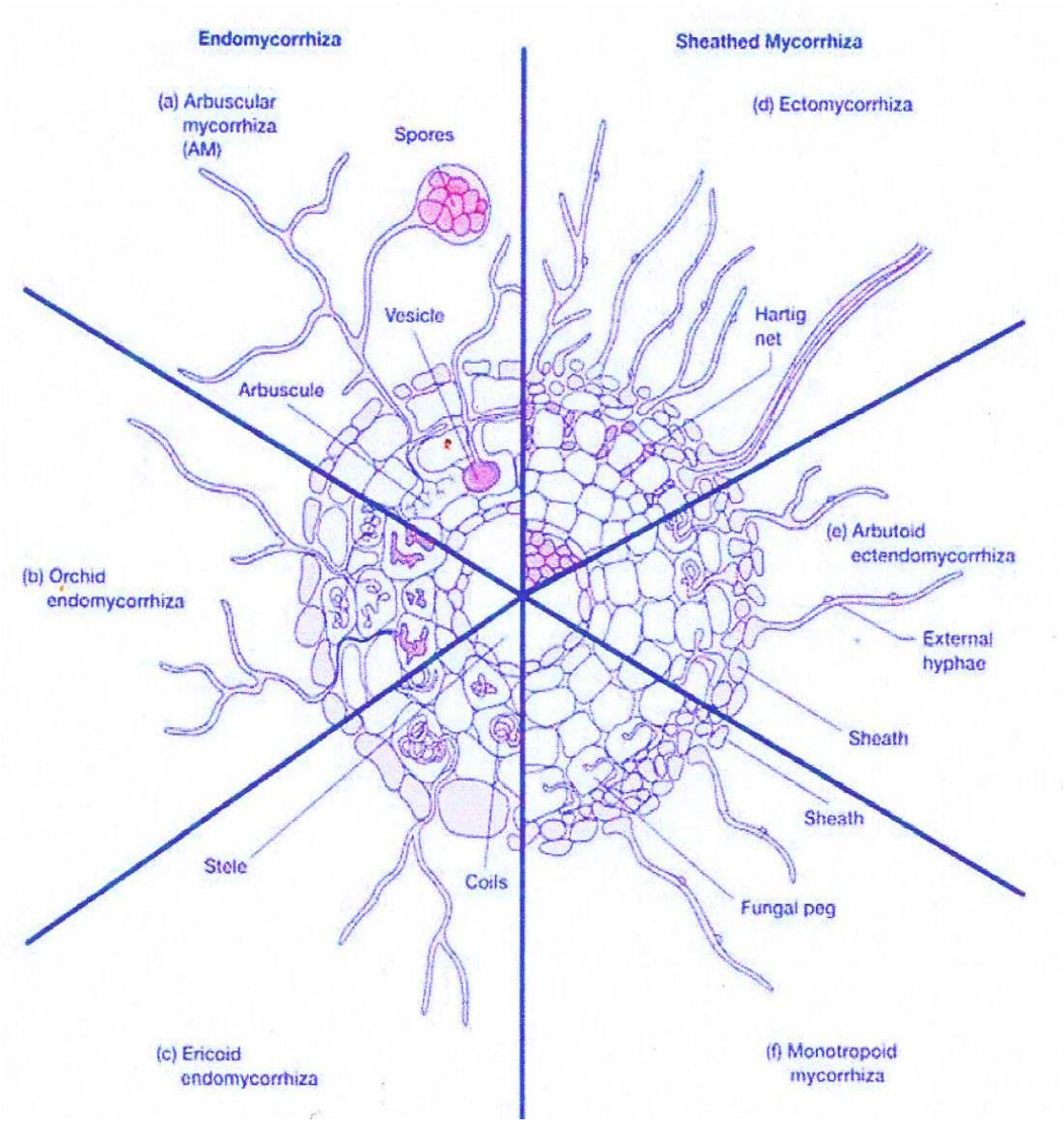


Figure 1.1 Cross section of root heir structure indicating various different Mycorrhizae groups (Prescott *et al*., 2005).

1.1.1 General Biology within Root Structure

Visually AM fungi are differentiated morphologically from other mycorrhizas according to the arbuscular structures found within the root cortices. Arbuscules are intracellular haustoria consisting of fine, dichotomously branched intraradical hyphae (Figure 1.2). Colonisation is divided into two different associations depending on the host plant species. In the Linear (*Arum*) series, hyphae grow longitudinally within intercellular air spaces whereas in the coiling (*Paris*) series, hyphae produce intracellular coils due to the lack of intercellular air spaces (Smith & Read 2008). Morphologically, intraradical hyphae differ within species. In the sub order *Glomus, Acaulospora* and *Entrophospora* hyphal branches are much smaller and thinner while in certain species intraradical hyphae may grow along the root axis or in a looped pattern. Intercellular hyphae then spread through the root cortices creating intercellular interfaces (Malla & Varma 2005). Within this area, the unique interface between plant cells and AM fungi is established. Arbuscules and vesicles are formed within these cell cortices forming a "compartment" and it is in this area within the plant cell cytoplasm that nutrients are exchanged (Azcón-Aguilar *et al.,* 2009). Once the host-fungi interface is established, nutrients are exchanged. Vesicles, oval to irregular shaped structures develop either inter- or intracellularly, acting as nutrient storage structures containing lipids and cytoplasm (BassiriRad 2005). However, vesicles are only produced by species in the order Glomales (Giovannetti & Avio 2002) and play an important role in fungal reproduction through propagule distribution.

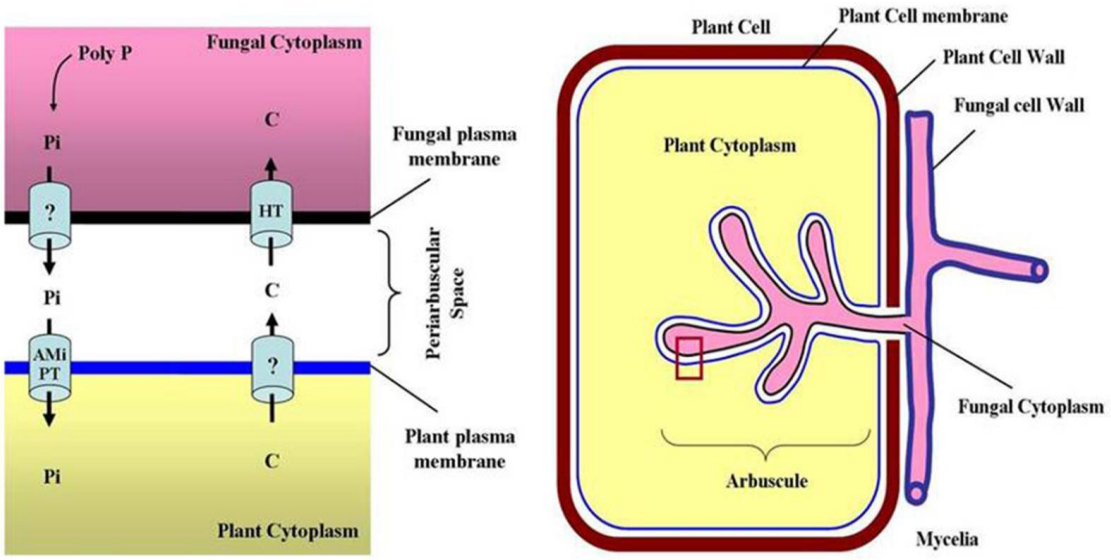


Figure 1.2 Nutrient exchange within root cell illustrating the arbuscular mycorrhiza symbiotic interface (Johri *et al*., 2015), carbon (C) from host plant and inorganic phosphate (Pi) from AM fungus.

1.1.2 Life cycle of AM fungi

Asymbiotic phase: The initial stage of reproduction occurs asexually relying only on germinating chlamydospores which are completely independent of the host (Waschke *et al*., 2012). AM fungal spores are thick-walled, round structures located in soil and occasionally in roots; and are mainly involved in the survival, dispersal and establishment of fungal structures. Spore germination occurs within hours, days or months depending on AM fungal species, and physical and chemical factors (Tommerup 1983a). Under unfavorable environmental conditions, spores undergo dormancy to protect against premature germination. After germination, runner hyphae (RH) 5 -10 µm wide develop, and continues to grow in a more or less straight line towards the intended host root branching off at perpendicular angles (Khasa *et al*., 2009). Further development of fine branching (FB) hyphal structures increase the exploratory surface area. In the absence of a host, asymbiotic

hyphal growth is delayed or cease completely causing protoplasm retraction and compartmentation (Giovannetti & Avio 2002).

Presymbiotic phase: Positive stimuli from root exudates, such as sesquiterpene, or in the presence of a host plant, root RH form a swollen hyphopodium structure (direct colonisation) on the surface of the roots. Dichotomous fan-like exploratory hyphae form, thereby increasing the probability of finding a root. The hyphopodium structure, 16 -80 µm in length, develops parallel with the root epidermis (Smith & Read 2008) with single or multiple hyphal peg structures developing from the pre-infection structure.

Symbiotic phase: The narrow paginated hyphae penetrate the root epidermal cells forming intraradical hyphal mycelium. Inter-or intraradical hyphae develop initiating intracellular arbuscular formation within the root cytoplasm. It is at this point, through the hyphal trunk, where the unique symbiosis between plant cells and AM fungi are established. After successful colonisation through symbiotic interface establishment, AM fungi produce extraradical symbiotic hyphae which facilitate the transportation of nutrient to the host plant and mycelium structures (Khasa *et al*., 2009). Extraradical hyphae undergo exponential growth after colonisation, increasing surface area by forming main and secondary hyphal networks promoting the colonizing of nearby plant or multiple plant species simultaneously. Sporulation emphasizes the final phase of AM fungi life cycle. New spores develop on FB "horse-tailed" structures (Khasa *et al*., 2009) produced from primary and lateral developmental hyphae. Collectively, the extraradical hyphal network and associated AM fungal structures within the soil form the mycorrhizosphere (Pinton *et al*., 2007). The mycorrhizosphere influence exudates which improves the microbial activity around the plant root. According to Smith & Read, (2008) the mycorrhizosphere is considered most important area for nutrient acquisition and exchange in the soil.

1.2 Plant root reaction during *Paris* and *Arum* colonisation

In order for AM fungi to successfully penetrate and be accepted by the host, biochemical and cytological events as well as intercellular modifications occur to initiate colonisation (Genre & Bonfante 2012). This also includes regulatory mechanisms through nutritional and hormonal plant defense regulation. At the preinfection developmental stage, signal transduction genes in the plant root is activated. As the respiratory activity of the AM hyphae increases, plant roots respond by producing strigolactones. (Smith & Read 2008). Prior to colonisation, some plant roots respond by thickening of epidermal wall, depending on the type of plant-fungus combination. During appressorium formation, cytological changes within the plant epidermal cell wall occur. As a defense strategy during the initial contact, the host plant initiates a series of induction and suppression functions as precursor to recognition and compatibility. These regulatory functions are poorly understood.

Plants and plant root morphology are variably influenced by environmental factors. The presence or abundance of sufficient water, sunlight, temperature and nutrients affect them directly and indirectly (Russell 1977; Smith & Read 1997). In addition, AM fungi also affect root systems and plant growth. Studies conducted on *Prunus cerasifera* seedlings showed an increase in root branching, root cap diameter as well as cell numbers (Berta *et al*., 1995). Furthermore, roots also experience cytological changes during mycorrhizal colonisation, such as epidermal thickening depending on the type of plant and fungus combinations.

1.3 Mycorrhizosphere effect

1.3.1 Biological

The rhizosphere contains vast amounts of microorganisms contributing to soil biological fertility and nutrient turnover. The microbial community constitutes 75-90% of the total soil

organic mass (SOM), whereas AM constitutes 5-50 % and P-solubilizing bacteria (PSB) 1-50% SOM (Johnson & Gehring 2007). These soil microbes form beneficial associations with AM fungi altering the plant root system, known as the "mycorrhizosphere effect" (Azcón-Aguilar & Barea 2015; Powell & Klironomos 2007). Within the mycorrhizosphere, tripartite associations could be formed with rhizobia; N-fixing bacteria (NFB) and phosphate-solubilizing bacteria (PSB) (Azcon-Aguilar *et al*., 1986), plant-growth-promoting-rhizobacteria (PGPR) and plant-growth-promoting-fungi (PGPF) (Dames & Ridsdale 2012). Mycorrhization helper bacteria (MHB) such as *Pseudomonas fluorescens, P. putida,* and *P. gladioliassist* associate with mycorrhizal fungus. They assist with soil fertility, promote spore germination in the rhizosphere (Azcón-Aguilar & Barea 2015; Powell & Klironomos 2007) and enhance AM fungi growth prior to colonisation. Bacteria from other genera, such as *Bacillus* and *Burkholderia,* have also been shown to associate with mycorrhizal structures in the mycorrhizosphere (Seneviratne *et al*., 2010).

1.3.2 Chemical

Soil microorganisms significantly alter the biochemical composition of soil (Smith & Read 2008) through solubilization, mineralization (Bano & Ilyas 2010) and the secretion of low molecular weight organic acids (LMWOAs). This allow mycorrhizas to exploit nutrients, especially element K through mineral weathering (Arocena *et al*., 2012), enhancing mycorrhiza-host symbiosis through "synergetic interaction" (Carvalho *et al*., 2010). Phosphate ions, a by-product of PSB are taken up by the AM mycelium thereby increasing plant growth and interaction. These interactions have been investigated with various host plants, for example soya bean legume (Azcon-Aguilar *et al*., 1986) and *Allium ampeloprasum* (Taktek *et al*., 2015). Legume soya grown in neutral-calcareous soil showed that interaction between PSB and AM fungi enhanced plant growth. Soil stability and quality

is enhanced by glomalin-related soil proteins (GRSP), an insoluble hydrophobic Fe-containing protein (Delian *et al*., 2011) produced by extracellular hyphae. It has been theorized that glomalin acts as a natural "glue" assisting as a soil binding agent within the mycorrhizosphere (Rillig & Mummey 2006). However, biochemical evidence for this is lacking since glomalin is difficult to solubilize and studies show inconclusive evidence to support this theory (Bedini *et al*., 2009; Cornejo *et al*., 2008). Based on hyphal biomass, glomalin contributes significantly to carbon storage. A study conducted on tropical forest soils showed a direct contribution of AM fungi and glomalin to SOM also indicating that the glycoprotein accumulates over millions of years in soil (Rillig *et al*., 2001). Glomalin is distributed across biomes (Rillig *et al*., 2003) constituting 4.5% of the total soil carbon (Rillig *et al*., 2001).

Nitrogen fixing bacteria (NFB) and crops associated with nitrogen cycling also contribute significantly to soil chemical fertility and this may indirectly allow AM fungi to further transfer N to nearby crops (Frey & Schüepp 1993). In legume-based cropping systems, most of the plant root residue and nodules are recycled (Cooper & Scherer 2012) into available N increasing soil fertility thereby acting as a "biological fertilizer" (Barea & Jeffries 2012).The amount of N_2 fixed differs according to legumes crops, but has been reported on *Arachis hypogaea* (100), *Trifolium* sp (250), *Lupinus* sp (150), *Calliandra calothyrsus* (24) and *Medicago sativa* (250) (kg N ha $^{-1}$) (Werner & Newton 2005). This indicates that the AM fungal partner enhances N_2 fixation rates and improves N cycling and uptake. However, successful legume performance depends on soil fertility levels (Azcón-Aguilar & Barea 2015). In a field experiment, Antunes *et al*., (2006), examined the effects of a tripartite combination of AM fungi, *Bradyrhizobium japonicum*, and soya bean *Glycine max*. The experiment showed that under high soil P conditions, no N_2 fixation occurred. This result is in an agreement with a previous experiment which showed that P levels higher than 100 mg

P kg $^{-1}$ decreased AM colonisation (Schubert & Hayman 1986). Their study further showed AM fungi favored soil P levels of 50 mg kg^{-1} and that the tripartite symbiosis at these levels, stimulated and enhanced nodulation activity. The results, indicated that excessive soil P levels, NFB were less effective in promoting N_2 fixation.

1.3.3 Physical

Arbuscular mycorrhiza contributes significantly to the physical properties of soil. Within the biophysical context, the AM hyphal network improves soil structure by forming macro-aggregates providing a skeletal structure which has a binding effect on the soil on a macro-level (Barea & Jeffries 2012) and which is further bound by glomalin. With regards to soil aggregation, through biosensing AM hyphae are able to use MHB to form a mycorrhizosphere which facilitates mineral weathering. For example, studies conducted on *Medicago sativa* (Bedini *et al*., 2009) showed that hyphal density and length of the AM fungus, Glomus, had a positive effect on soil aggregation, enhancing soil stability. The study further showed that the mean weight diameter (MWD) of AM plants were much higher than in uninoculated plants and there was a direct correlation between GRSP concentration, aggregate stability and root volume of AM plants. In AM fungal-rich soil, hyphal enmeshment and alignment modify soil particles microaggregation and water infiltration properties (Rillig & Mummey 2006). Studies on veritisols inoculated with AM fungi after disruptive wetting had lower water content (pore size of between 67 -75 µm) showed that pore indexes were much higher in soil with natural AM fungi (Bearden & Petersen 2000) improving root penetration.

1.4 Arbuscular Mycorrhiza benefits

The physical benefits of the arbuscular mycorrhizal/plant symbiosis are attributed to an increased surface area to form a common mycorrhizal network (CMN) which increases nutrient uptake, as opposed to uptake of nutrients via the direct pathway (plant roots). According to the *Law of the Minimum,* plant growth is directly associated with the amount of nutrients that are least available (Curl & Truelove 2012). Through the CMN, AM fungi provide a diverse range of benefits to most plants species: quality and quantity of seed production, alleviation of biotic and abiotic stresses, tolerance to drought (Allen 2006; Asrar & Elhindi 2011; Liu *et al*., 2015) and toxicity alleviation (Tao *et al.*, 2005). These benefits will be discussed further in section 1.4.1, 1.4.2 and 1.4.3.

1.4.1 Increased Flowering

Seed production rate and seed mass are linked to flower and bud formation (Koide 2010). AM association therefore enhances flower quantity (Smith & Read 2008) and duration (Koide 2010) which are an important consideration in post plant production (Nell *et al.*, 1997). To test the hypothesis that AM inoculation does affect flower quantity and duration, different tomato cultivars were inoculated with *Glomus etunicatum* (Bryla & Koide 1990). Seeds from two different cultivars were selected: Lycopersieon esculentum " Pixie" and large cherry and seeds from eight accessions (wild) species of tomato, and Lycopersicon esculentum var. cerasiforme from eight different Latin American countries. Bryla & Koide, (1990) reported that inoculated plants in accessions groups (seven out of ten) promoted early flowering. Furthermore, 50% of the inoculated accession plants produced on the average more flowers due to greater leaf area and P content. Flower duration was higher in seven out of ten groups inoculated with AM fungi but only one accession was significant at 95% confidence level. In a second study, AM fungi increased the reproductive phase (number of

flowers) of inoculated plants (Gaur *et al.*, 2000). *Petunia hybrida*, *Callistephus chinensis* and *Impatiens balsamina* were inoculated with *Glomus, Gigaspora* and *Scutellospora* spp. The study showed inoculated plants produced significantly greater number of flowers and improved flower initiate time.

1.4.2 Plant reproduction

Seed production is an important component of plant reproduction which relies on various input methods, and plants employ one of several strategies. Seed production is directly influenced by quantity, quality and proportion of flower formation (Koide 2010). Plants inoculated with mycorrhizal fungi can improve their seed production through increased flower output. For example, in a greenhouse study, the reproductive component of the annual *Abutilon theophrasti* inoculated with an AM fungus was investigated (Lu & Koide 1994). Plants were inoculated with *Glomus etunicatum* and growth parameters were studied at various intervals and at final harvest, 90 days after transplanting plants into pots. Lu & Koide (1994) showed that plants inoculated with AM fungi had increased seed production as a result of flowers. Inoculated plants increased their flower duration by flowering earlier than uninoculated plants which contributed to a higher number of flowers. This study did not only show that AM fungi-inoculated plants increased seed and fruit production but also that they produced much heavier seeds with higher P content.

Abutilon theophrasti seed from the above experiment were collected to investigate the hypothesis that seed from inoculated parental plants produce progeny with higher P content (Koide & Lu 1995). In another study, two separate experiments were conducted by Koide & Lu (1995). The first experiment consisted of a 45-day glasshouse experiment with two treatments groups; group one consisted of two groups treated with *Glomus intraradices* with

and without phosphorus (superphosphate 6g P m^{-2}) and a second group with similar P combinations but without an inoculant treatment. The second experiment consisted of only distilled water as growth medium. Seedlings were placed in seed growth pouches and root growth parameters such as tap root length and root densities were observed and recorded once developed and again on day 10. Results from experiment two showed that under nutrient-deficient conditions the growth rate of plants from AM fungi inoculated maternal plants were much higher than that of uninoculated plants due to inherent nutrient content. Biomass of first generation AM plants showed increased root branching (114%) and dry weight (24%). This finding indicates that root morphology traits of one generation is passed onto the next generation, allowing offspring of mycorrhizal plants to increase their growth much earlier and at a much faster rate than non-mycorrhizal plants.

1.4.3 Increased tolerance to drought

Plant-water-relations is key to plant development and sustained growth. A common denominator to all abiotic stresses is osmotic stress (Ruiz-Lozano & Aroca 2010). Hyphae of arbuscular mycorrhiza are able to alleviate osmotic stress through water uptake via an extended root network (Augé *et al.*, 2007; Augé *et al.*, 2008; Marulanda *et al.*, 2003) . To study the effects of drought stress, an experiment on lettuce was conducted (Ruiz-Lozano, Azcon, & Gomez, 1995). *Lactuca sativa* L. cv. Romana were inoculated with seven AM fungal isolates. This study showed that proline quantities in relation to drought stress were directly associated with the degree of mycorrhizal colonisation. The percentage *Glomus deserticola* and *G. caledonium* colonisation were highest in drought-stressed plants, 94.1% and 65.5%, respectively. The colonisation was lowest in the *G. occultum* drought-stressed group (32%) while inoculation was higher in the well-watered plant group (42%). Proline, an amino acid, produced by plants, accumulates in leaves when plants are under stress, was

higher in plants with lower colonisation. This was evident in plants inoculated with *G. occultum*, *G. fasciculatum*, and *G. mosseae* which produced 73%, 89%, and 107% proline respectively. In plants with higher values of colonisation, proline content was much lower. The proline content of plants colonised with *Glomus deserticola* and *G. caledonium* increased by 50%.

1.4.4 Toxicity alleviation

Soils heavily contaminated with Zn and Cd have a negative effect on plant biological processes (Powell & Klironomos 2007). Quantities exceeding tolerance levels of these metals impede the growth and loss of biomass (Påhlsson 1989). Studies showed that species of AM fungi are effective biosorbants that are capable of binding heavy metals in contaminated soils (Turnau *et al*., 2010). The removal of heavy metal involves the surface absorption via ion-exchange and crystallization within the microfibrillar cell wall of the AM hyphae. To test the absorptive capabilities of AM fungi, two plant species *Trifolium subterraneum*, cv. Mount Barker were inoculated with *Glomus mosseae* and *Lolium perenne*, cv. Barclay with *G. lamellosum* (Joner *et al*., 2000). The experiment demonstrated that *G. mosseae* biosorbtion by hyphal mycelium surface was the most effective means of eliminating Cd from the medium.

1.4.5 Nutrient Acquisition and Growth Response

Arguably the most important symbiotic benefit to plants is the acquisition of the major chemical elements, P and N, in exchange for carbon (Watkinson *et al*., 2015) contributing directly to plant growth. In some instances, up to 80% of P and N is acquired via AM fungi (Azcón-Aguilar & Barea 2015). This is due to the cost effectiveness of acquiring P via the

extraradical mycelium (ERM) which is much faster than diffusion through plant roots (direct pathway) (Smith & Read 1997). AM fungi acquire P through the ERM via the mycorrhizal pathway. P is translocated through the ERM and transferred to the host-fungal interface by Pi transporters (Smith *et al*., 2011). Any excess P is converted into polyphosphates (polyP) (Figure 1.2). AM colonised plants benefit mostly from orthophosphate or inorganic Pi ($H_2PO_4^-$), which is considered the most important nutrient acquired by the host plant through AM symbiosis (Smith & Smith, 2012). Since mycorrhizas are naturally acidophilic, P uptake in the rhizosphere is accomplished by decreasing soil pH around hyphae structures. External hyphae release oxalic, citric and malonic acids transform complexed P into soluble form (Watkinson *et al*., 2015). Different uptake and diffusion rates allow net import of P into the root system through the AM pathway to the host plant. The spread of the ERM is limited by the size of the hyphal compartment in root cortical cells as well as hyphal longevity. According to Bucher (2007), root architecture affects mycorrhizas ability to mine P in the depletion zone.

In high rainfall areas, P deficiency is a common problem due to leaching of nutrients leading to phosphorus deficiency. Host plants in P deficient soils benefit greatly from symbiosis with AM fungi (Ahmad & Prasad 2011). The amount of phosphates available in the mycorrhizosphere affects AM fungal ability to acquire P. Experiments conducted on *Kolapanax septemlobus* indicated that an increase of only 2 mg P kg $soil^{-1}$ significantly increased root, shoots and total plant dry weight. However, the addition of 16 to 32 mg P kg $soil^{-1}$ had no effect on growth, biomass yield and P uptake (Aggangan & Moon 2013; Smith *et al*., 1986). Once in oversupply, AM fungi have a limited functional capacity to absorb P. Arbuscular mycorrhiza is completely reliant on host organic C. These include, energy (Smith *et al*., 2011), development and maintenance of intra- and extraradical mycelial structures (Jones *et al*., 2009), respiration and uptake of nutrients (Smith & Read 2008). As

such, AM growth is severely limited without host carbohydrate supply, which can metabolize up to 30-35% of host photosynthates (Jennings & Lysek 1999). Smith & Read (2008) estimated that an average of 15% of net C produced by plants is allocated to mycorrhizal development. AM fungi acquire C either through intracellular hyphae or at the fungal-host interphase where it is converted to lipids (Figure 1.2). The transfer of C to the fungus is made possible through proton motive force (Smith & Read 2008) through a series of effluxes and uptake synchronized by the bidirectional movement of C-P trade. The exact order of importance and 'signals' responsible for this transfer are still unknown (Smith & Smith 2012).

Nutrient P deficiency affects plant functioning and sustained growth. Limited access to available P affects respiration and photosynthesis thereby reducing reproduction (Bucher 2007; Schachtman *et al*., 1998). At low P conditions, plants initiate a P-starvation strategy and form interactions with other soil microorganisms such as AM fungi. P is readily taken up by plants in solution in the root zone. This source quickly becomes depleted outside the depletion zone. Phosphates are inaccessible to most plant roots (Suriyagoda *et al*., 2014). Through the symbiotic relationship with AM fungi, plants are able to supplement their P requirements.

After successful colonisation, both host plant and AM fungi show mutual growth benefits. Plants inoculated with AM fungi display vast improvements compared to untreated plants (Bonfante & Genre 2010a). The supply of nutrients to treated plants and water uptake are readily available and in greater quantities compared to plants acquiring nutrients only through their root system. Hyphal growth rate increases, thereby increasing nutrient translocation and influx capacity, accelerating as the internal-external hyphae growth and cellular interface development (Hause & Fester 2005). Post-colonisation by AM fungi

increases nutrient supply to host plants resulting in net growth increase of root, shoot and P content (Smith & Read 2008).

Nzanza *et al.*, (2011) showed that inoculated *Solanum lycopersicum* improved elongation and dry biomass. AM inoculation enhanced growth of seedlings *Amorpha crenulata* and *Jacquemontia reclinata* indicating a moderate improvement of nodule numbers and shoot-dry weight (Fisher & Jayachandran 2016).

1.4.6 Disease Control

AM host plants show increased resistance to soil pathogen attack due to enhanced plant vigor and health (Whipps 2007). Studies have shown that root architecture undergoes significant changes under disease stress (Dugassa *et al*., 1986; Sharma *et al*., 1992) caused by pathogens such as *Phytophthora, Aphanomyces,* and *Verticillium* (Barea & Jeffries 2012). Various AM fungal species have been shown to reduce disease incidence. Some examples are as follows: *Glomus intraradices,* inoculated *Linum usitatissimum* (Linseed) challenged by the pathogen *Fusarium oxysporum.* L. (Dugassa *et al*., 1986), *Glomus intraradix* inoculated potato *Solanum tuberosum* and the pathogens *Fusarium sambucinum* (tuber rot) (Niemira *et al*., 1996), *Glomus fasciculatum* and *Glomus etunicatum* inoculated strawberry (*Fragaria x ananassa)* and root pathogen *Phytophthora fragariae* (Norman *et al*., 1996). Furthermore, *Glomus intraradices* and G. *claroideum,* inoculated *Pisum sativum* (host plant) reduced the incidence of pathogen *Aphanomyces euteiches* (root rot) (Thygesen *et al*., 2003).

The soil borne pathogen *Pythium* causes root rot, a common growth debilitating plant disease affecting seeding cycles and seedling production and is present in virtually all soils

around the world. The infection cycle of *Pythium* is initiated by above or below ground germ tubes and infects root systems of newly sown seed. During the post-emergent germination stage, infection occurs either above the ground (Agrios 2005). In a recent study, *Glomus intraradices, G. mosseae and G. claroideum* were inoculated onto tomato *Lycopersicon esculentum* Mill to analyze the effect on the soil pathogen *Pythium aphanidermatum* (Larsen *et al*., 2011). Three-day-old *Lycopersicon esculentum* seedlings were transplanted into pots containing isolates of all three AM fungi mixed into the sand/soil medium. After 14 days, seedlings were infected with the pathogen *P. aphanidermatum*. Root subsamples were collected at seven and fourteen days. AM fungal colonisation was assessed and *P. aphanidermatum* infection was quantified by measuring the quantity of protein (μg protein mg^{-1}) of fresh roots. The study showed that the effects of *P. aphanidermatum* varied at both harvesting periods. At the first harvesting period, colonisation percentage of *G. clariodeum* was 74%, *G. intraradices* 63% while *G. mosseae* were unaffected by *P. aphanidermatum*. In contrast, in the second harvesting period, none of the AM fungi were affected by soil pathogen. The concentration of *P. aphanidermatum* protein extraction was lowest in *G. clareodium* inoculated plants showing greater suppressive qualities, while *G. mosseae* indicated least antagonistic response. Dry and wet biomass weights of *G. mosseae* were lower compared to *G. clareodium*. Results showed positive suppressive responses to *P. aphanidermatum* pathogen.

1.5 AM fungi in Horticultural Systems

Ornamental annuals are widely used in commercial and domestic gardens due to their functional and aesthetical qualities. They are important bedding plants providing instant color throughout the year. These plants also have a huge economic importance. In the United States of America, ornamental plants contribute over 10% to the total agricultural sector

valued at over $2 billion (Nell *et al*., 1997). According to an interview on 10 June 2012, Mr. R Goodwin, manager of a wholesale seedling production nursery (Peebles Plants, Johannesburg) confirmed that the total annual sales of cavity trays (six packs) for their nursery was estimated at R 800 000, and cavity containers 48, 104 and 200 trays has a combined monthly average of over R100 000. Quality and health of ornamental annuals are important factors in the nursery production and landscape sectors. Transplant survival of seedlings (germination trays to cavity containers) directly affects the quality and health of annuals during the production phase. During the germination stage alone, mortality rate of seedlings transplanted from growing to cavity trays is 20-30% (Goodwin 2012, pers.comm.,10 June).

1.5.1 Growth responses and survival rate after transplanting

Sexual reproduction is the most cost effective means of growing ornamental plants and attaining greater species selection and diversity. However, reproduction via seed is limited by high transplant mortality rates (Fenner & Thompson 2005). Due to relative immature root development, nutrient availability affects transplant success rate of newly established seedling. An integrated inoculation system at establishment stage could provide enhanced growth-related benefits (Smith & Read 2008). For instance, increased survival rate and growth response were observed in olive trees under saline conditions inoculated by *Glomus mosseae, G. intraradices* or *G. claroideum* (Porras-Soriano *et al*., 2009) and micropropagated hardwood tree species, *Kalopanax septemlobus,* inoculated with *Glomus* sp. (Aggangan & Moon 2013). Similar benefits were observed by *Allium cepa L., cv. 'VSetana'* (Vosatka 1995) and *umbrella pine* root cuttings, *Sciadopitys verticillata,* inoculated with *Glomus intraradix* (Douds *et al*., 1995).

To study the effects of inoculation at different intervals - sowing and transplanting stages, experiments under controlled environments were conducted on six bedding plants. The experimental setup included *Tagetes patula, Impatiens walleriana, Salvia splendens, Petunia × hybrida, Coleus × hybridus,* and *Viola × wittrockiana* inoculated with *Glomus intraradices* (Koide *et al*., 1999). In the first experiment *Tagetes, Impatiens, Petunia, Coleus* and *Viola* seeds were sown in flats and inoculated. Once true double leaves appeared, roots of randomly selected plants were assessed for colonisation. The remainder of the seedlings was harvested at different intervals to determine shoot dry weights and AM fungal colonisation. To determine colonisation percentage on the plant *Salvia,* known for low inoculation success rate, a second experiment was conducted. Seeds of *Salvia* were sown into flats and inoculated with G. *intraradices.* Roots were harvested after 25 and 32 days to test the percentage of colonisation. Results indicated that the percentage of colonisation varied among species. Results from ANOVA indicated that plant shoot dry weights varied among plants inoculated at different intervals. Inoculation at sowing did not affect *Tagetes* and *Petunia,* while *Impatiens* was significantly affected. Inoculation at transplant stage was highly significant in four plant species; *Tagetes, Impatiens, Petunia* and *Viola.* Results from the second experiment showed *Salvia* percentage root colonization was significantly higher on day 32.

Vosatka *et al*., (1999) conducted a greenhouse experiment to study the effects of inoculation of AM fungi on transplanted seedling. Seeds of *Cyclamen persicum* var. 'Rosa mit Auge' were inoculated with *Glomus fistulosum, G. mosseae and G. intraradices* in peat medium flats and transplanted at 10 weeks into 12 cm pots and grown for 6 months. Mortality rates in uninoculated plants were almost 50% higher than in inoculated plants, 33.3% and 16.3% respectively. Growth parameters showed varied responses: *Cyclamen* plants inoculated with AM fungi showed higher mean scores for number of buds, 27.5 and leaf area, 2750 cm^2

compared to 20.3 and 2477 cm^2 of the uninoculated plants. Number of Flowers/Leaves, dry weights (leaves and flowers) were higher in the inoculated plants but were not significant. According to Barea & Jeffries, (2012) the use of AM fungi in plant production systems may improve productivity practices. The selection of appropriate AM fungi and host combinations are required to elicit plant growth benefits (Smith & Read 2008) and would result in economic benefits in terms of the overall production and implementation costs.

CHAPTER 2
GENERAL INTRODUCTION

CHAPTER 2

2. GENERAL INTRODUCTION

2.1 Motivational Considerations

Recently, interest in beneficial soil microorganisms as an alternative biostimulant to improve soil fertility and plant growth have received much attention. The possibility of soil microbes to reduce plant dependence on high intensity fertilizer programs and chemical disease control, while reducing environmental stress and degradation, have far reaching implications. Necessity to improve nutrient is further compounded by higher nutrient demand of modern cultivated plants species (Mengel 1983; Tawaraya 2003). A proposed alternative of "green" biotechnology for introducing arbuscular (AM) fungi to nursery based production systems, as a biofertiliser, to improve crop yield, minimize loss and increase profitability of high-end value crops have been suggested. Furthermore, seedling loss under nursery conditions is a major limiting factor especially at post-transplant stage (Porras-Soriano *et al.*, 2009). In principle, studies show that AM fungi enhances seedling survival and establishment rates. However, in practice, integrated fertilizer programs are the preferred method to increase plant fitness and growth (Smith & Read 2008). Moreover, the production carbon footprint and the increasing cost of fertilizer is an important consideration. Current agrobiotechnological advances into the benefits of AM-host symbiotic have greatly improved, however, benefits relating to AM fungi-horticultural species have received less attention. Moreover, nutrient dynamics associated with the inclusion of AM fungi into large scale production system are limited by "poor knowledge" and benefits of indigenous AM fungi to ornamental crop species and cultivars (Marschner 1995) especially in transplant based horticultural systems.

2.2 Hypothesis

The application of commercial AM fungal-inoculants can improve plant growth and survival of the ornamental annuals, *Dianthus chinensis x barbatus, Impatiens wallerana, Petunia x hybrida* and *Viola x wittrockiana.*

2.3 Study aim and objectives

Aim: The aim of this study was to investigate the effect of inoculation of selected annual seedlings with AM fungi. The annuals selected are commonly grown in South Africa for their use in the horticultural landscape industries.

Objectives: To demonstrate any benefits of AM fungal inoculation; the study objectives were to:

1. Investigate the effects of AM fungal inoculation on the growth and development of seedlings.
2. Determine the effects of AM fungal inoculation on survival of seedlings after transplanting and colonisation success rate.

2.4 Experimental plants

Four ornamental annuals plant species were selected as experimental plants. All of the selected plants have fairly similar cultural conditions and require moderate to specialist knowledge to grow. All experimental plants, except for *Impatiens wallerana,* are not susceptible to disease. Damping-off is usually associated with post-production *I. wallerana* seedling, however proper production management will reduce disease incidence.

D. chinensis x barbatus – The Genus *Dianthus* consist of over 300 species and thousands of cultivars and numerous subgroups. Flowers are red which are produced in summer, with short tubular bases with up-to five spreading tepals, bearded either toothed or fringed. *Dianthus* prefer well drained neutral to alkaline soils except for the species *D. microlepis, D. glacialis* and *D. pavonius*. *D. chinensis x barbatus* (Common name Sweet William) which is a cross between *D. chinensis* and *D. barbatus,* has a slightly larger canopy spread producing single flowers with terminal clusters 8-12 cm across, leaves are lanceolate 10 cm long with entire margins. *D. chinensis* produces terminal cymes in summer, leaves lanceolate shaped up to 8 cm long. Flowers are complexly patterned producing up-to 15 single fringed picotee shaped flower heads. *D. chinensis x barbatus* leaves are similar to *D. barbatus* however size of the flower cluster is similar to *D. barbatus.* Growth habit of *D. chinensis x barbatus* is considerably smaller than D. *chinensis and D. barbatus,* height 20-35 cm and width 23 cm (Brickell 1996).

I. wallerana – Common name garden balsam. Native to East Africa and a member of the Balsaminaceae. *I. wallerana* is hairless herbaceous perennial herb, growing mainly under shade to semi-shade conditions. Leaves are spirally arranged or ovate to oblong-elliptical 4-13 cm in length and 2.5 – 5.5 cm wide (Grey-Wilson 1980). Leaf margins are coarsely toothed and lanceolate shaped. Flowers have 5 petals 2-4 cm in diameter. *I. wallerana* 'Super Elfin White' produce pure white flowers. Upper petals are crested and laterals fused at the base (Grey-Wilson 1980).

Petunia x hybrid – Common name Petunia is an herbaceous perennial of the Solanaceae. They prefer full sun to partial shade. Leaves are simple with solitary trumpet-shaped flowers with viscid-pubescent weak or straggling growth (Brickell 1996). *Petunia x hybrida* 'Dreams Red Series' produce deep red flowers; the corolla is fused with undulating margin.

Stems are alternate on the basal stems, however, it becomes opposite near the top. Mature plants grow up to 10 cm in height and 40 cm diameter (Brickell 1996).

Viola x wittrockiana – Common names; pansy, viola, or violet. Native to Europe and Asia Minor. *V. wittrockiana* has two petals facing upwards and three pointing down wards. Flowers are zygomorphic consisting of five flowers with the bottom having a spurred shape. *V. wittrockiana* grows 10-15 cm tall, flowers 2.5 – 7.5 cm diameter (Brickell 1996). Plants tolerate full to partial shade but with less flowers are produced under shaded conditions.

CHAPTER 3
METHODS

CHAPTER 3

3. METHODS

3.1 Plant materials

Dianthus chinensis x barbatus, *Impatiens wallerana*, *Petunia x hybrid* and *Viola x wittrockiana* (Figure 3.1) were selected due to their versatility, functional and decorative qualities, and diverse range of habits. The selected annuals, contribute significantly to the horticultural and landscape industries (Brickell 1996). All seeds were obtained from Ball Straathof (Johannesburg, South Africa).

Figure 3.1 Annuals of (a) *Impatiens wallerana* 'Super Elfin White', (b) *Viola x wittrockiana* 'Matrix Series', (c) *Dianthus chinensis x barbatus* 'Ideal Red Series' and (d) *Petunia x hybrida* 'Ideal Red Series'. Source: Photos taken by author.

3.2 Experiment design and layout

The trial consisted of one experiment using the following plants, *Petunia* x *hybrida* 'Dreams Red Series ', *Impatiens wallerana* 'Super Elfin White' and *Dianthus chinensis* x *barbatus* 'Ideal Red Series' and *Viola* x *wittrockiana* 'Matrix Series' obtained from Ball Straathof (Johannesburg, South Africa).

Seed germination phase. Seeds of *I. wallerana*, *V. wittrockiana*, *P. hybrid* and *D. chinensis* x *barbatus* were surfaced-sterilized for 5 minutes with sodium hypochlorite (20%, v/v). Seeds of each species were sown in 20 cm x 30 cm seed germination trays (Figure 3.2) which were pre-sterilized with sodium hypochlorite (20%, v/v) and rinsed with distilled water. Klasmann Plug Mix obtained from Greenhouse Technologies (Johannesburg, South Africa) was used as the germination medium. The medium consisted of equal parts fine 1-7 mm sterile White Sod Peat and White Peat with pH 6.0. Treatment groups were inoculated with Mycoroot Super Booster®, a product containing mycorrhiza fungi. The following nutrients were present in the medium; Nitrogen (mg N/l): 100, Phosphorus (mg P_2O5/l): 115, Potassium (mg K_2O/l): 125, Magnesium (mg Mg/l): 100 (Klasmann-Deilmann 2012).

The temperatures within the greenhouse were kept at 22-24^{0}C. Radicales emerged after 3-7 days and stem and cotyledon emerged after 10-14 days. Grow medium were kept moist and saturated during this period. True leaves emerged 15-21 days after germination. Humidity was kept at 97% until cotyledons appeared. Light levels were kept at 35,000 – 45,000 lux to promote shoot and root growth. All plants received Nitrosol foliar spray, 50 ppm twice a week. After one week, dosage was increased to 100 ppm, until plants were ready for transplanting.

Glasshouse experimental set-up. After three weeks, seedlings of similar maturity were transplanted into seedling trays, six cavities per tray (Six packs). A completely randomized design (see Fig. 3.3) consisting of one treatment inoculated with Mycoroot Super Booster® a product containing mycorrhiza fungi. The trial design was a 4x2 factorial with factors, four plants species *I. wallerana, Viola* x *wittrockiana, Petunia* x *hybrid* and *D. chinensis* x *barbatus* and two treatments mycorrhiza (Myc^- = uninoculated control and Myc^+ = inoculated with mycorrhiza fungi). The 8 treatment combinations (4 x 2) were regularly rotated. Non-destructive data were recorded at regular intervals and time or periods are considered as a third factor (Snedecor & Cochran 1967). The experiment was conducted in a greenhouse facility at the ARC Roodeplaat Vegetable and Ornamental Plant Institute, Pretoria, South Africa.

3.2.1 Mycorrhizal Inoculation

At the seed germination phase, four seed germination trays were each filled 4000 grams of Grow Plug Mix ® and 100 grams of Mycoroot Super Booster® were mixed into medium and seeds of the four species were sown into each tray. For the glasshouse experiment, standardized individual plantlets were transplanted into six pack trays according to size, amount of true leaves and plant maturity. Growth media consisted of pre-sterile “Grow Plug Mix ®” 60 grams per cavity (dry weight), and inoculant Mycoroot Super Booster® mixed into grow medium 2.5% of the total volume, 260 propagules per cavity (50 propagules per ml). Seedlings were placed in the planting hole and covered with growth medium-inoculant mixture leaving young shoots exposed and hand watered.

MYCOROOT™ products have been developed using southern African isolates of Arbuscular Mycorrhizal fungi. The isolates have not been genetically altered in any way. The product is environmentally-friendly and produced in an inert clay carrier. Mycoroot products contain a combination of arbuscular mycorrhizal isolates which include *Glomus clarum* (new name *Rhizophagus clarus*), *Gigaspora gigantea*, *Glomus mosseae* (new name *Funneliformis mosseae*), *Glomus etunicatum* (new name *Claroideoglomus etunicatum*) and *Paraglomus occulum* (identifications are molecular determinations), (Mycoroot 2013, pers.comm., 2 May).

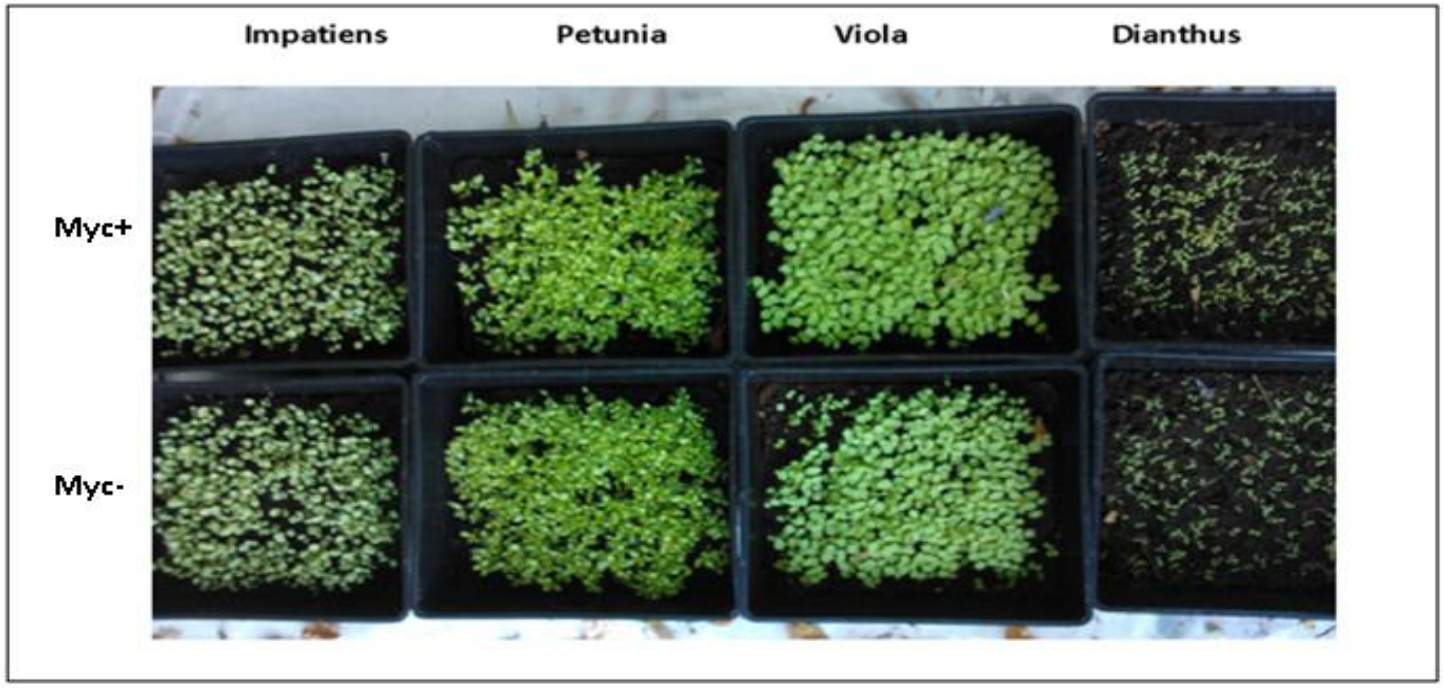


Figure 3.2 Seed germination trays with plantlets *Dianthus chinensis* x *barbatus*, *Impatiens wallerana*, *Petunia* x *hybrida* and *Viola* x *wittrockiana* before transplanting. Experimental Groups; Treatment (AM^+) and Control (AM^-).

Source: Photo taken by author.

Figure 3.3 Experimental design layout according to complete randomized design. Four plant species with groups inoculated (AM^{+}) and un-inoculated Control (AM^{-}), where T_1 – T_{10} = Treatment.

3.2.2 Cultural Conditions

Seedlings trays were randomized on a greenhouse bench and rearranged on a weekly basis to expose all plants to similar conditions. Plants were watered daily below the foliage by hand to minimize leaf contact, plants with low growing habit were carefully watered to avoid contact where possible and absolute care was taken when watering to eliminate any accidental inoculation across the two groups. The temperature within the glasshouse was controlled by an automated cooling and ventilation system. Temperatures during the day were kept at 17^0-18^0, night temperatures 19-20^0 at 60% humidity. Plants were dehumidified to allow airflow from above, enhance evapotranspiration and dry out the growing medium assisting root-oxygen exchange more efficiently. Under low light conditions, supplemental lighting HID (320 mmol/s) were provided when necessary. Plants received fortnight application of soluble foliar fertilizer at a rate of 100 ppm (Nitrosol).

3.3 Growth Parameters

During the three-month trial, plant growth parameters of *Viola x wittrockiana* 'Matrix Series', *Petunia x hybrida* 'Dreams Red Series', *Impatiens wallerana* 'Super Elfin White' and *Dianthus chinensis x barbatus* 'Ideal Red Series' were recorded through visual observations, destructive and non-destructive measurements at various intervals.

3.3.1 Non-destructive Measurements

Plant growth parameters per plant were measured every 15 days until 90 days. Parameters measured were plant mortality, plant height and plant canopy (length x width) measured in mm, number of leaves, number of flowers and number of buds.

3.3.2 Destructive Measurements

Leaf Area: Leaves of all four plant species were randomly selected and harvested on day 26 and day 90. In total 160 leaves were sampled, two samples (S1+S2) per tray were randomly selected and harvested and measured (cm^2) with a leave area meter (LI-COR 3100C). The mean leaf area of each tray was recorded and the difference in leaf area for each tray was calculated.

Final Harvest: At harvest, 90 days after the start of the experiment, plants were severed from plant roots. Shoots, flowers, buds and roots were weighed using Highland® scale (HCB602) and oven dried for 10 hours at 50^0C after which dry weights were recorded. Roots were weighed before being washed under running tap water to calculate the total weight before and after removing any soil medium from the root systems to minimize root material loss. Subsamples were taken 1-2 mm from root tips and placed in a container with an ethanol solution (50%, v/v) for staining. Roots were again weighed to correct subsample weight, oven dried and re-weighed.

To calculate the shoot:root ratio, total dry biomass in the phyllosphere (leaves, flower and buds) was divided by total dry biomass of below ground (roots) (Nouri *et al*., 2014). The following abbreviations apply; SDB = shoot dry biomass and RDB= root dry biomass.

Mycorrhizal dependency (DM) was calculated based on the total dry biomass content of above and below ground plant structures (Plenchette *et al*., 1983).

$$DM = \frac{\text{total dry biomass mass of myc}^{+}\text{ plant - total dry biomass of myc}^{-}\text{ plant}}{\text{total dry biomass of myc}^{+}\text{ plant.}} \times 100$$

Mycorrhizal dependency was further divided into either shoot mycorrhizal dependency (SMD) or root mycorrhizal dependency (RMD) to assess the MD of above and below ground plant structures.

$$SMD = \frac{\text{shoot dry biomass mass of myc}^{+}\text{ plant - shoot dry biomass of myc}^{-}\text{ plant}}{\text{shoot dry biomass of myc}^{+}\text{ plant.}} \times 100$$

$$RMD = \frac{\text{root dry biomass mass of myc}^{+}\text{ plant - root dry biomass of myc}^{-}\text{ plant}}{\text{root dry biomass of myc}^{+}\text{ plant.}} \times 100$$

The following scale was used to categorize mycorrhizal dependency of plants (Habte & Manjunath 1991).

i. Moderately dependent: plant with MD of 25- 50%.

ii. Marginally dependent: plants with MD of less than 25%.

iii. Independent: plants unaffected by AM fungi with MD of less than 0%.

3.4 Mycorrhizal Assessment

Uninoculated control and inoculated treatment sub-root samples were sent to Mycorrhizal Research laboratory, Rhodes University (Grahamstown, South Africa) to assess the total amount of arbuscular mycorrhizal root colonization. Roots were colored and stained with Trypan Blue (Sigma Cat No - T8154) (0.05%) (Varma & Oelmüller 2007). Root samples were first viewed under a Dissection Stereo microscope (LEICA S4E) to assess arbuscular mycorrhizal colonization of the treatment group and to establish any possible inoculation of the control group. The percentage of AM inoculation was determined using a modified Line Intersect Method to calculate the percentage of root colonization (Smith & Dickson 1997). Roots were placed on a microscopic slide, covered with a cover slip and inspected under a light compound microscope (LEICA CME), using 400 x magnification. Samples were inspected for any AM structure; intercellular hyphae and coils, appressorium structures and vesicles.

$$\text{Percentage of root colonization} = \frac{\text{colonized field of view}}{50} \times 100$$

3.5 Statistical Procedures

The non-destructive data was analyzed by applying a 3 factor factorial analysis of variance (ANOVA) test. Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro & Wilk 1965). In cases where significant deviation from normality and it was due to skewness, outliers were removed until data was normalized or symmetric distributed. (Glass *et al.*, 1972). Student's t-LSD were calculated at a 5% significance level to compare means of significant source effects. Contingency Rows x Columns frequency tables were performed for association between factors (plant species,

inoculation and periods), number of flowers, mortality and buds (Snedecor & Cochran 1967). Chi-Square (χ^2) tests were performed for association (Patterns) and where significant evidence was found graphs were constructed to demonstrate difference. To distinguish between the (eight plant species x treatments) combinations or only between plant species a Multivariate Discriminant Analysis has been performed with dependent variables (plant height, canopy width, canopy length, number of leaves, number flowers, buds and leaf area) as explanatory variables to identify the drivers for discrimination (Rencher 2003).

Dry biomass data were subjected to a 2 factor factorial analysis of variance (ANOVA) test, with random replication adding plant parts as a subplot factor. Shapiro-Wilk's test was performed on the standardized residuals to test for deviations from normality (Shapiro and Wilk 1965). Student's t-LSD (Least significant difference) were calculated at a 5% significance level to compare means of significant source effects.

A one-way ANOVA was used to analyze the percentage of root colonization.

All the above data analyses were performed with SAS version 9.3 statistical software (SAS Institute 1999). All Multivariate statistics were performed using XLSTAT software which is a statistical add-on module to Excel (XLSTAT 2015).

CHAPTER 4
RESULTS

CHAPTER 4

4. RESULTS

4.1 Growth Parameters

Growing conditions within the glasshouse was kept constant for optimal growth. No issues were experienced during the germination and transplantation processes.

4.2 Non-destructive Measurements

The 3 factor interaction (Plant Group x Mycorrhiza x Period) source effect was highly significant ($P<0.0001$) for all variables except for the number of leaves ($P = 0.13$) as illustrated in Table 4.1. The coefficient of variance was less than 15% (11.5%) therefore the data measured in one or two dimensions were considered to be reliable. The 4 x 2 treatment combinations over period for Plant Height, Plant Width, Plant Length, Number of Flowers and Number of Leaves (Figures 4.1, 4.2, 4.3, 4.4 & 4.5), were graphically interpreted based on 3 factor interaction.

Table 4.1 Three factor factorial Analysis for plant growth and development parameters; plant height, plant width, plant length, number of leaves, number of flowers and number of buds.

	Plant Height		Plant Width		Plant Length		Number of Flowers		Number of Leaves		Number of Buds		
Source	**DF**	**MS**	**Pr>F**	**MS**	**Pr>F**	**MS**	**Pr>F**	**MS**	**Pr>F**	**DF**	**MS**	**Pr>F**	**DF**	**MS**	**Pr>F**
PlantGroup	3	515039.8	<.0001	144749.4	<.0001	64267.6	<.0001	155.82	<.0001	3	15316.0	<.0001	3	278.6	<.0001
Mycorrhiza	1	48013.6	<.0001	8399.6	<.0001	10457.9	<.0001	0.20	0.4552	1	3955.3	<.0001	1	18.0	<.0001
Plant Group*Mycorrhiza	3	4933.4	<.0001	2458.3	<.0001	3839.2	<.0001	0.46	0.2755	3	31.8	0.1572	3	7.2	<.0001
Period	6	370701.5	<.0001	415467.3	<.0001	298090.8	<.0001	64.54	<.0001	6	16354.6	<.0001	6	70.4	<.0001
PlantGroup*Period	18	32334.3	<.0001	10302.2	<.0001	36145.2	<.0001	35.56	<.0001	18	895.8	<.0001	18	44.5	<.0001
Mycorrhiza*Period	6	2867.5	<.0001	506.2	<.0001	788.0	<.0001	0.87	0.0232	6	329.3	<.0001	6	5.5	<.0001
PlantG*Mycorrhiza*Period	18	417.9	**0.0004**	251.8	**<.0001**	364.2	**<.0001**	1.63	**<.0001**	18	25.3	0.1341	18	3.6	**<.0001**
Experimental Error	504	163.315		87.165		116.142		0.3523		504	18.266		504	0.4082	
Sample Error	2703	26.103		39.119		54.004		0.2066		2701	2.149		2705	0.2058	
Corrected Total	3262									3260			3264		
Coefficient of Variance		11.5%		13.1%		12.3%		n/a			n/a			n/a	

DF = Degrees of Freedom, MS Mean Squares, Pr>F = F probability
n/a = Not applicable
(P<0.01)

The three factor interaction analysis indicated that plant height increased dramatically from day 16-30 with relative consistent interaction between Treatment and Control groups. Least interaction occurred in plant groups *Petunia x hybrida* and *Viola x wittrockiana*. However, significant height differences were observed in the *Dianthus chinensis x barbatus* group at day 73 or 12.3% difference, with the highest height difference of 26.2% in the *Impatiens wallerana* group on day 90. Both plant species indicated a significant height increase when treated with mycorrhiza as compared to the control group (Figure 4.1).

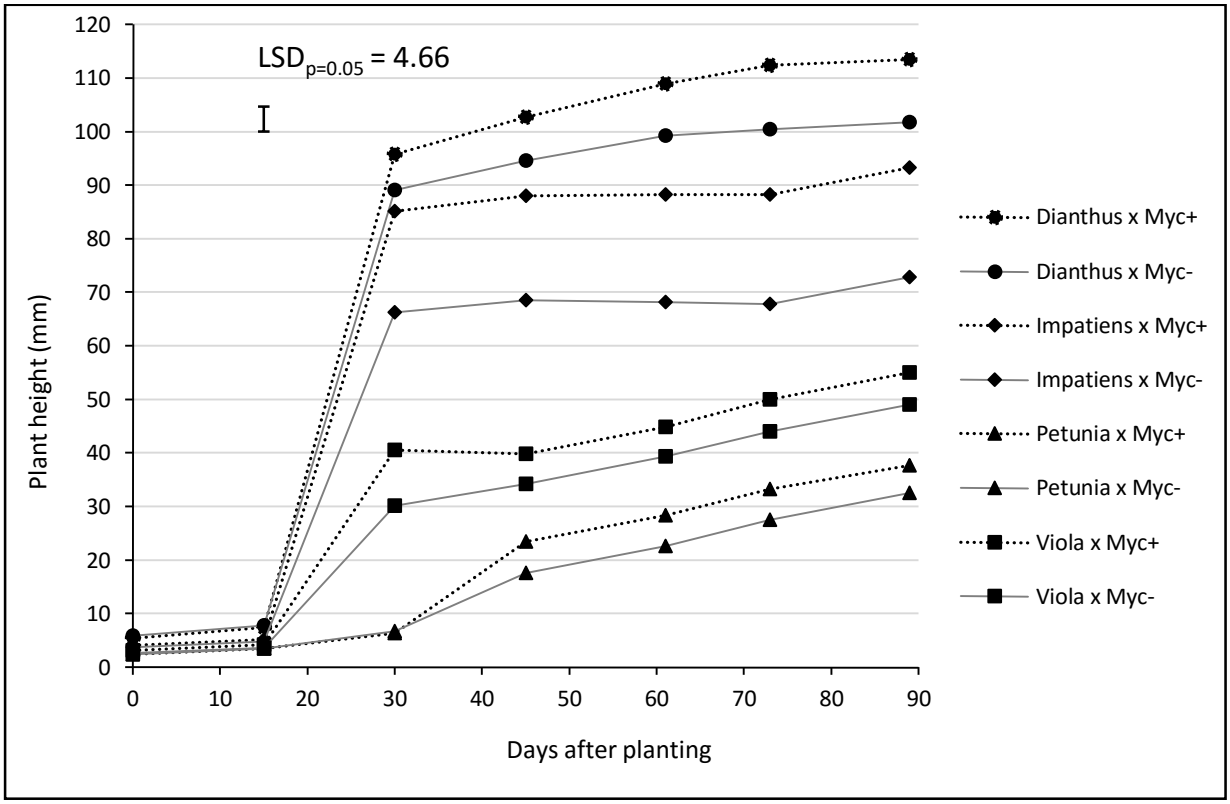


Figure 4.1 Three factor interaction (Plant Group x Mycorrhiza x Period) means for plant height.

The plant width increased dramatically from 16-30 and levelled off up to day 90 as illustrated in Figure 4.2. There was no significant difference in width observed in plants *Dianthus*, *Impatiens* and *Petunia*. However, 26.3% width difference at day 45 was recorded in the *Viola* group indicating a significant increase in plant width of inoculated plants compared to non-inoculated group.

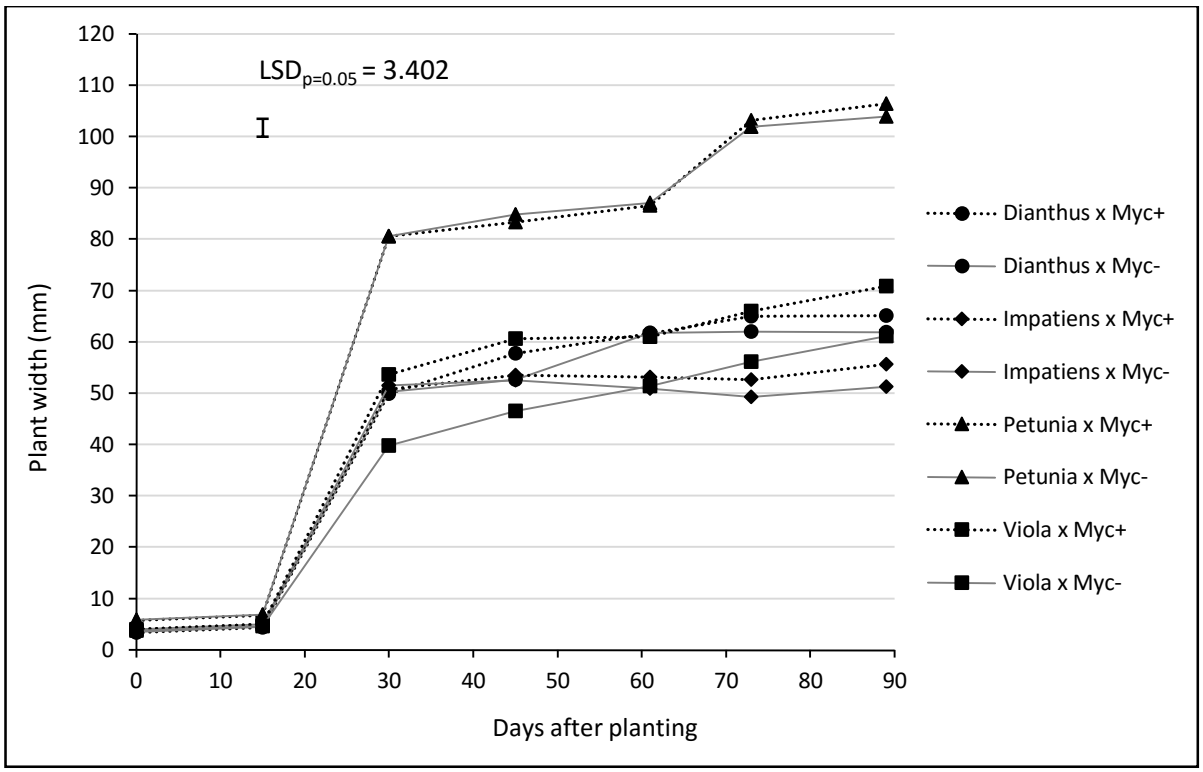


Figure 4.2 Three factor interaction (Plant Group x Mycorrhiza x Period) means for plant width.

Plant length increased differently for all plants (Figure 4.3). Significant difference occurred in inoculated plants *Dianthus* and *Impatiens* (day 90) and *Viola* (day 30 to day 90). On day 90, plant length of inoculated plants *Dianthus* and *Impatiens* increased by 6-7%. Mycorrhiza had a significant effect on *Viola*. On day 30, plant length of inoculated plant *Viola* increased by 30% (see Appendix A), but on day 90, decreasing gradually by 13%. Mycorrhiza had least effect on *Petunia,* showing no difference in plant length between inoculated and uninoculated plants.

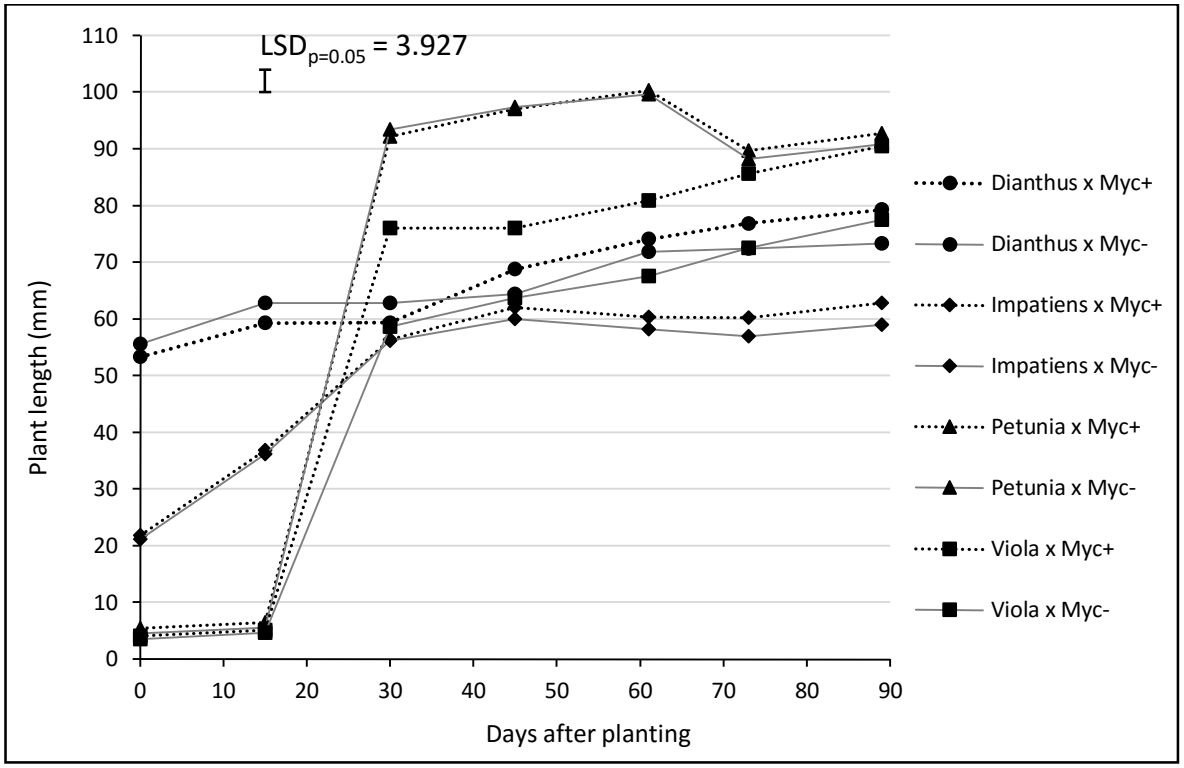


Figure 4.3 Three factor interaction (Plant Group x Mycorrhiza x Period) means for plant length.

The number of leaves show different trends over the experimental period for the different plant groups, (see Appendixes A and B). Mycorrhiza treated plants produce significantly more leaves from 45 days after planting for plant groups. At day 90 mycorrhizal treated plants showed a significantly increased number of leaves (Figure 4.4). Significant percentage difference from the 1.558; *Impatiens* 4.6 (74.5%), *Petunia* 4.3 (91.5%), *Dianthus* 4.2 (91.5%) and *Viola* 4.6 (96.8%).

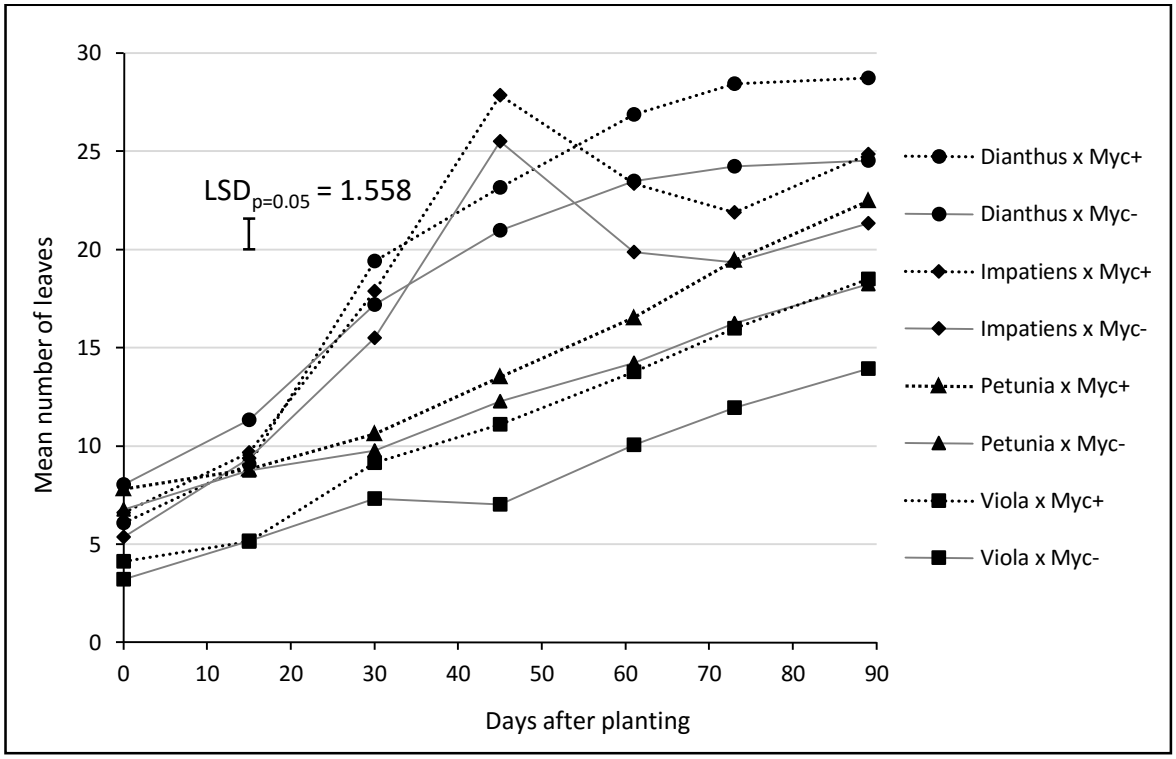


Figure 4.4 Three factor interaction (Plant Group x Mycorrhiza x Period) means for number of leaves.

The number of flowers produced show different trends over the experimental period for the different plant groups. Mycorrhizal treatment showed no significant effect within each plant group and period. In the *Impatiens* control group a noticeable increase in the number of flowers occurred compared to the treatment group 0.7 at day 45 but no difference occurred afterwards up to day 90. However, mycorrhiza did effect *Dianthus* on day 30 to 45, 0.6 and 0.4 respectively (Figure 4.5).

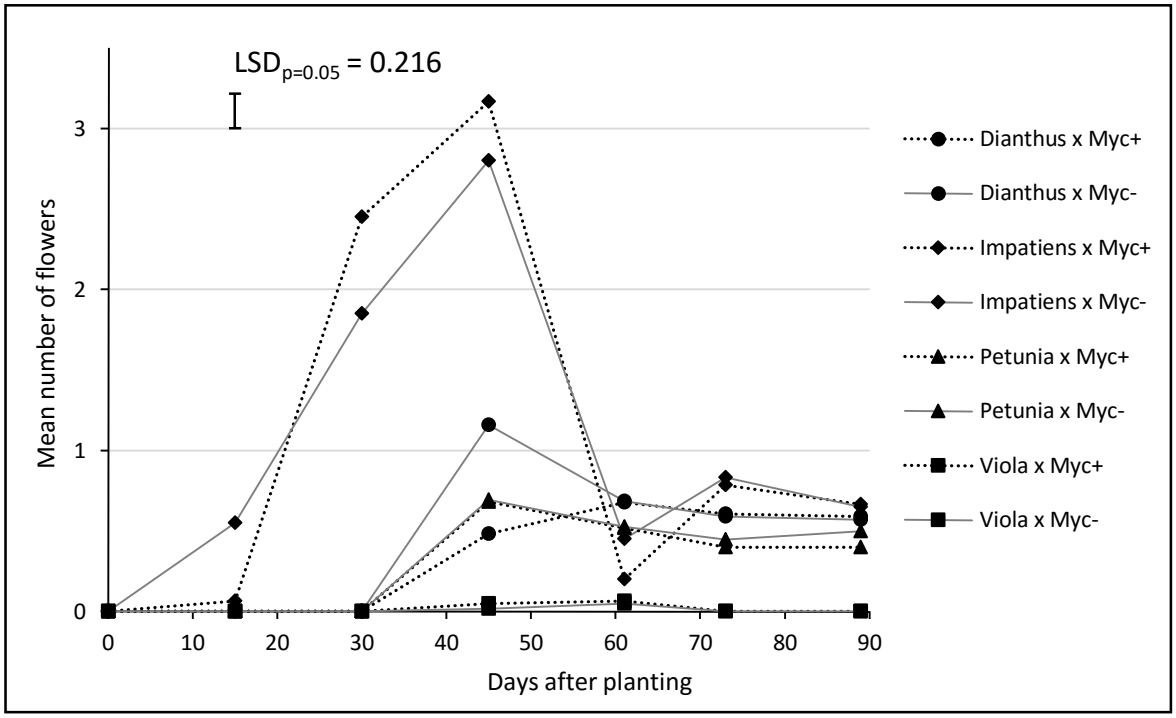


Figure 4.5 Three factor interaction (Plant Group x Mycorrhiza x Period) means for number of flowers.

4.2.1 Discriminant Analysis

Discrimination analysis was conducted between the eight treatment combinations (Plant Group x Mycorrhiza) using all variables measured on day 90 (Table 4.2). From Figure 4.6 there is clear separation between plant groups which are larger than that between

Mycorrhiza and the control. A further Discrimination analysis between the Plant Groups using all variables measured on day 90, is presented in Figure 4.7. From Figure 4.7 there is a clear separation between plant groups with, *Impatiens* and *Dianthus* grouped close together, however, *Viola* and *Petunia* are far apart (Table 4.2). From the total variation, 95.7% are declared by the drivers in Table 4.3.

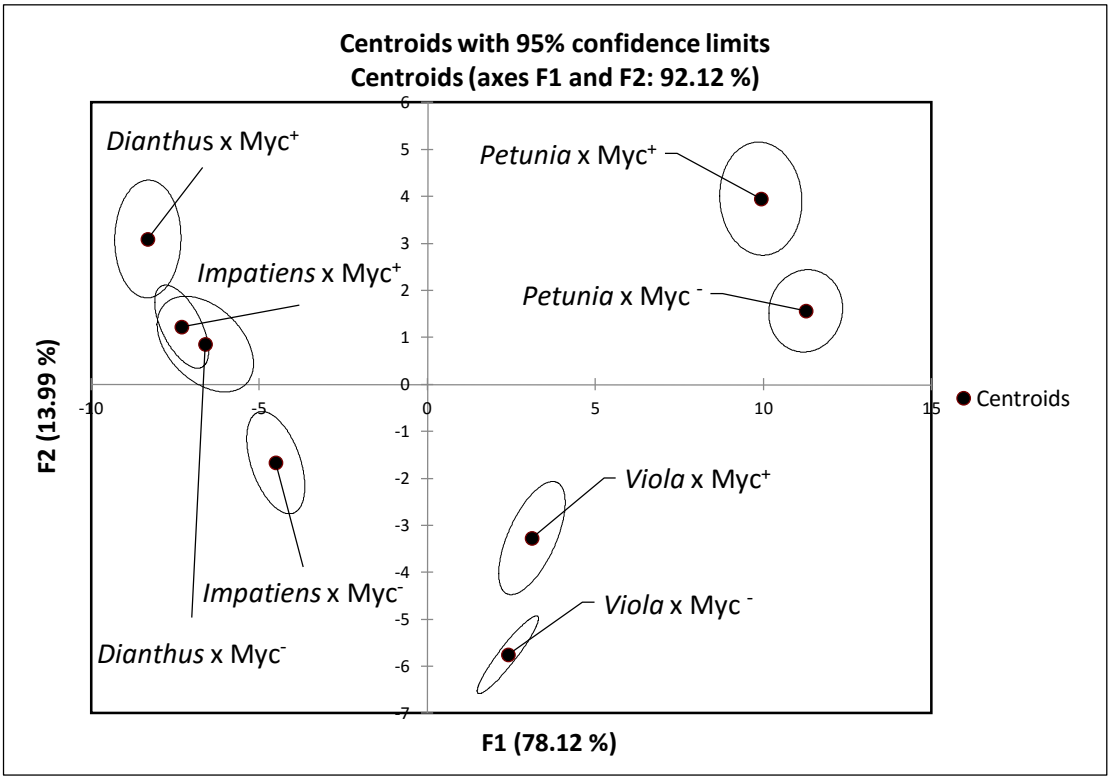


Figure 4.6 Discrimination association plots of the eight treatment combination (Plant x Group x Mycorrhiza).

Factor 1 distinguish between three groups 1 = *Impatiens and Dianthus*, 2 = *Viola* and 3 = *Petunia* with 82%. While for factor 2 there are only 2 groups 1 = *Petunia*, *Impatiens* and *Dianthus* and Group 2 = *Viola*.

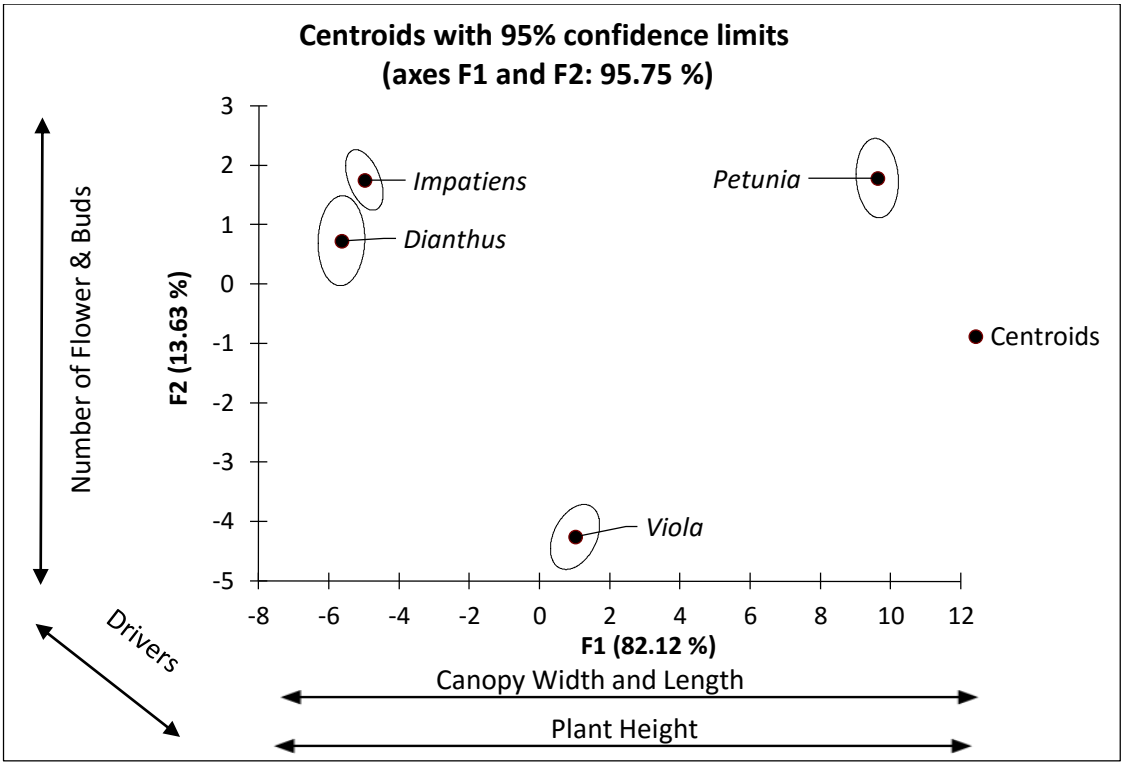


Figure 4.7 Discrimination association plots of the Plant Group indicating the drivers with each factor.

Table 4.2 Variable Factor correlations indicating the drivers for each variable in red

	F1	F2
Height	-0.940	0.244
Width	0.855	0.490
Length	0.732	0.174
Leaves	-0.575	0.723
Flowers	-0.360	0.559
Buds	0.048	0.658

Table 4.3 Variables Factor indicating the drivers for each variable in red

	F1	F2
Height	-0.891	0.237
Width	0.920	0.243
Length	0.751	-0.271
Leaves	-0.463	0.607
Flowers	-0.282	0.768
Buds	0.159	0.751

4.3 Destructive Measurements

Leaf Area: The mean leaf area (cm^2) of each tray was recorded and the difference in leaf area for each tray was calculated. If the leaf areas did not vary between day 15 and day 90 of the experiment, it can be assumed that the mean differences for each tray would vary around zero. The coefficient of variance was less than 15% (12.95), therefore the data measured in one or two dimensions were considered to be reliable. The two factor interaction (Plant Group x Mycorrhiza) source effect was significant (P<0.001) for all variables (Table 4.4). There was a significant increase in leave area between the start and end of the experimental period. Mycorrhizal treatment had no significant effect at the start and end periods on *Dianthus, Impatiens* and *Petunia.* However, leave area of *Viola* was affected by the mycorrhizal treatment with 101.3% difference between treatment and control groups, (see Appendix B), but decreased at the end of the experimental to 17.9% (Figure 4.8).

Table 4.4 Three factor Analysis of Variance table for destructive variable leaf area.

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Plant Group	3	358.42	119.4725	79.29	<.001
Mycorrhiza	1	105.67	105.6735	70.13	<.001
PlantGroup*Mycorrhiza	3	106.84	35.6124	23.64	**<.001**
Date	1	5639.51	5639.5092	3742.85	<.001
PlantGroup*Date	3	14.99	4.9980	3.32	0.022
Mycorrhiza*Date	1	0.82	0.8232	0.55	0.461
PlantGroup*Mycorrhiza*Date	3	12.26	4.0866	2.71	0.047
Experimental Error	**144**	**216.97**	**1.5067**		
Sample Error	160	144.18	0.9012		
Corrected Total	319	6599.67			
CV	12.95%				
Shapiro-Wilk PR<W	<0.001				
Skewness	0.000				
Kurtoses	2.584				

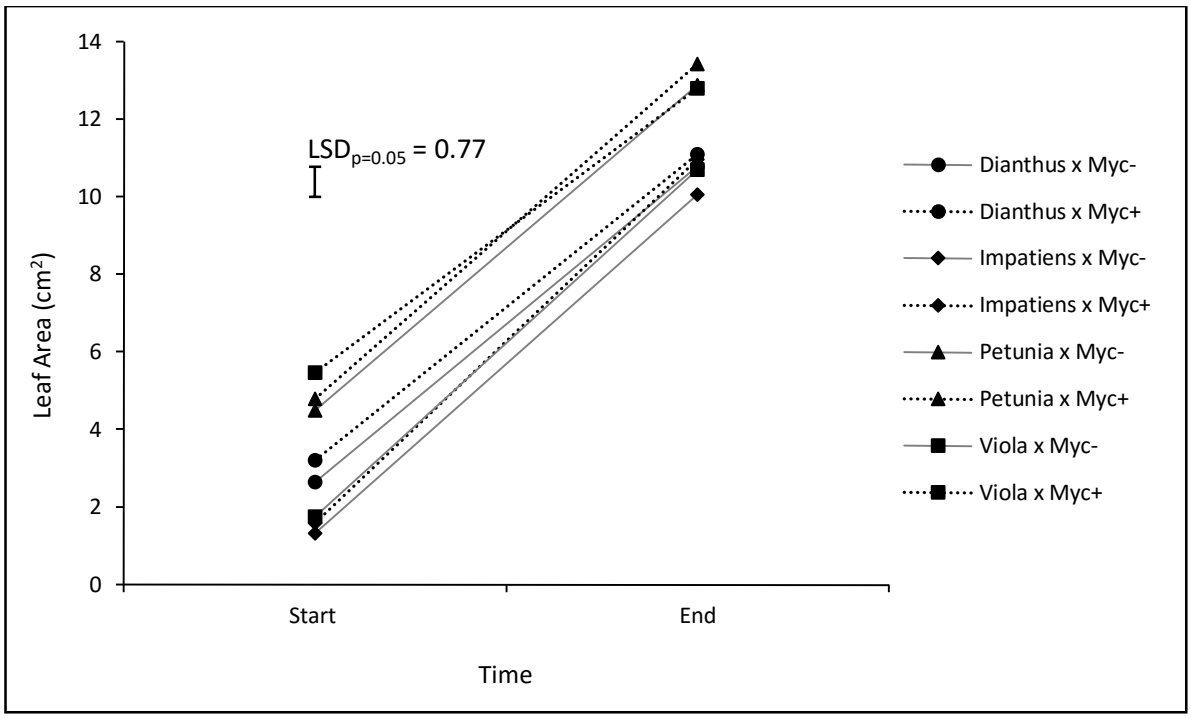


Figure 4.8 Three factor interaction (Plant Group x Mycorrhiza x Period) means for leaf area.

Dry Biomass: At 90 days after transplanting, dry biomass for roots and shoots were measured from the experimental unit. The coefficient of variance was less than 20% (18.1%), therefore the data measured in one or two dimensions were considered to be reliable. The three factor interaction (Plant Type x Mycorrhiza x Plant Part) source variation effect was highly significant (P<0.0001) for all variables (Table 4.5). Significant differences in shoot and root dry biomass weights were found in inoculated plants *Dianthus, Impatiens* and *Viola* in comparison with plants in control group (P=0.080) (Figure 4.9). However, *Petunia* recorded an inverse growth response to inoculation treatment. Shoot and root biomass dry weights of colonised plants decreased significantly compare to uncolonised plants (-23.4% and -40.0%, respectively).

Table 4.5 Combined Analysis of Variance with plant part as subplot factor for destructive variable dry biomass.

Variation Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
PlantType	3	5.7815	1.9272	241.62	<.0001
Mycorrhiza	1	0.0838	0.0838	10.51	0.0018
PlantType*Mycorrhiza	3	0.7168	0.2389	29.95	<.0001
Error a	72	0.5743	0.0080		
PlantPart	2	1.3148	0.6574	158.79	<.0001
PlantType*PlantPart	6	1.1032	0.1839	44.41	<.0001
Mycorrhiza*PlantPart	2	0.0538	0.0269	6.5	0.002
PlantType*Mycorrhiza*PlantPart	6	0.3308	0.0551	13.32	<.0001
Error b	144	0.5961	0.0041		
CorrectedTotal	239	10.5551			
CV = 18.1%					

DF Degrees of Freedom
Pr>F

Table 4.6 Three factor (Plant x Mycorrhiza x PlantPart) interaction means for dry biomass.

		PlantPart		Shoot/Root Ratio (SRR)	Total Dry Biomass (TDB)	MD (%)		
Plant	**Treatment**	**Shoot (SDB)**	**Root (RDB)**			**SMD**	**RDB**	**MD**
Dianthus	My-	1.002 b	0.505 d*	1.977 b	1.507 b			
	My+	1.091 a	0.796 a	1.368 b	1.887 a	8 ++	37 +	20 ++
Impatiens	My-	0.296 e	0.127 lm	2.355 a	0.422 f			
	My+	0.406 d	0.228 ijk	1.769 cd	0.634 e	27 +	44 +	33 +
Petunia	My-	1.020 a	0.518 cd	2.005 b	1.538 b			
	My+	0.781 g	0.311 gh	2.484 a	1.092 c	- 31+++	- 67 +++	- 40 +++
Viola	My-	0.269 i	0.302 gh	0.904 d	0.571 e			
	My+	0.459 h	0.416 e	1.099 cd	0.874 d	41 +	27 +	34 +
$LSD_{(p=0.05)}$		0.080		0.345	0.138	–		

* Means with the same letter/s (Column) do not differ significantly at a 5% significance level. **MD** (%) = mycorrhizal dependency. **SDB** = shoot dry biomass (shoots and leaves). **RDB** = root dry biomass. AM dependency categories; + = 25-50% moderately depended, ++ = 0-25% marginally depended, +++ = <0% independent. **SMD**=shoot mycorrhizal dependency. **RMD** = root mycorrhizal dependency.

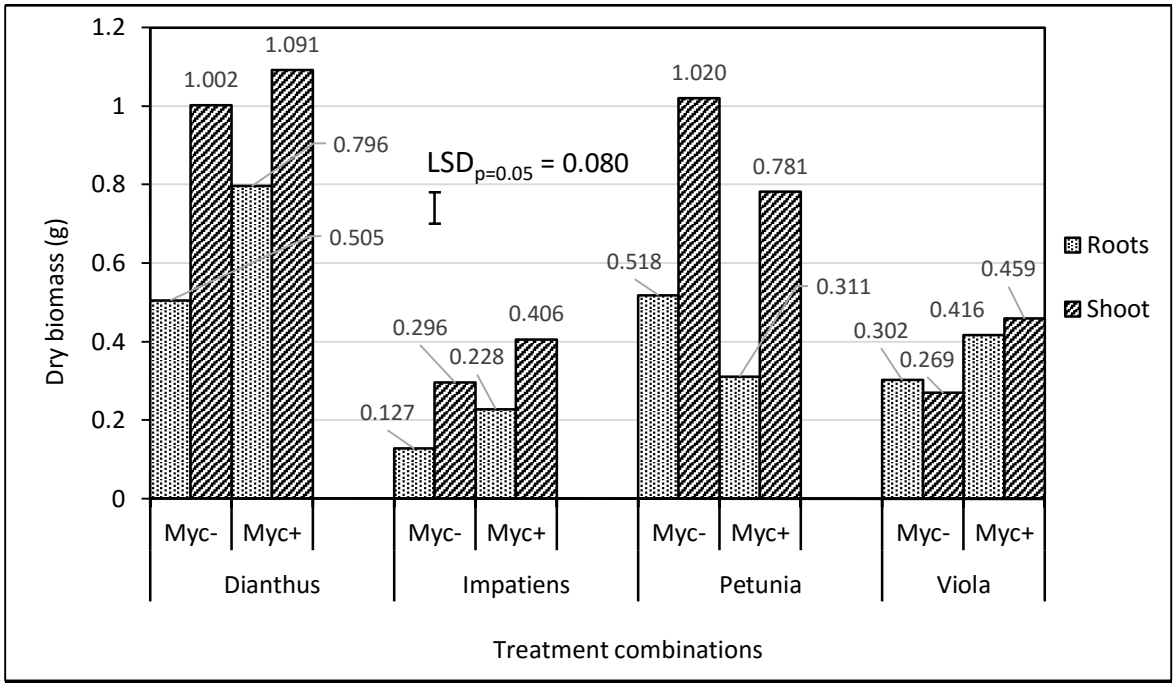


Figure 4.9 Three factor (Plant x Mycorrhiza x Plant Part) interaction means for dry biomass.

Shoot and root weight ratios were determined by dividing shoot and root weights expressed as percentage. Shoot/root ratios varied among inoculated and uninoculated plant groups. Ratios of inoculated plants *Dianthus* and *Impatiens* decreased significantly (30.8% and 24.8%, respectively) compare to uninoculated plants (Figure 4.10). However, ratios of inoculated plants *Petunia* and *Viola* increased compare to uninoculated plants. Only *Petunia* recorded a significant increase (19.3%) (P=0.355).

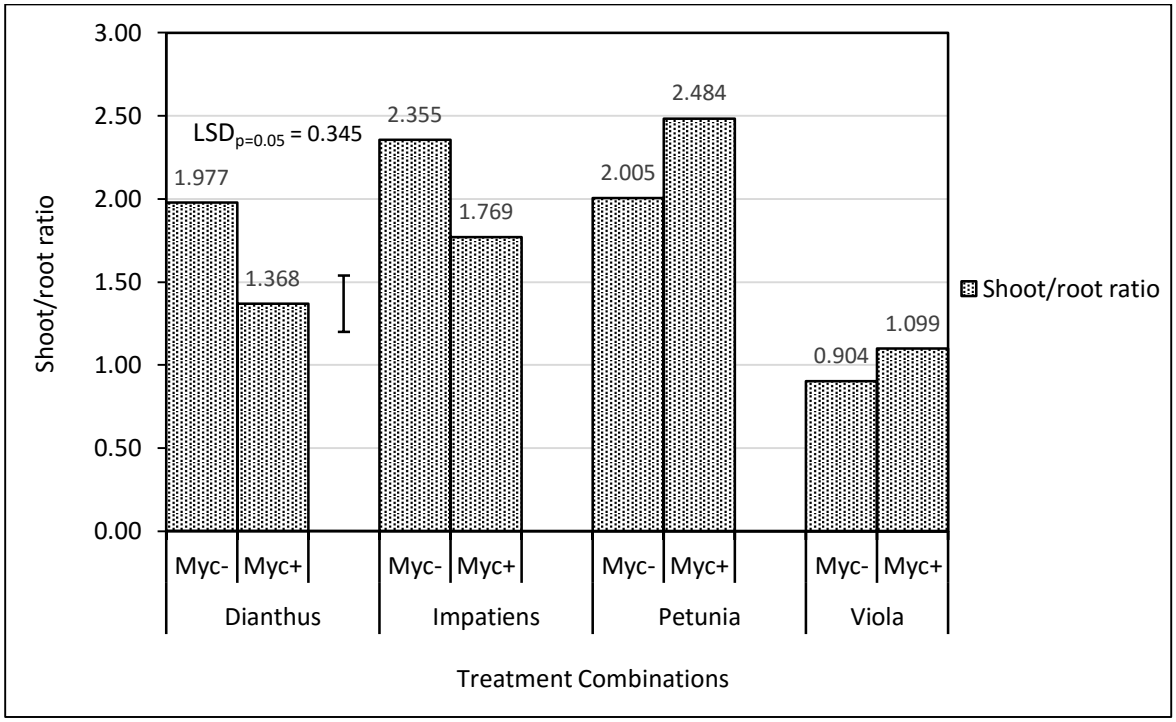


Figure 4.10 Two factor (Plant x Mycorrhiza) interaction means for shoot /root ratio.

There was a significant interaction between inoculated and uninoculated total dry biomass (Table 4.6). Dry biomass of *Dianthus, Impatiens* and *Viola* increased significantly compare to uninoculated plants (Figure 4.11). Biomass weights of inoculated plants *Dianthus* increased by 25.2%, *Impatiens* 50.2% and *Viola* 53.1%. However, dry biomass weight of uninoculated plant *Petunia* increased significantly (26.6%) compare to inoculated plants.

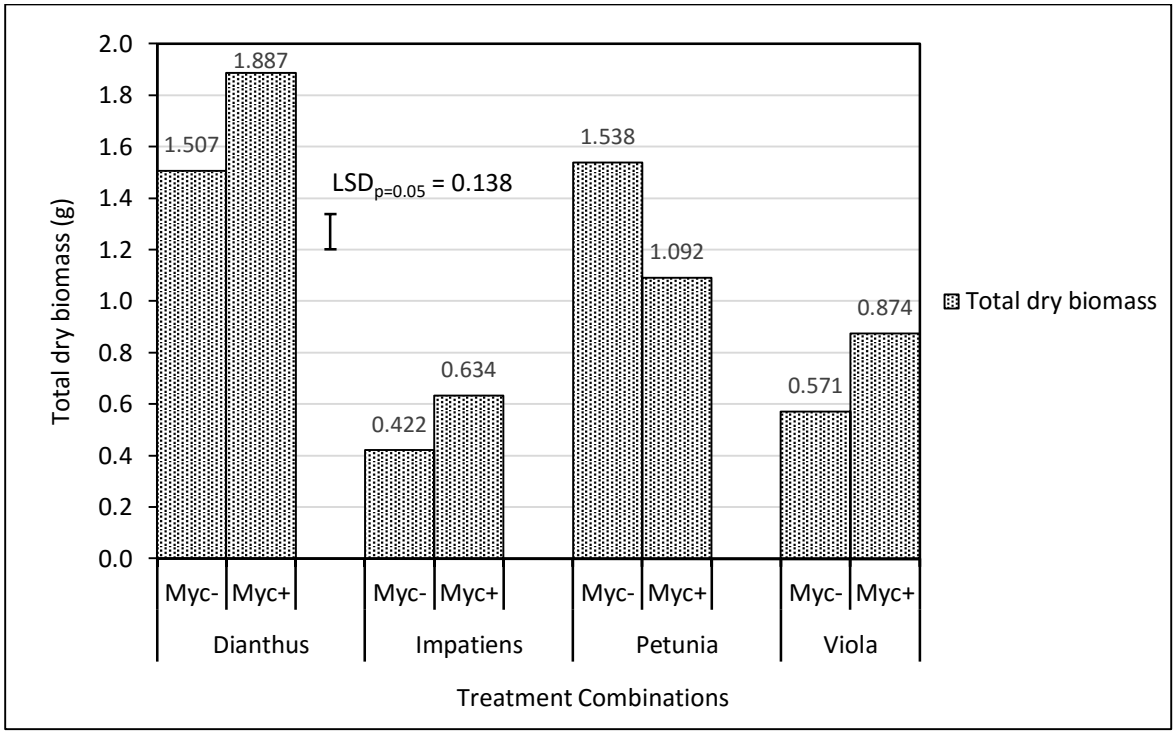


Figure 4.11 Two factor (Plant x Mycorrhiza) interaction means for total dry biomass.

At day 90, the dependency of above and below ground vegetative parts on AM fungi varied considerable (Figure 4.12). MD ratings fluctuated according to plant type; *Impatiens* and *Viola* (Moderately dependent), *Dianthus* (Marginally depended) and *Petunia* least dependent (Independent). According to the SMD ratings, the shoots of *Viola* responded more positively to AM fungi (Moderately dependent) with respect to roots (Marginally dependent). However, the roots of *Dianthus* and *Impatiens* showed a greater dependency on mycorrhiza (Moderately depended) compare to shoots (Marginally dependency), increasing root production in favor of shoot development. The SMD and RMD of *Petunia* showed a similar trend. However the dependence on mycorrhiza showed a negative trend.

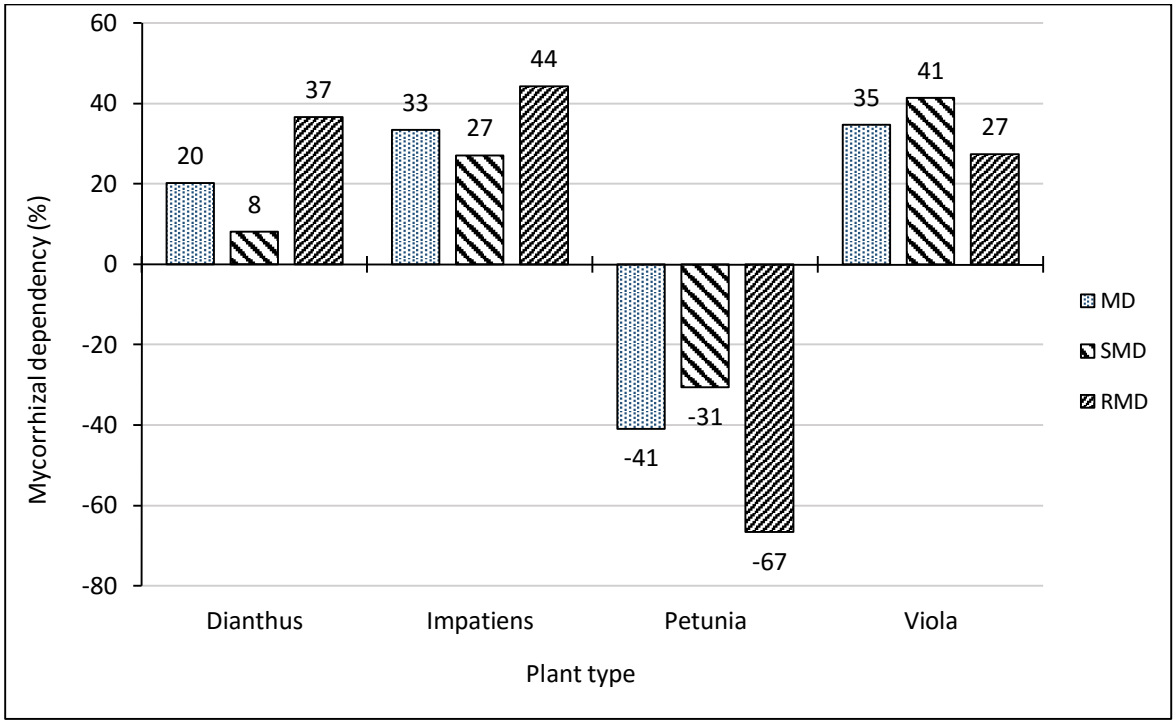


Figure 4.12 Mycorrhizal dependency. **MD** % = (dry biomass of myc^{+}) – (dry biomass of myc^{-}) / (dry biomass of myc^{+}) x 100. **SMD** = shoot mycorrhizal dependency. **RMD** = root mycorrhizal dependency.

4.4 Mortality Rate

The overall plant mortality over time showed 97 out of 3360 plants (2.9%) plants. Mortality in the Control group were 2.5 times more than the mycorrhiza treated plants ($\chi^2_{df=1}$ = 17.8 p <0.01) (Figure 4.13). The mortality association for *D. chinensis x barbatus*, n=83, is significantly more than (n=7 or 0) of the other species ($\chi^2_{df=3}$ = 196 p <0.01) see Figure 4.14.

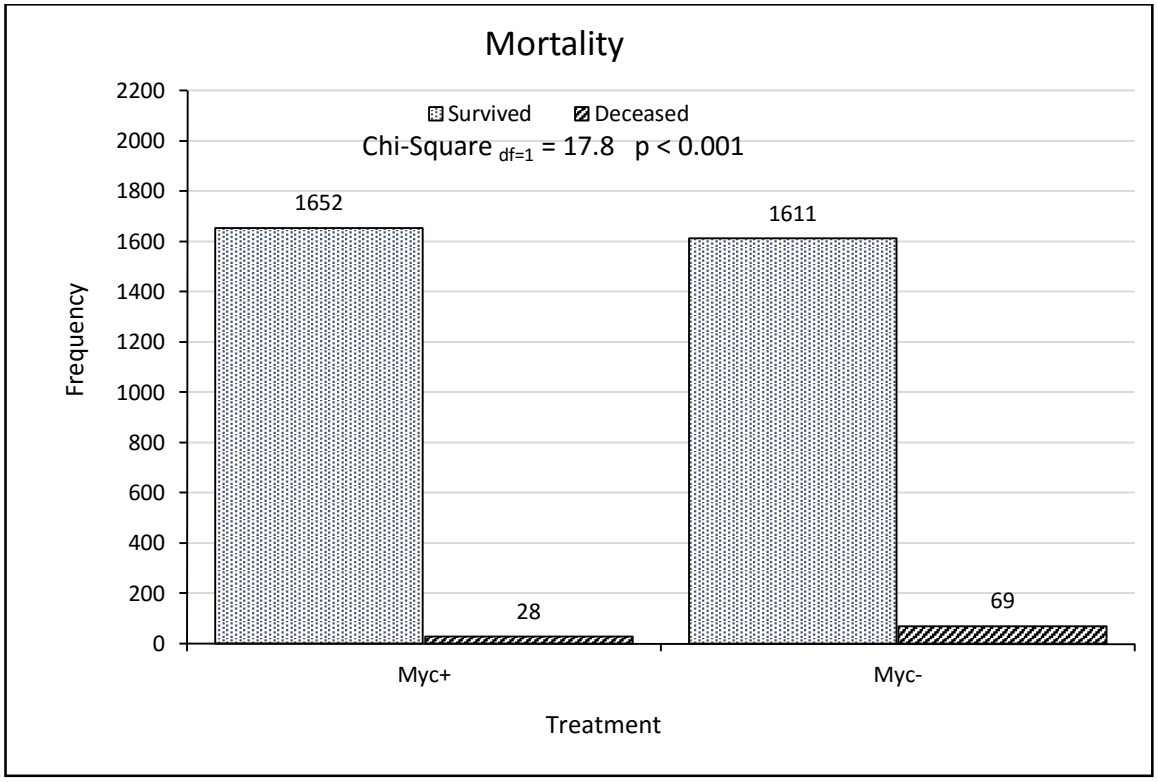


Figure 4. 13 Frequency of mortality between mycorrhizal and the uninoculated control.

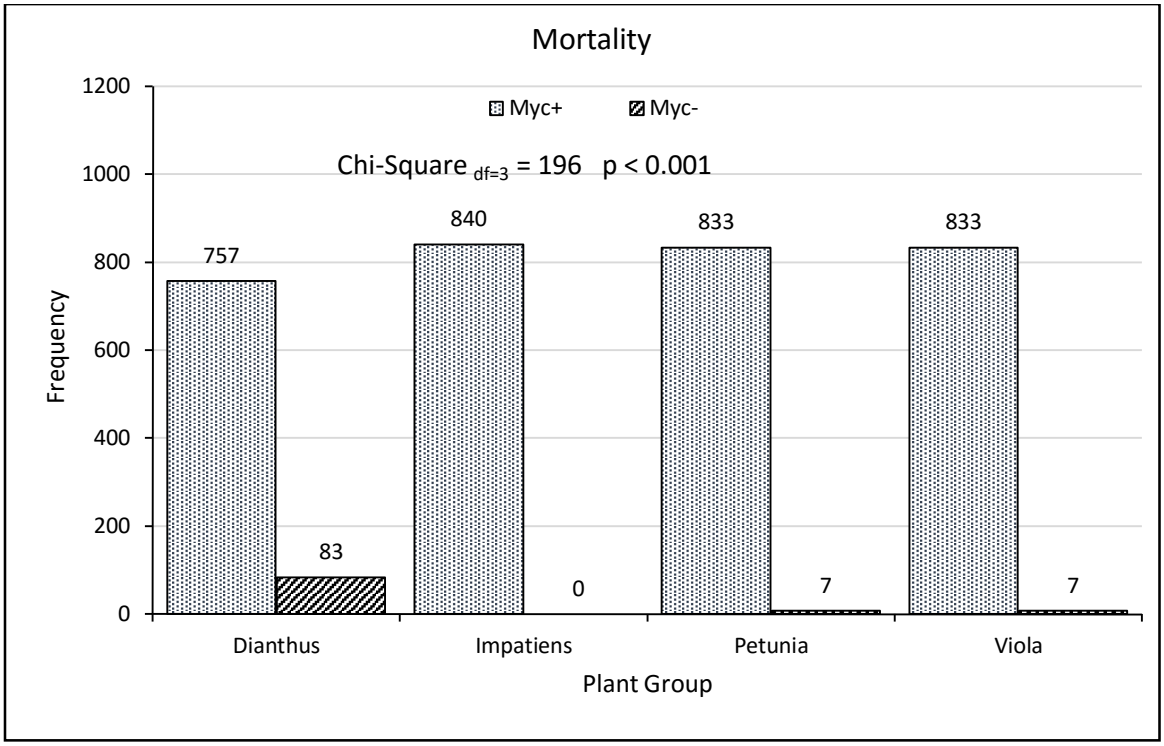


Figure 4.14 Mortality association between plant species and treatments.

4.5 Mycorrhizal Assessment

The percentage AM fungal colonization was based on microscopic observation of mycorrhizal structures; inter- and intra-cellular hyphae, arbuscules and vesicles (Figure 4.16). Un-inoculated plants in the Control group were not colonized therefore only the inoculated treatment groups were analyzed. Significant difference occurred between plant species ($P<0.0001$) with *Viola* showing significantly higher colonization percentage ($LSD_{p=0.05} = 5.85$) (Figure 4.15). Colonization of *Dianthus, Impatiens* and *Petunia* was not significantly different (Figure 4.15).

Table 4.7 Analysis of variance on percentage of mycorrhizal colonization.

Source	DF	Sum of squares	Mean squares	F	Pr > F
Plant Species	3	7003.400	2334.467	56.065	< 0.0001
Error	36	1499.000	41.639		
Corrected Total	39	8502.400			

Table 4.8 Analysis of the difference between categories at 95% confidence level.

Contrast	Difference	Standardized difference	Critical value	Pr > Diff	Significant
Viola vs Dianthus	32.600	11.297	2.028	< 0.0001	Yes
Viola vs Petunia	30.700	10.638	2.028	< 0.0001	Yes
Viola vs Impatiens	27.500	9.529	2.028	< 0.0001	Yes
Impatiens vs Dianthus	5.100	1.767	2.028	0.086	No
Impatiens vs Petunia	3.200	1.109	2.028	0.275	No
Petunia vs Dianthus	1.900	0.658	2.028	0.514	No
LSD-value:			5.853		

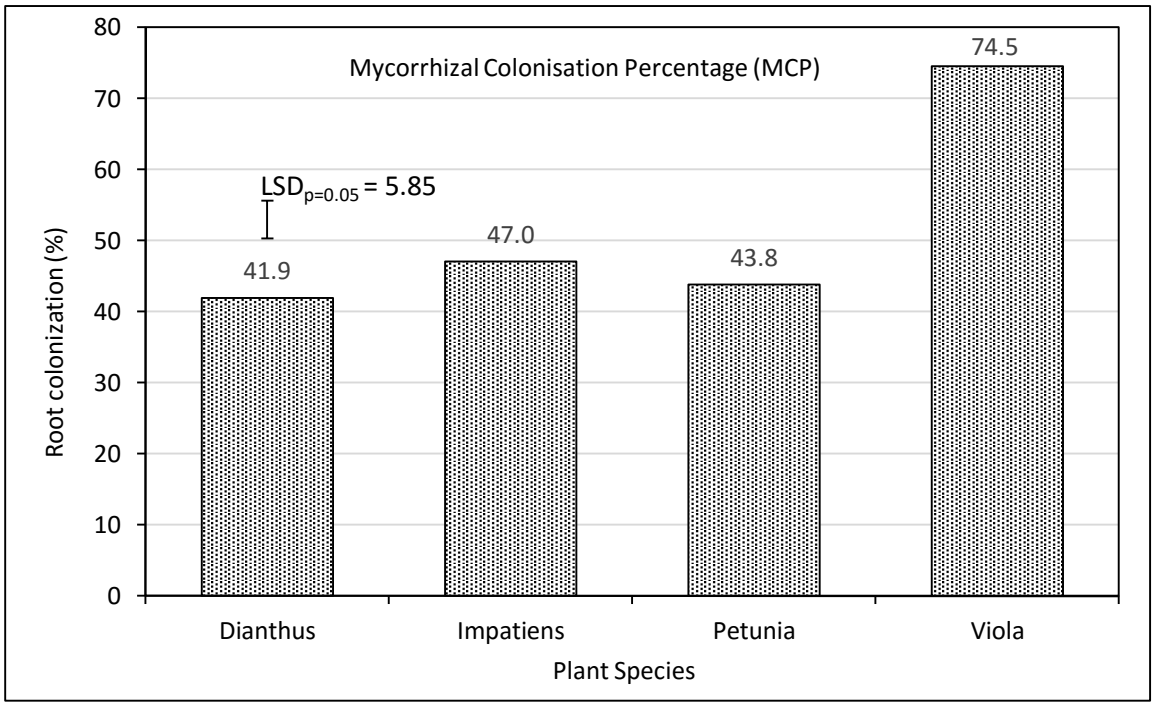
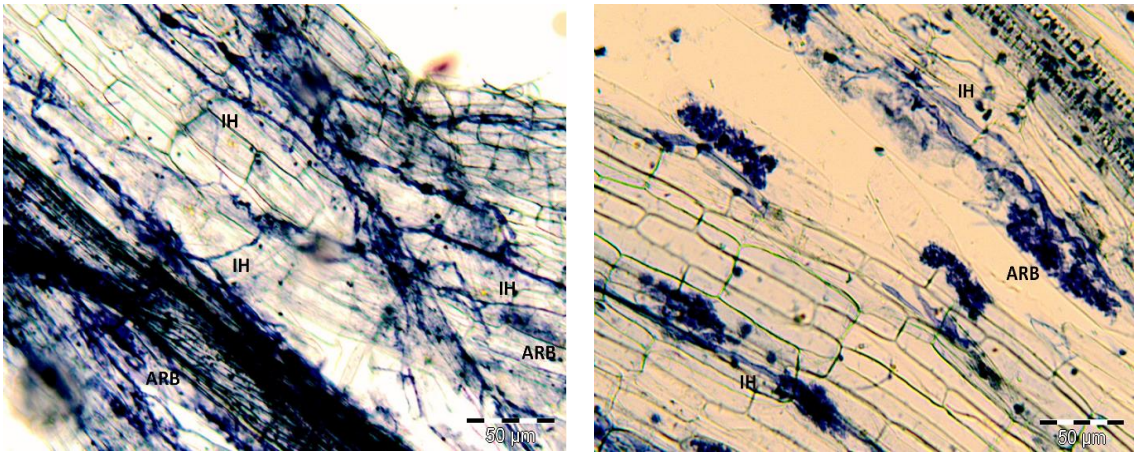
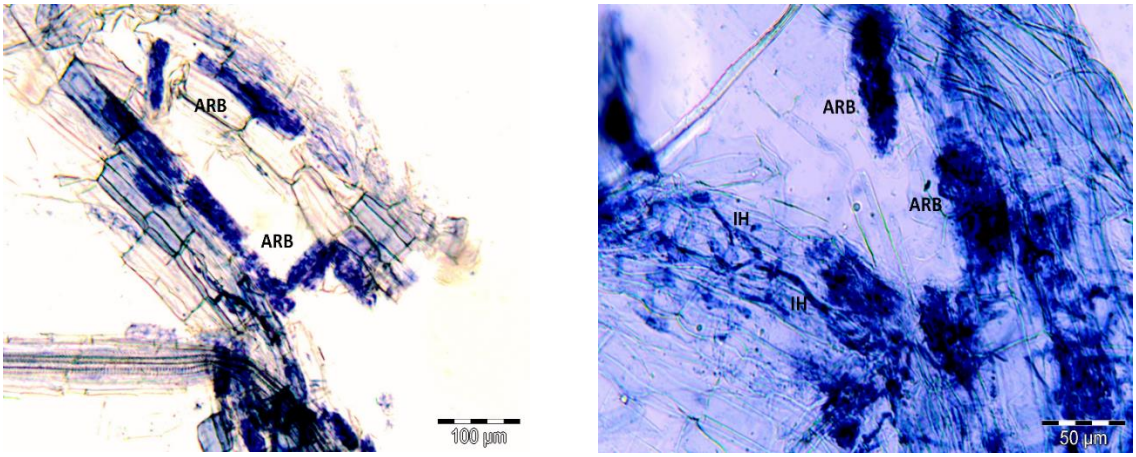


Figure 4.15 Percentage mycorrhizal root colonization of inoculated plants *Dianthus*, *Impatiens*, *Petunia* and *Viola*.



(a) (b)



(c) (d)

Figure 4.16 Micrographs showing mycorrhizal colonised root sections of (a) *Dianthus*, (b) *Impatiens*, (c) *Petunia* and (d) *Viola* stained with trypan blue showing intercellular hyphae (IH), Arbuscules (ARB).

CHAPTER 5
DISCUSSION

CHAPTER 5

5. DISCUSSION

Arbuscular mycorrhizal fungi have the potential to colonise the roots of 80% of all plant species and the aim of this study was to investigate whether this microbial symbiosis do enhance seedling growth and survival of four selected ornamental annuals. Since mycorrhizal associations was discovered, extensive research on the subject has been conducted by Mycologists and Soil Scientists, however further research into the early stages of mycorrhization is required especially on horticultural plants (Smith & Read 2008). In this study it was important to understand how the different plant species respond to arbuscular mycorrhizal fungal inoculation and the significance it has on plant growth and survival as a biofertiliser (Azcón-Aguilar & Barea 1996).

5.1 Non-destructive Component

During the initial stages (day 1-15) of the glasshouse experiment *Dianthus, Impatiens, Petunia* and *Viola* showed very little visible response to AM fungal inoculation with minimal plant development and growth difference between groups indicates the non-aggressive nature of AM fungi and the duration required to establish symbiosis. However, over time, results confirmed that all plants inoculated with AM fungi produced more leaves (14-24%, increase) and grew taller (12-28%, increase) compare to plants in the uninoculated control group at day 90 (Figures 4.1 & 4.4). This indicates that *Dianthus, Impatiens, Petunia* and *Viola* have moderate to high response to mycorrhiza. This may be attributed to increase P and N uptake which influences shoot and leaf development (Corrêa *et al.*, 2015). In a greenhouse study conducted by Püschel *et al.*, (2014), was shown that plants such as *Capsicum annuum*, *Pelargonium zonale*, *P. peltatum* and *Gazania splendens,* inoculated

with AM fungi when observed over a three-month period, significantly increased the number of leaves and plant height of these ornamental crops. These results are in agreement with other studies which showed significant increase in plant height of the AM inoculated horticultural crops, *Dianthus* (Bhatti *et al*., 2013; Gaur *et al*., 2000), *Zinnia elegans* and *Tagetes erecta* (Aboul-Nasr 1995), *Chrysanthemum morifolium* (Sohn *et al*., 2003). Furthermore, AM fungi are able to increase flower production of inoculated plants by increasing uptake of P and K under low soil nutrient conditions. In a study by Gaur *et al.,* (2000), AM fungi increased the number of flowers of inoculated plants *Petunia hybrida, Callistephus chinensis* and *Impatiens balsamina*. Results from Gaur *et al.,* (2000) study showed that AM inoculated plants *P. hybrida, C. chinensis* and *I. balsamina* produced significantly higher concentrations of shoot P and K content, initiated earlier flower formation and significantly increased the number of flowers compare to uninoculated plants.

On the other hand, AM fungi showed no significant effect on other growth parameters, plant width, plant length (except *Viola*) and number of flowers (Figures 4.2, 4.3 & 4.5) due to reallocation of nutrients for leaf and shoot development or nutrient possible growth depression. Excessive AM colonization at seedling stage may result in a carbon drain which manifests as growth depression (Smith & Read 2008). Buwalda & Goh (1982) conducted a study on ryegrass which resulted in negative growth when excessively inoculated. Results showed competition for available C resources between AM fungi and host plant caused by a reduction in C-N ratio levels of inoculated plants since excessive inoculum density lowered growth output of inoculated plants (Clapperton & Reid 1992). Inoculated *Viola* plants increased width and length significantly from day 30 (Figures 4.2 & 4.3). This may be due to high percentage mycorrhizal colonisation (Figure 4.15). *Viola* also developed more leaves consistent with increased plant width and length as interpreted visually by discriminative analysis (DA).

In contrast, AM inoculation of *Petunia* had an opposite effect resulting in an invert response (plant length, width, number of flowers and buds) due to possible root architecture incompatibility (Koide 1991) or C drain from the host plant to AM fungus (Gaur & Adholeya 2005). However, inoculant Mycoroot, is a mix of isolates that may not be compatible at the early stages of growth and benefits may only be evident at later stage of growth. In a similar study Hayek *et al*., (2012) showed that AM fungi, *Glomus mosseae* were able to enhance the growth of *Petunia* at lower mycorrhizal colonization percentage (9.7%), and significantly increased biomass and P content of shoots. The results in Hayek *et al*., (2012) study were consistent with a similar experiment conducted on *Petunia* by Gaur *et al*., (2000). These results from Gaur *et al*., (2000) experiment, confirmed that *Petunia* produced significantly higher concentrations of shoot P content and dry biomass and increased shoot height compared to uninoculated plants, with a colonisation percentage of 63.3%. This indicates that AM fungi are able to enhance the growth of *Petunia* and produce healthier and more vigorous plants.

5.2 Destructive Component

In general, prolonged periods of above-ground growth increases leaf area size as a result of sustained supply of nutrients to leaves (Smith & Read 2008). This was evident in inoculated *Viola* which produced significantly larger leaves at both periods measuring (Figure 4.8) showing that AM inoculation have a significant effect on *Viola* leaf area (Table 4.4) as a consequence of higher mycorrhizal colonization (Figure 4.8). According to Sohn *et al*., (2003), *Chrysanthemum morifolium* plants inoculated with AM fungi produced leaves with larger leaf areas due to higher concentrations of P compared to uninoculated plants indicating that there is a positive correlation between the rate of P uptake and leaf size of

inoculated plants. The effect of AM fungi on leaf area as a result of increased supply of P and N was also confirmed by Jia *et al*., (2004), who reported that N accumulation in, broad bean, increased leaf area in response to increase P uptake associated with AM fungi. Interestingly, two distinct growth trends are evident (Figure 4.8). The first trend, a decrease in leaf area occurs at the end of the measuring period (*Dianthus and Viola*) as a result of a rapid increase (precipitous gradient) by uninoculated plants compare to inoculated plants (gradual gradient). The second trend, a decrease in leaf area at the start of the measuring period (*Impatiens* and *Petunia*) as a result of an increase (precipitous gradient) by inoculated plants. In the first trend, inoculated plants *Dianthus* and *Viola* reached maximum growth potential maturing much earlier than uninoculated plants. This may be a result of efficient P supply or possible senescence causing reduction of leaf P by reallocating resources (Koide 1991). In contrast, according to the second trend, increase in leaf area experienced by inoculated plants, *Impatiens* and *Petunia*, showed a lower initial response to AM fungi due to a possible lowering of inherent growth rate. However, AM fungi had no measurable effect on *Dianthus* and *Petunia* (Figure 4.8) suggesting lower or unsustained uptake of P because mycorrhizal colonisation percentage was also found to be insignificant (Table 4.8).

Recent studies have reported the effects of AM fungi on plant dry biomass (Sohn *et al*., 2003; Aggangan & Moon 2013). Aggangan & Moon, (2013) showed that AM fungi had a significant effect on dry biomass of six month old *Kalopanax septemlobus* seedlings. In this study shoot and root dry biomass increased (52% and 57%, respectively) and total dry mass increased by 50% compare to uninoculated plants. In another study, AM fungi, inocululated rooted cuttings of *Chrysanthemum morifolium* were assessed after eight weeks (Sohn *et al*., 2003). Results from this study revealed that plants inoculated at transplanting stage significantly increased stem and root dry biomass (130% and 327%, repectively), but leaf dry biomass, though insignificant, was 44% higher compared to uninoculated control.

Results from the above experiments showed that biomass of below and above ground plant structures are directly affected by AM fungi (Marschner 1995).

In the present study, inoculated plants experienced similar increases in biomass (Figure 4.9) which were highly interactive (Table 4.6). Three months after inoculation *Dianthus, Impatiens* and *Viola* significantly increased root and shoot biomass by 38-80% and 9-71% respectively, and total dry biomass by 22-50% compared to uninoculated plants. The increase in total dry biomass further substantiate the effect of AM fungi on plant growth because P uptake in inoculated plants has been reported to be 2.5 times higher than uninoculated plants (Marschner 1995). In a similar study, *G. mosseae*, *Acaulospora laevis* and *Gigaspora* sp. increased the shoot dry biomass and root dry biomass of the host plant *Dianthus caryophyllus* when assessed at 90 days by 318% and 15% respectively (Bhatti *et al*., 2013). Further, these researchers reported combination and individual inoculation treatments showed a consistently higher shoot dry biomass compared to root dry biomass showing shoots were heavier than roots in all treatments. Higher shoot mass in relation to lower root mass indicates a healthy root/shoot relationship, which is a common growth trend amongst inoculated plants (Marschner 1995) due to a more efficient exchange of nutrients between AM fungi and host plant. Interestingly, the above and below ground plant biomass of *Viola* did not differ showing that shoots and roots development were almost equal, as indicated in Figure 4.9. Unfortunately, dry biomass was only assessed once and therefore it was not possible to determine whether AM fungi increased shoot dry biomass or root dry biomass at other stages of development. However, results indicate that inoculated *Viola* produced shoots with 10% higher shoot dry biomass compare to root dry biomass which suggests shoots responded more favorable to AM fungi. This growth trend supports previous reports showing plants inoculated with AM fungi improves shoot development due to increase root efficiency (Hetrick 1991). In contrast, the shoot dry biomass of uninoculated

plants decreased by 11% compare to an increase in below ground root production. This suggests a possible growth imbalance as a result of nutrients being diverted from shoot to roots due to insufficient nutrients resulting in increased root production. In contrast, *Petunia* showed a negative growth response to AM inoculation. Shoot dry biomass and root dry biomass were reduced by 40% and 23%, respectively compared to inoculated plants (Figure 4.9). The negative growth response may be attributed to ineffective nutrient exchange at the fungal-host interface or underdeveloped extraradical mycelium resulting in decreased uptake of nutrients (Marschner 1995). These results contradict previous studies by Gaur & Adholeya (2005) and Gaur *et al*., (2000) which showed that AM fungi were able to successfully colonise *Petunia* and significantly increase shoot dry biomass and root dry biomass by 31% and 61%, respectively when assessed at 90 days compare to uninoculated plants. The results further show AM inoculated plants developed shoots with higher dry mass compare to lower root dry mass. These finding are consistent with the growth trend observed in *Dianthus* and *Impatiens* (Figure 4.9) which showed the positive influence of AM fungi on shoot development (Figure 4.1) by increasing shoot dry biomass.

The study further revealed varied response to AM fungi in terms of shoot: root ratios. AM fungal inoculation had no significant effect on shoot: root ratio of host plant *Viola* and significantly decreasing shoot: root ratios of *Dianthus* and *Impatiens* (Figure 4.10). Plants that experience no change or decrease in shoot: root ratio is indicative of AM fungi symbiosis. According to Smith & Read, (2008), reduction in shoot:root ratio are associated with increased nutrition and enhanced growth. Several studies have reported the effect of AM fungi on decreasing shoot:root ratio (Smith 1980). Crush (1974) reported that the effect of AM fungi and plant nutrition were mostly responsible for shoot: root ratio fluctuations. In his study, tropical legume crops, *Centrosema* and *Stylosanthes,* were treated with 3 inoculation combinations. Plants were treated with either AM fungi inoculum, phosphorus

(+P) (0.4 g/kg) or void of any P and AM fungi inoculum (control group). After 4 weeks, the control groups of both legume crops (*Centrosema* and *Stylosanthes*) showed higher shoot: root ratios compared to plants that received inoculation or +P treatments. The study revealed a reducing of shoot: root ratios, by both inoculation and +P treatments (50% and 60%, respectively), and significantly increased total fresh weight (132% & 196%, respectively) compare to control group. These results indicate the effect of AM fungi and P on plant nutrition by reducing shoot: root ratios and an alternative strategy plants employ to survive by increasing shoot: root ratios under low nutrient conditions.

Furthermore, variation in shoot: root ratio is generally associated with the degree of mycorrhizal dependency (Hetrick 1991) which is a function of soil fertility (Gerdeman 1975). This was true for *Dianthus*, *Impatiens* and *Viola* which displayed marginal to moderate dependence on AM fungi (Table 4.6) in response to lower shoot: root ratio as compare to *Petunia* that had lower MD (independent) but higher shoot: root ratio (Figure 4.10). Hetrick *et al*., (1988) showed that MD of mycotrophic warm-season (C_4) plants with high MD (over 90%) had lower shoot: root ratio and higher total dry biomass when compared to uninoculated plants. This was comparable to the MD displayed by *Dianthus* and *Impatiens*, *Petunia* and *Viola* and aligned with the MD of most cultivated plants. According to Tawaraya (2003), cultivated plants are generally less dependent on mycorrhiza compare to accession species. Furthermore, Tawaraya (2003) reported that the average MD of 250 cultivated species assessed were less than 60% compared to the average MD of trees and wild grasses (70 - 79%, respectively). These finding indicate that the rate of MD are related to selection of mycorrhizal species, availability and amount of soil P and plant metabolic rate (Habte & Manjunath 1991; Janos 2007; Smith 1980). Moreover, shoot and root MD of above and below ground plant structures varied compared to MD of the overall plant which may be influenced by the type of AM species. (Figure 4.12). The roots of

Dianthus, *Impatiens* and *Viola* were moderately MD and except for *Viola*, which showed higher shoot MD but lower root MD, which indicate that shoots were less depended on AM fungi compare to roots. High root MD (*Dianthus* and *Impatiens*) indicate a possible shortage of soil nutrients, and as a consequence, root dependency on fungal partner increases, simultaneously decreasing shoot production to compensate for nutrient deficiency. In contrast, the shoots of *Viola* showed increased MD compare to roots which suggest shoot production increased at the expense of roots in response to increased uptake of nutrients. However, the inverse dependency (shoot MD, root MD and MD) displayed by *Petunia* indicates possible competition for nutrients due to low soil P levels affecting MD (Tawaraya 2003). Furthermore, low light intensity resulting in an increase in shoot:root ratio (Smith 1980) which may explain the significantly higher shoot:root ratios of inoculated *Petunia* (Figure 4.10).

5.3 Mortality Rate

Although the aim of this study was to assess AM fungi as a potential biostimulant to enhance the growth of selected annuals it was also important to investigate the possibility of AM fungi to improve the survival rate during critical transplanting phase. Mortality rate was assessed at the onset when plantlets were transplanted from growing tray into commercial six pack containers until the end of the experiment. Results showed that mortality was more frequent in uninoculated control group ($P<0.01$) compare to inoculated treatment group (Figure 4.13). Mortality between plant groups were low except for uninoculated plant *Dianthus* (Figure 4.14). Results show colonization was lowest in *Dianthus* (Figure 4.15) indicating possible reason for higher mortality due to high degree of non-significant growth resulting in less vigorous plants and therefore unable to cope with transplant shock. Studies

have shown significant increase in survival rate, when inoculated with AM fungi of horticultural crops such as *Cyclamen* (Vosatka *et al*., 1999), micropropagated plantlets *Prunus cerasifera* (100%) and *Podophyllum peltatum* L. (57%) (Moraes *et al*., (2004). This reduced mortality was a result of plants having a highly developed root systems and enhanced plant vigor thereby increasing plant survival rate thereby enabling plants to better cope with transplant shock. Furthermore, enhancing water absorption capabilities improves transplant shock (Marschner 1995). Menge & Davis (1978) reported avocado plantlets inoculated with the AM fungus, *Glomus fasciculatus* significantly increased plant growth rate and reduced plant wilt after transplant. During the assessment period (185 days), root weights, plant height and canopy weight significantly increased (120%, 250% and 80%, respectively). After transplanting, only two plantlets showed signs of wilting and fully recovered after two days. However, only two plantlets of uninoculated plants survived after transplanting. Results indicated, that plants inoculated with AM fungi increased survivability and decreased transplant wilt by improving root functionality and plant vigor.

5.4 Mycorrhizal Assessment

The ability of AM fungi to successfully colonise host plant depends on individual fungal (Klironomos & Hart 2002) and specific plant species (Smith & Read 2008). In this study isolates of *Rhizophagus clarus, Gigaspora gigantea, Funneliformis mosseae, Glomus etunicatum, Claroideoglomus etunicatum* and *Paraglomus occulum* successfully colonised the root systems of all four experimental plants (Figure 4.15). Colonisation of *Viola* by AM fungi was highly significant (Table 4.7) indicating high rate of fungal-host compatibility. This result is in agreement with Koide *et al*., (1999) who conducted a similar experiment on host plant *Viola* attaining 73.3% colonisation when assessed 35 days after transplanting,

compared to 74.5% colonisation in this study. However, colonisation was lower in *Dianthus, Impatiens and Petunia* (Figure 4.15) with no significant differences occurring between these plants (Table 4.8). When compared to similar studies colonisation percentage does vary. Results from similar studies show colonization of *Dianthus* roots were much lower (Bhatti *et al*., 2013; Gaur & Adholeya 2005), (37 and 33.9%, respectively) compare to 41.9% colonization achieved in this study. In contrast, results from other studies show higher AM fungal colonization of *Impatiens* (Gaur *et al*., 2000; Koide *et al*., 1999), and *Petunia* (Gaur & Adholeya 2005; Koide *et al*., 1999) (59.8, 48.3% and 85.5, 54.5%, respectively). Results from the above studies do contradict observed colonisation in this study. According to the study by Gaur & Adholeya (2005) when *Petunia* roots were assessed (90 days after transplant), colonization increased almost twofold compare to results in this study (Figure 4.15). However, it is important to note that the experiment was conducted under field conditions therefore temperature played a possible role resulting in increase sporulation and mycorrhizal development (Tommerup 1983b) due to sustained favorable environmental condition (Schenck & Smith 1982). In contrast, Gaur *et al*., (2000) reported, *Petunia* grown under glasshouse conditions showed 18.2% reduction in colonisation when roots were assessed (120 days after transplanting) compared to *Petunia* grown under field conditions. Interestingly, variation in growing conditions also impacted on the colonisation of ornamental crop, *Callistephus chinensis*, indicating possible trend in the above two studies by the same researchers. *C. chinensis* increased colonisation when grown in open field by 13.4% compare to same crop grown under glasshouse conditions suggesting horticultural crops inoculated with AM fungi may attain higher colonisation when planted under field conditions.

5.5 Conclusion

The purpose of this study was to determine the extent to which AM fungi, whether positively or negatively, were able to influence inoculated ornamental plants. This was achieved primarily by assessing the growth and development of plants under controlled environmental conditions over a period of 3 months and by evaluating the above and below ground vegetative plant parts at the end of the experimental period. With the results obtained, AM fungi had a general overall positive effect on the development and growth of inoculated plants notably between 30-90 days, between plant species and treatment groups. All AM inoculated plants grew taller and produced more leaves that had a pronounced effect on plant weight. Inoculated, *Dianthus*, *Impatiens* and *Viola* plants, were heavier which translated into a significant increase in dry biomass yield in comparison to uninoculated plants. Furthermore, in view of this result, the growth and development of *Petunia* does raise important questions with regards to "shelf life" of inoculated plants in containers, growth depression and physiological age of plants. AM inoculated *Petunia* plants are known to mature early compared to uninoculated plants (Daft & Okunanya 1973) resulting in premature increase in root density leading to competition for resources between symbionts and increase in shoot:root ratio and root dry biomass. Inoculated plants such as *Petunia* should therefore be planted out sooner enabling the mycorrhizal network to exploit a larger area of soil or increasing the size of the container.

Overall, AM fungi improved the survival rate between plant species and treatment groups. The results of this study showed that inoculated plants, notably *Dianthus*, were less susceptible to transplant shock compare to uninoculated plants. During the experimental period all plants received adequate supply of water and no inherent signs of disease were detected. The combination of all these factors may have assisted in lowering the mortality rate of uninoculated plants and a less controlled environment may have affected the plants

differently. However, the benefits of mycorrhiza fungi in reducing mortality rate are consistent with the results indicating AM fungi played a significant role in increasing survival rate of inoculated plants.

All inoculated plants were successfully colonised and showed marginal mycotrophic qualities. The degree of mycorrhizal colonisation presented an interesting insight into the dynamics of AM fungi-host symbiosis and the extent to which plants are willing to accept a fungal partner. For example, the net growth output under low colonisation rates were found to be similar in plants with equal or higher colonisation rate. Having said that, increase in colonisation rate did not necessarily result in a net growth increase and that optimal mycorrhization and plant phenotype may have determined the rate of colonisation.

In terms of production cost, it can be concluded that a single AM fungal plant cost 33.2% less than chemically produced plant, see Appendix C. The cost was based on single plant expressed as recommended treatment dosage, growing medium and packaging cost. The cost of single chemically produced plant was based on average cost price from three different commercial nurseries. In view of the information presented, AM fungi should be fully or at least partly incorporated into horticultural production system or in combination with a revised fertilizer program to reduce the dependence on chemical based nutritional programs.

5.6 Recommendations

Based on the findings and observations made in this study, to fully assess the advantages of AM fungi as a biostimulant, factors such as inoculation dosage and the duration that seedling are kept in container based systems may require further research. The application of AM fungi ensures plant vigor and improves plant health. To fully benefit from AM fungi as a biostimulant, future research should be directed at developing plants that are less dependent

on existing artificial growth stimulants and focus on developing plant species that are more dependent on mycorrhiza. Introducing mycorrhiza into seed packaging prior to distribution may also prove advantageous. This system may prove beneficial to introduce mycorrhiza to the retail, commercial and agricultural sectors.

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APPENDICES

APPENDIX A: Plant growth, 27 days after transplant. 1(a, b) *Dianthus*, 2(a, b) *Impatiens*, 3(a, b) *Petunia* and 4(a, b) *Viola.* * = myc^{-}, ** = myc $^{+}$. Source: Photos taken by author.

1(a)*

(b)**

2(a)*

(b)**

3 (a)*

(b)**

4 (a)*

(b)**

APPENDIX B: Plant growth, 90 days after transplant. 1(a, b) *Dianthus*, 2(a, b) *Impatiens*, 3(a, b) *Petunia* and 4(a, b) *Viola.* * = myc $^{+}$, ** = myc $^{-}$. Source: Photos taken by author.

1 (a) * (b)**

2 (a)* (b)**

3 (a) * (b)**

4 (a)* (b) **

APPENDIX C: Cost analysis of AM plant compare to chemically produced plant.

		AM Plant					
				Total		Six pack tray	
Discription	Unit Price/Rc	Per unit	QTY	AM	CPP	AM	CP
Inoculum	49.95	0.050	1.5	0.07	-	0.44	-
Soil Medium	430.58	0.002	60	0.13	-	0.78	-
Packaging	23.00	3.800	1	3.80	-	23.00	-
Production cost per tray	sum	*1.200	1	0.20	-	1.20	-
Total/Rc				4.20	5.600	25.42	*32.25

* Average cost based on 3 nurseries

All total prices exclude markup

AMP - arbuscular mycorrhiza plant

CP/P - chemically produced/ plant