# ARBUSCULAR MYCORRHIZAL FUNGI (AMF) FOR DROUGHT TOLERANCE IMPROVEMENT IN TOMATO (*SOLANUM LYCOPERSICUM*)

**ABDULKAREEM, MUHAMMAD TAOHEED**

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## DECLARATION

**Candidate:**

This Thesis is my original work and has not been submitted to any other University for examination.

Signature: _____ Date: _____

**ABDULKAREEM Muhammad Taoheed**

**Supervisors:**

This thesis report has been submitted for examination with our approval as University supervisors.

Signature: _____ Date: _____

**Prof. Losenge Turoop**

Department of Horticulture

Jomo Kenyatta University of Agriculture and Technology

Signature: _____ Date: _____

**Prof. Elijah Ateka**

Department of Horticulture

Jomo Kenyatta University of Agriculture and Technology

## DEDICATION

This PhD thesis is dedicated to:

ALLAH, the Mighty and Majestic, who knows everything that was, everything that is, and everything that will be and Whose knowledge is comprehensive, extending to and containing all things {We raise in degrees whom we will, but over all those endowed with knowledge is the All-knowing (Q12: 77)}

and

my entire family, for their consistent support of my educational career, their prayers and well-wishes.

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## LIST OF ABBREVIATIONS

| Abbreviation | Full Meaning |
|---|---|
| $^1O_2$ | Singlet oxygen |
| ABA | Abscisic acid |
| AMF | Arbuscular mycorrhizal fungi |
| APX | Ascorbate peroxidase |
| AsA | Ascorbate or ascorbic acid |
| CAT | Catalase |
| cDNA | Complementary DNA |
| Chl | Chlorophyll |
| CK | Cytokinin |
| DHAR | Dehydroascorbate reductase |
| DNA | Deoxyribonucleic acid |
| DNTB | 5,5’-dithio-bis-(2-nitro-benzoic acid) |
| DR | Drought resistance |
| DRW | Dry root weight |
| DSW | Dry shoot weight |
| DT | Drought tolerance |
| DW | Dry weight |
| EDTA | Ethylenediaminetetraacetic acid |
| EM | Ectomycorrhizal fungi |
| FW | Fresh weight |
| GA | Gibberellic acid |
| GLM | General linear model |
| GPX | Glutathione peroxidase |
| GR | Glutathione reductase |
| GSH | Glutathione (reduced form) |
| GSSG | Disulfide glutathione |

| | |
|---|---|
| $H_2O_2$ | Hydrogen peroxide |
| IAA | Indole-3-acetic acid |
| $KH_2PO_4$ | Monopotassium phosphate |
| LRWC | Leaf relative water content |
| MDA | Malondialdehyde |
| MDHAR | Monodehydroascorbate reductase |
| NAD(P)H | Nicotinamide adenine dinucleotide phosphate |
| NBT | Nitroblue tetazolium |
| $O_2^{\bullet-}$ | Superoxide anion |
| OA | Osmotic adjustment |
| $OH^{\bullet-}$ | Hydroxyl radical |
| PCR | Polymerase chain reaction |
| qPCR | Quantitative polymerase chain reaction |
| RNA | Ribonucleic acid |
| ROS | Reactive oxygen species |
| RT-PCR | Real time polymerase chain reaction |
| RuBisCO | Ribulose-1,5-bisphosphate carboxylase/oxygenase |
| RuBP | Ribulose-1,5-bisposphate |
| SA | Salicylic acid |
| SL | Strigolactones |
| SOD | Superoxide dismutase |
| TBA | Thiobarbituric acid |
| TCA | Trichloracetic acid |
| TDR | Time domain reflectometry |
| TNB | 2-nitro-benzoic acid |
| TW | Turgid weight |
| WUE | Water use efficiency |

## ABSTRACT

Drought stress is the single most important abiotic factor in tomato (*Solanum lycopersicum*) production. It affects quantity and quality yield depending on the growth stage at which the plant is subjected to the stress. The use of rhizosphere microbes, such as arbuscular mycorrhizal fungi (AMF), offers an alternative or complementary approach to conventional plant breeding for improving tomato plant drought tolerance. Studies were therefore carried out to investigate the effect of two species of AMF, *Glomus intraradices* and *Glomus mossea*, in single and combined application, on growth, physiological, biochemical and molecular aspects of tomato plants grown under drought stress. In these studies, the establishment of AMF within the tomato plants was determined by assessing the root colonization in plants grown in cocopeat with or without phosphorus application. The impact on growth, water status, pigment content, proline concentration, oxidative stress markers, catalase activity, abscisic acid (ABA) related genes and aquaporin genes were also assessed under drought stress. Omission of phosphate in the growth media significantly ($P \leq 0.001$) enhanced root colonization in all AMF treatments and the highest root colonization (76.67%) was observed in mixed inoculation without phosphate addition. Plant inoculated with either AMF, but without phosphate addition produced significantly higher dry shoot weight (DSW) compared to non-inoculated plants with phosphate addition. DSW was significantly lowered in all drought stressed plants, but plants inoculated with AMF had higher DSW under watered and drought stress conditions ($P \leq 0.001$). Plants inoculated with AMF showed less pigment damage, maintained higher leaf relative water content and accumulated significantly ($P \leq 0.028$)

higher free proline in their tissues under drought stress. The levels of hydrogen peroxide and malondialdehyde (MDA) were significantly lower in tissues of plants inoculated with *G. intraradices* ($P \leq 0.033$) while catalase activity was significantly ($P \leq 0.001$) higher in plants inoculated with AMF under drought stress. The expression of abscisic acid (ABA) related genes, *LeNCED1* (3-fold) and *Le4* (13-fold) was upregulated under drought stress in non-inoculated plants, but unaffected in plants inoculated with *G. intraradices* and downregulated in plants inoculated with either *G. mossea* or mixed AMF ($P \leq 0.002$). Under watered condition, expression of tomato aquaporin genes was generally increased in plants inoculated with AMF. Under drought stress however, the expression of aquaporin genes was reduced or unaffected in plants inoculated with AMF, but enhanced in non-inoculated plants. The results of this study indicate the potential of AMF in improving the growth of tomato plants under normal conditions (of watering and phosphate) as well as under stress conditions (drought and phosphate deficiency). The AMF induced drought stress tolerance is associated with enhanced accumulation of free proline, increased antioxidant enzyme activities and differential regulation of ABA biosynthetic gene and aquaporin genes.

**Keywords**: *Solanum lycopersicum*, *Glomus intraradices*, *Glomus mossea*, phosphate, drought, proline, antioxidants, reactive oxygen species, abscisic acid, aquaporin

# CHAPTER ONE

# INTRODUCTION

## 1.1. Background information

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop globally after potato. Tomato is cultivated widely and global production has been on a steady rise (Heuvelink, 2005), estimated at 177 million tonnes per year with a total production area of about 5.0 million ha (FAO, 2016). Tomato production systems can either be open field or greenhouse production systems. Field grown tomato is commonly found in tropical, subtropical and warm temperate climates. Field produced tomato is frequently exposed to unfavourable environmental conditions, such as drought, waterlogging or excess water caused by heavy rainfall, and extreme temperatures. These environmental stresses together can claim up to 50% of global production of major crops (Mahajan & Tuteja, 2005).

Under natural environmental conditions, plants are continuously exposed to biotic stresses such as pests (herbivores) and pathogens, as well as abiotic stresses such as extreme temperatures, metal toxicity, nutrient imbalances, salinity and drought, which negatively impact plant survival development and productivity. Drought is considered the most important abiotic factor limiting plant productivity (Bray, 1997). It can be defined as a period of insufficient precipitation that results in water deficit (Tuberosa, 2012). In recent years, the severity and harmful effects of drought are increasing (Dai, 2012). In addition, global climate change is contributing to its spread worldwide

(Trenberth *et al*., 2014). Water shortage is currently affecting more than 70% of arable lands globally, and could lead to expansion of agricultural activities to less fertile areas to satisfy food demands in the near future (Foley *et al*., 2011). Crop plants grown in high potential agricultural lands will be exposed to random short-term drought stress of days to weeks, from which they must quickly respond to limit damage caused by short-term drought stress while they continue to grow and yield in the stressful environments (Basu *et al*., 2016). With increasing global population, which is expected to exceed 9 billion by 2050 (DESA, 2015), an immediate priority for agriculture is to maximize crop productivity. This will require improvement of crops for optimal productivity under normal as well as drought stress conditions.

Water is a vital component of plant cycle and regular physiological processes. As a result, its deficiency affects almost all plant processes directly or indirectly (Basu *et al*., 2016). Water deficiency induces morphological, physiological, biochemical and molecular changes in plants. Plant responses to water deficiency are complex and diverse (Osakabe *et al*., 2014). Although different plant species vary in their sensitivity and response, all plants have innate capability for drought stress perception, signalling and response (Golldack *et al*., 2014; De Vasconcelos *et al*., 2016).

**'**Drought resistance' (DR) is a broad term which applies to plant species with adaptive features that enable them to escape, avoid, or tolerate drought stress (Tuberosa, 2012). 'Drought escape' is the ability of a plant species to complete its life cycle before onset of drought. Such plants do not experience drought stress as they are able to modulate their vegetative and reproductive growth according to water availability. 'Drought avoidance' is the ability of plants to relatively maintain higher tissue water content

despite reduced water content in the soil through minimization of water loss (water savers) and optimization of water intake (water spenders). 'Drought tolerance' (DT) is the ability of plants to endure low tissue water content through adaptive traits such as osmotic adjustment and cellular elasticity (Basu *et al*., 2016). Plants have developed diverse array of mechanisms for drought escape, avoidance and tolerance that can be exploited to improve DR and maintain yield in crop plants. They include morphological adaptations, osmotic adjustments, improvement of antioxidant system and hormonal regulations, all of which are aimed at optimizing water use efficiency (Osakabe *et al*., 2014). The understanding of the mechanisms that enhance plant DT is crucial in development of new strategies to cope with drought and to guarantee world food production (Chaves & Oliveira, 2004).

In addition to their intrinsic protective systems against environmental stresses, plants can establish beneficial association with a number of microorganisms present in the rhizosphere that can alleviate the stress symptoms (Ahanger *et al*., 2014). Plant rhizospheres are repositories for specialized microbial population (bacteria, fungi, viruses), which provide various beneficial inputs to plants through processes such as enhanced nutrient uptake, protection against pathogens, adaptation to harsh abiotic conditions like drought, high temperature and heavy metal contamination (Morgan *et al*., 2005; Mayer, 2014). One of the most common and ubiquitous mutualistic plant-microorganism association is that established with arbuscular mycorrhizal fungi (AMF) (Smith & Read, 2008). Hence, its attraction in sustainable plant production system.

Arbuscular mycorrhizal fungi belong to the phylum Glomeromycota. Although not very diverse, they are amongst the most abundant and widespread of all fungi. This group are among the oldest of fungi, as their spores and hyphae have been discovered in the fossil roots of plants known to be as old as 450 million years (Redecker *et al*., 2000). Unfortunately, changes in soil conditions such as pH, nutrient or contamination with toxic elements, as a result of human activities especially during the 'green revolution', have affected the balance of these beneficial microorganisms (Dudal *et al*., 2002). AMF occur in the rhizosphere of a wide spectrum of temperate and tropical plant species, and are absent in less than 30 plant families (Smith & Read, 2008). Because, they are obligate symbionts, they require the presence of actively growing plants during their reproduction. Most plants, including globally important food crops, form symbiotic relationship with AMF. Through this mutualistic association, the AMF obtain photoassimilates from the host plant to complete its life cycle, and in turn, they help the plant in acquisition of water and mineral nutrients. Hence, AMF plants generally show an enhanced growth, improved nutrient uptake ability and stress tolerance (Schüßler & Walker, 2011). In the case of drought, AMF symbiosis alleviates the negative effects induced by the stress, making the host plant more drought tolerant (Augé, 2001, 2004). The signalling and transduction pathways involved in these effects are not well understood yet (Ruiz-Lozano *et al*., 2012).

Tomato is very sensitive to drought stress, especially during vegetative development and at reproductive stages (Wudiri & Henderson, 1985). The effect of drought or water deficit on various aspects of tomato morphology, physiology, biochemistry and gene expression has been studied (Torrecillas *et al*., 1995; Rahman *et al*., 1999). However, there are fewer studies on the effects of water deficit on these aspects in AMF

inoculated tomato. The model plant *Arabidopsis thaliana*, like all members of the *Brassicaceae*, cannot be used for investigating AMF symbiosis due to its inability to be colonised by AMF (Harrison, 1997). Members of the *Solanaceae* family, including important crops such as tomato, potato, eggplant, tobacco and petunia, are used as model systems in research on many plant biology topics including plant-microbe interactions (Arie *et al*., 2007). Tomato has become a model plant for understanding mechanisms underlying AMF symbiosis, and numerous AMF up-regulated phosphate transporters have been characterized from tomato (Nagy *et al*., 2005; Xu *et al*., 2007). In addition, tomato has been used to study AMF protection against biotic and abiotic stress (Song *et al*., 2015; Chitarra *et al*., 2016). This study therefore specifically aims at: (i) assessing root colonization and growth enhancement of tomato by AMF; (ii) determining the effect of AMF on leaf water content, pigment concentration and proline accumulation in tomato under drought stress; (iii) assessing the production of reactive oxygen species and antioxidants in AMF tomato under drought stress; and (iv) assessing the expression of drought related gene in AMF tomato under drought stress.

## 1.2. Problem statement

Drought is the most common abiotic stress that affects the survival and productivity of numerous plant species, including economically important crops like tomato. It is a worldwide environmental problem which is unrestricted to desert regions due to global climate change (Trenberth *et al*., 2014). Tomato is one of the most widely grown vegetables worldwide (Passam, 2008). The amount of water required daily for tomato in different growing systems varies from 0.89 to 2.3 L/plant/day (Tiwari, 2003). Papadopoulos (1991) estimated the water consumption for tomato to be 0.5 – 0.9 $m^3$

/ $m^3$ greenhouse area/year. Most commercial tomato cultivars are drought sensitive at all stages of their development, with seed germination and early seedling growth being the most sensitive stages (Foolad *et al*., 2003). The increasing vulnerability to drought requires development of more resilient crop varieties capable of surviving drought conditions while maintaining yields. This will require new technologies to complement traditional methods, which are often unable to prevent yield losses. Several strategies, including traditional breeding and targeted genome editing, have been employed to improve drought tolerance in crops. However, large gaps remain due to the complex nature of drought tolerance and the numerous number of genes involved in drought stress response (Fleury *et al*., 2010). The use of rhizosphere microorganisms to improve plant tolerance to drought stress has been relatively less studied. This study therefore aims at investigating the effect of one of such rhizosphere microbes, the arbuscular mycorrhizal fungi, on various physiological, biochemical and molecular aspects of tomato plant adaptation to drought stress.

### 1.3. Justification

Plant growth and health are supported in many ways by the rhizosphere microbes, and key among these microbes is the AMF. AMF can enhance water uptake in host plants (Augé, 2001). The symbiosis can spread extraradical mycelia outside the roots of host plants to increase access to greater quantity of water and soil minerals, increasing water uptake and nutrient absorption for the host plants. Studies have shown that AMF can alter water uptake by plants in both extremes of water availability: drought and waterlogging. AMF-inoculated plants show higher stomatal conductance, increased transpiration rates and increased water stress tolerance by increasing root hydraulic

conductivity (Augé, 2004).. It has also been shown that AMF-inoculated tomato plants have higher transpiration and stomatal conductance compared to non-inoculated plants, when subjected to drought conditions (Subramanian *et al*. 2006).

AMF can exert significant effects on plant growth and survival under drought conditions. They can be exploited as an eco-friendly and sustainable strategy for agriculture and could replace or complement the need for genetic modification of plants, as well as reduce the use of organic fertilizers.

### 1.4. Objectives

AMF are capable of protecting host plants from deleterious effects of drought stress. The symbiosis regulates several physiological and biochemical processes involved in direct uptake and transfer of nutrients, osmotic adjustment, water use efficiency and protection against oxidative stress.

This study therefore primarily aims to investigate the effect of AMF inoculation on physiology, biochemistry and gene expression of tomato under drought stress conditions.

The specific objectives therefore are:

1. To assess the effects of inoculation with *G. intraradices* and *G. mossea* on tomato root colonization and growth.
2. To determine the effect of AMF on water status, pigment concentration and proline accumulation of tomato under drought stress.
3. To assess production of reactive oxygen species (ROS) and antioxidants in AMF tomato under drought stress.

4. To determine the expression of ABA-related genes and aquaporin genes in AMF tomato under drought stress

## 1.5. Research questions

1. Does inoculation with AMF improve plant growth under low or reduced phosphate level?
2. How does inoculation with AMF improve plant physiological response to drought stress?
3. Does AMF influence ROS production and antioxidant enzyme activity in drought stressed tomato?
4. How does AMF modulate the expression of ABA-related genes and aquaporin gene in drought stressed tomato?

# CHAPTER TWO

# LITERATURE REVIEW

## 2.1. Tomato

Tomato (*Solanum lycopersicum*) is the second most important vegetable crop globally after potato. It originated from South America and has been widely cultivated from Asia to Europe and America (Jenkins, 1948). The plant belongs to the nightshade family, *Solanaceae*, together with potato, eggplant and capsicum (Punt & Monna-Brands, 1977). Global production of tomato and production area have been on a steady rise and were estimated at 177 million tonnes per year and about 5.0 million ha respectively. Asia accounted for more than 50% of the world tomato production, followed by America (about 18.5%), Europe (16.4%) and Africa (11.8%). In Africa, Egypt is the largest tomato producer (5th in the world) with an average production of 7.4 million tonnes (FAO, 2016).

The tomato plant is cultivated majorly for its fruits which are consumed in diverse ways: fresh (raw) or processed (as an ingredient in many dishes, sauces, and salads, or as drinks). Tomato is a rich source of nutrients, such as vitamins A and C, and antioxidants. They are high in water and low in calories (Davies *et al.*, 1981).

Numerous varieties of tomato are cultivated across the world. Tomato production systems can either be open field production systems or greenhouse production systems. Field grown tomatoes are commonly produced in the tropical, subtropical and warm temperate climates. In cooler climates, tomatoes are often grown in greenhouses. In

open field, fruit yield and quality may be poor due to prevailing low temperature during winter seasons. Greenhouse is the best alternative for quality and quantity production of tomato because in addition to higher yield; the production is free from dust, insects and pests. Greenhouses also allows all year round tomato production (Mahajan & Singh, 2006).

Field grown tomato are often exposed to unfavourable environmental conditions, such as flood or waterlogging caused by heavy rains, drought, extreme temperatures and metal toxicity. These environmental stresses are major causes of severe crop yield loss globally and in combination can claim up to 50% of global major crop production (Mahajan & Tuteja, 2005).

## 2.2. Drought stress

Drought is the most common environmental stress factor in reducing crop yields globally. The word drought is a meteorological term for a period of insufficient precipitation, resulting in plant water deficit (Taiz & Zeiger, 2010). Jaleel *et al*. (2009) considers drought as a moderate loss of water, which leads to stomatal closure and limitation of gas exchange. Desiccation on the other hand is a much more extensive loss of water, which can potentially lead to gross disruption of metabolism and cell structure and eventually to cessation of enzyme catalysed reactions. Drought is of increasing global concern due to increased rate of evapotranspiration losses from plants and soils as a result of climate change and global warming (Dai *et al*., 2004). Drought is closely associated with high temperature stress and is estimated to affect crop production in approximately 64% of the global land area (Cramer *et al*., 2011).

Water is a major factor influencing plant productivity. It is essential for vital plant processes such as photosynthesis. When water is insufficient in the soil, drought stress occurs. Plants adapt to survive and maintain their growth and development through mechanisms such as drought avoidance and drought tolerance (Tuberosa, 2012). Drought avoidance is the ability of plants to retain high tissue water potential either through increased water absorption from roots or reduced evapo-transpiration from their aerial parts, while drought tolerance is the ability of the plant to sustain normal functions even at low water potential (Basu *et al.*, 2016). Drought can affect the plant's morphology, physiology and biochemistry, leading to a reduction in plant growth and productivity. Plant responses to drought stress are complex and diverse (Osakabe *et al*., 2014). While different plant species may have different family-specific responses to cope with drought, it is believed that all plants have the inherent ability for drought stress perception, signalling and response (Golldack *et al*., 2014).

The water requirement for tomato ranges from 400 to 600 mm within a period of 75 – 125 days growing period and tomatoes can tolerate drought to some degree (Jensen *et al*., 2010). Wudiri & Henderson (1985) on the contrary reported that tomatoes were very sensitive to water stress, particularly during their vegetative and reproductive stages, while Foolad *et al*. (2003) reported that most commercial cultivars are drought sensitive at all stages of their development, with seed germination and early seedling growth being the most sensitive stages.

Drought stress may induce morphological, physiological and biochemical changes in plants. Generally, the consequences of drought stress are generation of reactive oxygen species (ROS), disruption in membrane stability, increased protein denaturation,

perturbation and physical injury (Taiz & Zeiger, 2010). Plant strategies to cope with drought normally involve a combination of stress avoidance and tolerance strategies (Basu *et al*., 2016). Plants adapt to protect themselves from drought through various physiological, biochemical, anatomical and morphological changes, including alterations in gene expression patterns (Figure 2.1) (Shao *et al*., 2008). If plant response is insufficient however, there could be irreversible damage to cells, the destruction of functional and structural proteins and membranes, resulting in cell death and eventually plant death (Lisar *et al*., 2012).

### 2.2.1. Morphological adaptations

Drought stress triggers a wide array of anatomical traits expressed to different levels and patterns in different species and even in different cultivars within species (Henry *et al*., 2012). Drought stress is perceived first by the root system, triggering different plant structural changes or morphological adaptations, such as decline in growth rate, deep rooting system, and modification of root to shoot ratio for desiccation avoidance (Spollen & Sharp, 1991). Poor plant growth may be caused by slower cell division, induced by a decline in cyclin-dependent kinase activity (Mahajan & Tuteja, 2005).

Under drought stress, plant growth is generally reduced, as manifested by a reduction in stem elongation, leaf expansion and number of leaves. However, Mahajan & Tuteja (2005) suggested that the reduction in leaf expansion is a form of response, termed leaf area adjustment, and not an effect caused by drought stress. Prolonged exposure to severe drought stress leads to wilting, leaf curling and rapid senescence in the old leaves, leading to plant death (Torrecillas *et al*., 1995).

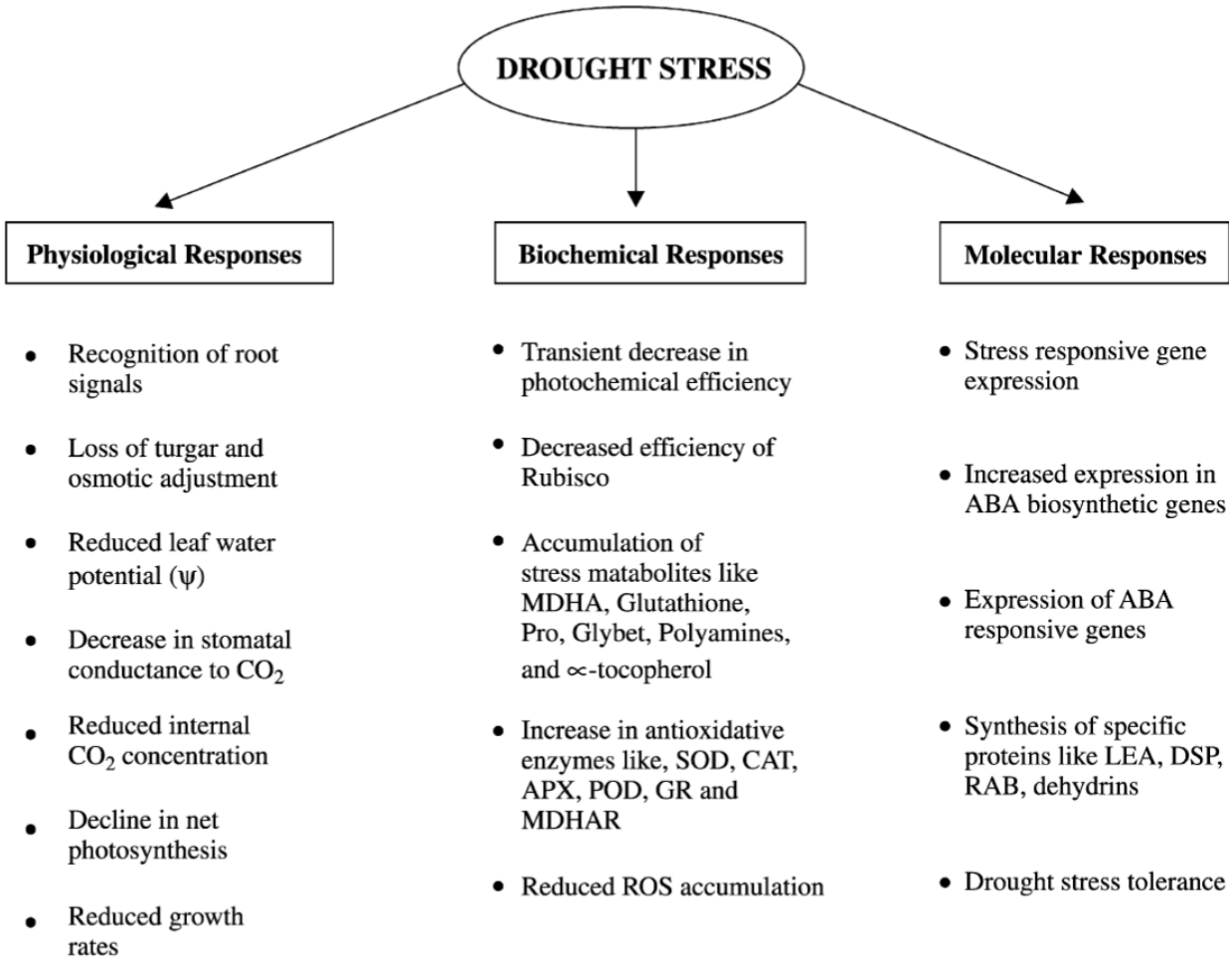


**Figure 2. 1: Physiological, biochemical and molecular basis of drought stress tolerance**

**(Source: Shao *et al.* (2008))**

While the growth of the aerial parts of the plant may be significantly reduced, primary root growth is maintained in order to extract more water from deeper soil layers (Torrecillas *et al.*, 1995; Deak & Malamy, 2005). The presence of lateral and small roots is considered as an adaptive strategy to increase water uptake by providing more absorptive surface. The presence of specialized tissue like rhizodermis, with a thickened outer cell wall or suberized exodermis, or reduction in the number of cortical layers are considered an adaptive advantage for drought stress survival (Basu,

Ramegowda, Kumar, & Pereira, 2016b). Hydrotropism, the plant's growth response towards water sources, is another adaptive measure taken by plants to counter stress. During drought stress, the degradation of amyloplasts in the columella cells of plant roots increases hydrotropism (Ponce *et al.*, 2008; Takahashi *et al.*, 2003).

### 2.2.2. Physiological responses

The immediate response of plants upon exposure to drought is stomatal closure to diminish water loss through transpiration and also reduce $CO_2$ uptake, hence altering metabolic pathways such as photosynthesis (Figure 2.2). Reduction in stomatal size and number on exposure to drought is an adaptation for survival under drought conditions (Xu & Zhou, 2008). Such adaptations reduce the negative impacts of drought stress on photosynthesis and thereby have a positive effect on water use efficiency (WUE), which in turn will result in high yield (Blum, 2005).

Reduced water loss through transpiration during drought stress can also be achieved through leaf shedding (as in deciduous plants) as well as decrease in leaf number, leaf size and branching. Sclerophylly, the exceptional development of sclerenchyma in the leaves resulting thickening and hardening of the foliage, is another adaptation to counter drought stress. It prevents water loss, and prevents permanent damage due to wilting, enabling the leaves to restored to full functionality when normal conditions resume (Basu *et al.*, 2016).

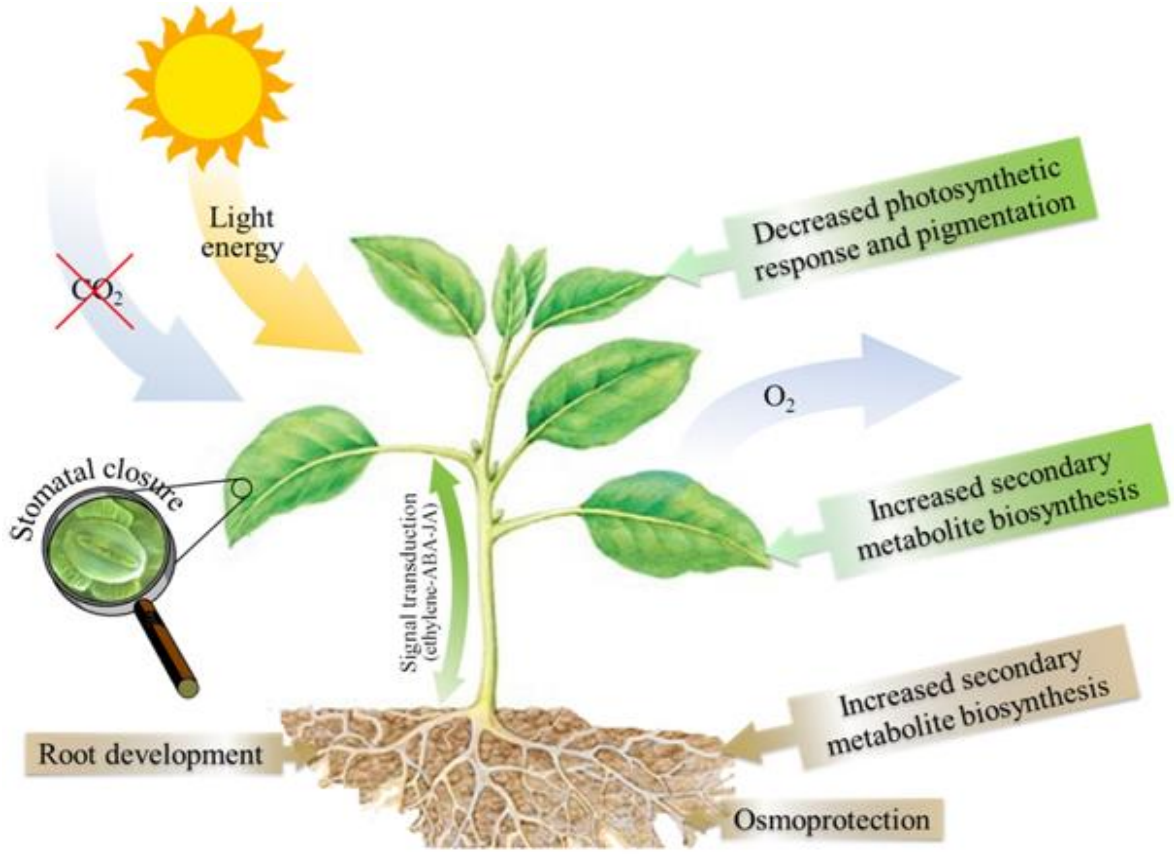


**Figure 2. 2: Plant physiological mechanisms to cope with drought stress (Source: Arve (2011))**

Drought stress reduces photosynthesis mainly through stomatal closure and metabolic impairment (Tezara *et al.*, 1999). During drought stress and under limited $CO_2$ concentration, continued photosynthetic light reactions results in the accumulation of reduced photosynthetic electron transport components, which can potentially reduce molecular oxygen, resulting in the production of reactive oxygen species (ROS). ROS can cause severe damage to photosynthetic apparatus (Lawlor & Cornic, 2002). The adaptive responses that plants have developed to reduce drought induced damage to photosynthesis include thermal dissipation of light energy (Demmig-Adams & Adams, 2006), the xanthophyll cycle (Demmig-Adams & Adams, 1996), the water-water cycle

and dissociation of light-harvesting complexes from photosynthetic reaction centres (Niyogi, 1999). The metabolic impairment during drought stress is mainly caused by changes in photosynthetic carbon metabolism (Lawlor & Cornic, 2002). The biochemical efficiency of photosynthesis under drought stress mainly depends on ribulose-1,5-bisphosphate (RuBP) regeneration and the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Medrano *et al.*, 1997; Lawlor, 2002) The C4 pathway of carbon assimilation has been suggested to be the major adaptation of the C3 pathway to limit water loss, reduce photorespiration, and improve photosynthetic efficiency under drought stress (Jensen, 1983). However, many important crops use the C3 pathway of photosynthesis.

Osmotic adjustment (OA), the process of solute accumulation in dividing cells when water potential is reduced, helps in maintaining the turgor. Under drought stress, OA has been implicated in maintaining leaf water volume, stomatal conductance, photosynthesis and growth (Chaves & Oliveira, 2004). Inorganic cations, organic acids, carbohydrates, and free amino acids are the known predominant solutes that accumulate in response to water stress. In some plants however, sugars such as sucrose, trehalose, glucose and fructose are the main osmolytes that play a significant role in OA. Studies have shown that drought-resistant wheat varieties have a greater capacity for accumulation of osmolytes than the less resistant varieties (Serraj & Sinclair, 2002). The accumulation of compatible solutes such as proline and glycine betaine help in protecting the plants from detrimental effects of drought stress, not only by OA, but also by detoxification of ROS, protection of membrane integrity, and stabilization of enzymes and proteins (Ashraf & Foolad, 2007).

Free proline accumulation is an indication of disturbed physiological conditions triggered by biotic or abiotic stress conditions (Hayat *et al.*, 2012). Accumulation of free proline has been reported in plants exposed to drought stress (Yamada *et al.*, 2005). Proline has been proposed to act as an important compatible osmolyte and osmoprotective compound. It acts as a molecular chaperone in OA, protecting cellular structures, proteins, and membranes during stress. It protects proteins by stabilizing their structures and preventing aggregation during refolding (Samuel *et al.*, 2000). Proline is also considered as a scavenger of ROS, able to reduce the damage of oxidative stress induced by drought and other environmental stress factors (Mohanty & Matysik, 2001). Proline levels during and after osmotic stress is controlled by the reciprocal regulation of two genes: *P5CS,* encoding delta-1-pyrroline-5-carboxylate synthetase, which catalyses the rate-limiting step in proline biosynthesis from glutamate; and *PDH*, encoding proline dehydrogenase, an important enzyme in proline catabolism (Peng *et al.*, 1996). Metabolic engineering of proline levels either by overexpression of *P5CS* or reducing expression of *PDH* results in increased proline accumulation and protection of plants from osmotic stress (Nanjo *et al.*, 1999; Hong *et al.*, 2000). Exogenous application of proline at low concentration provided osmoprotection and also enhanced the growth of plants exposed to osmotic stress (Ali *et al.*, 2007; Ali *et al.*, 2008; Kamran *et al.*, 2009).

### 2.2.3. Biochemical responses

Drought induces oxidative stress in plants, which occurs as a result of excessive production of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide ($H_2O_2$) and hydroxyl radical ($OH^{\bullet-}$) (Apel & Hirt,

2004; Sharma *et al*., 2012). Oxidative stress can lead to lipid peroxidation, protein oxidation and DNA damage in plants (Figure 2.3) (Arora *et al*., 2002; Miller *et al*., 2010). Excessive ROS need to be removed to maintain normal plant growth (Eshdat *et al*., 1997). ROS accumulation as a result of environmental stress factors is a major cause of loss of crop productivity worldwide (Gill & Tuteja, 2010).

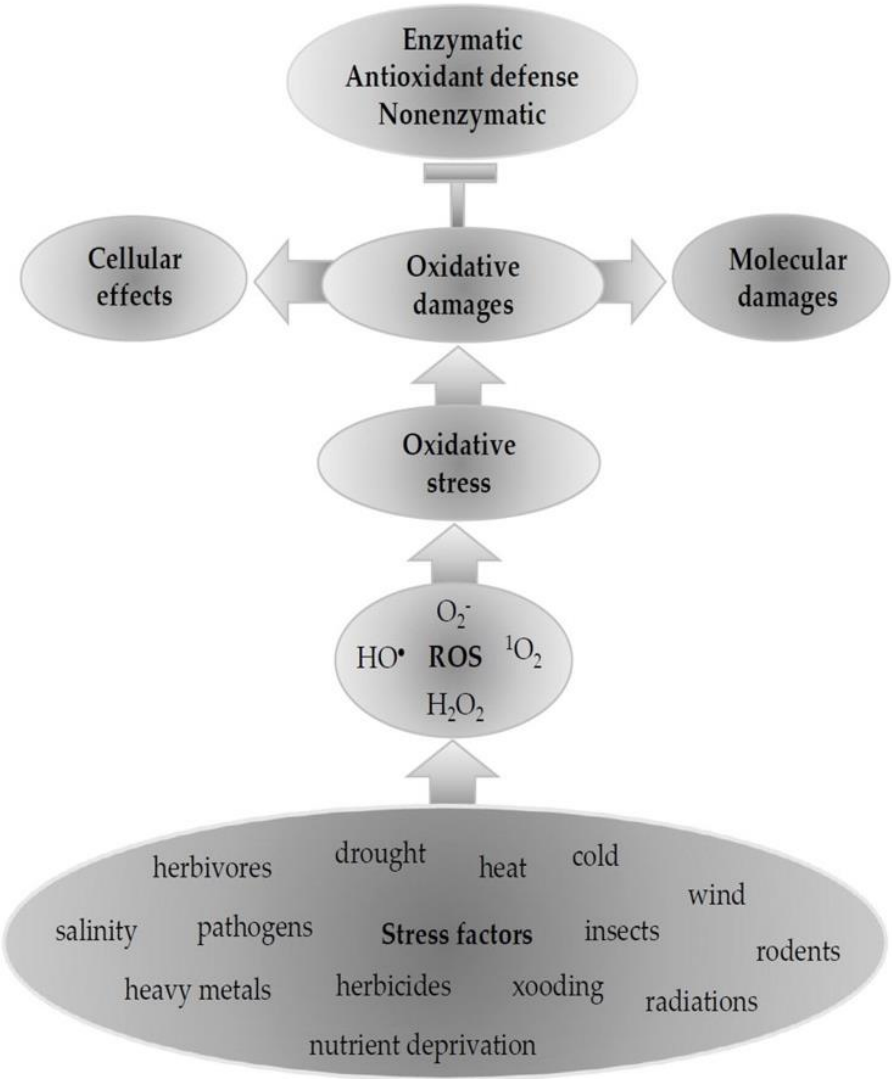


**Figure 2. 3: Stress factors, reactive oxygen species generation, oxidative damage and antioxidant defence**

**(Source: Caverzan *et al.* (2016))**

Reactive oxygen species are normally produced as by-products of various metabolic reactions including photosynthesis, photorespiration and respiration (Foyer & Noctor, 2011). Hence, ROS may be found in metabolically active cell, particularly in organelles like mitochondria, chloroplasts and peroxisomes (Gill & Tuteja, 2010; Miller *et al*., 2010). In higher plants, photosynthesis takes place in the chloroplasts and the oxygen generated in the process can accept electrons passing through the photosystems, thus forming $O_2^{\bullet-}$ (Gill & Tuteja, 2010).

To protect themselves against these toxic ROS, plants have evolved antioxidant defence mechanisms (Figure 2.4) (Gill & Tuteja, 2010). These include both enzymatic and non-enzymatic antioxidants. The enzymatic components of the antioxidant defence system (Table 2.1) comprise several antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), which catalyse ROS degradation, as well as enzymes of the ascorbate-glutathione (AsA-GSH) cycle, such as ascorbate peroxidase (APOX), monodehydroascorbate reductase (MDAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), that regenerate soluble antioxidants (Sanchez-Rodriguez *et al*., 2012; Caverzan *et al*., 2016). Plant stress tolerance may therefore be improved by the enhancement of *in vivo* levels of antioxidant enzymes (Gill & Tuteja, 2010). The non-enzymatic antioxidants are compounds which may act directly in the detoxification of ROS and radicals, or they can reduce substrates for antioxidant enzymes. They include ascorbic acid (AsA), glutathione (GSH) and carotenoids (lycopene and β-carotene) (Foyer & Noctor, 2011; Murshed *et al*., 2013).

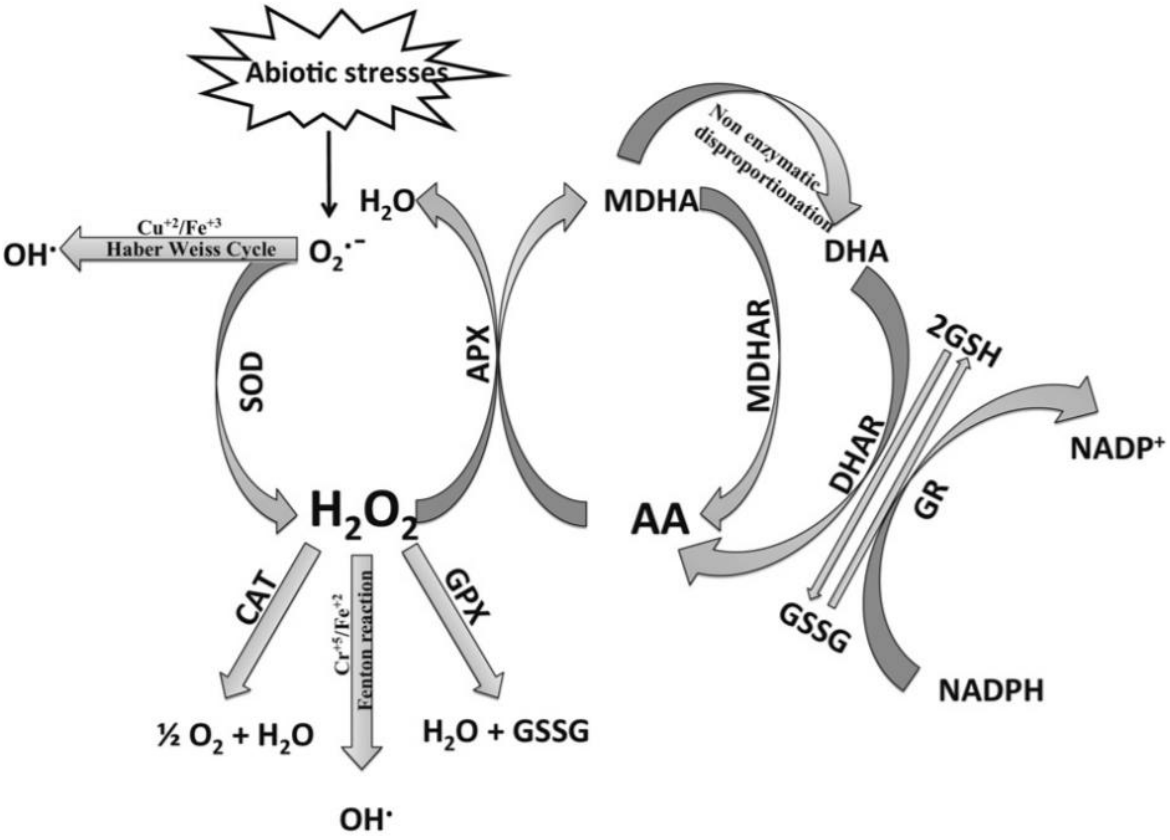


**Figure 2. 4: Reactive oxygen species and antioxidant defence mechanism in plants**

**(Source: Gill & Tuteja (2010))**

In addition, proline can now be added to the list of non-enzymatic antioxidants plants need to counteract the inhibitory effect of ROS (Mohanty & Matysik, 2001). The equilibrium between ROS production and scavenging may be perturbed by various biotic and abiotic stress factors such as drought, salinity, extreme heat and cold. These perturbations in equilibrium lead to sudden increase in intracellular ROS levels, causing significant damage to cell structures (Gill & Tuteja, 2010). It has been estimated that 1-2% of $O_2$ consumption leads to the formation of ROS in plant tissues. Through a variety of reactions, $O_2^{\bullet -}$ leads to the formation of $H_2O_2$, $OH^{\bullet -}$ and other

ROS (Caverzan *et al*., 2016). Bartels (2001) proposed that prevention of oxidative stress and the elimination of ROS species are the most effective approaches used by plants to gain tolerance against several abiotic stresses, including drought.

**Table 2. 1: Major reactive oxygen species scavenging antioxidant enzymes**

| Enzymatic Antioxidant | EC Number | Reaction catalysed |
|---|---|---|
| Superoxide Dismutase (SOD) | EC 1.15.1.1 | $O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2$ |
| Catalase (CAT) | EC 1.11.1.6 | $H_2O_2 \rightarrow H_2O + 1/2O_2$ |
| Ascorbate Peroxidase (APX) | EC 1.11.1.11 | $H_2O_2 + AA \rightarrow 2H_2O + DHA$ |
| Monodehydroascorbate reductase (MDHAR) | EC 1.6.5.4 | $MDHA + NAD(P)H \rightarrow AA + NAD(P)^+$ |
| Dehydroascorbate Reductase (DHAR) | EC 1.8.5.1 | $DHA + 2GSH \rightarrow AA + GSSG$ |
| Glutathione Reductase (GR) | EC 1.6.4.2 | $GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$ |
| Glutathione Peroxidase (GPOX) | EC 1.11.1.9 | $2GSH + 2LOO^{\bullet} \rightarrow 2LOOH + GSSG$ |

Adapted from Gill & Tuteja (2010)

SOD (EC 1.15.1.1) are the most effective intracellular enzymatic antioxidant. They are the first line of defence against the toxic effect of elevated ROS. SOD removes $O_2^{\bullet-}$ by catalysing its dismutation, reducing one molecule of $O_2^{\bullet-}$ to $H_2O_2$ and oxidizing the other to $O_2$ (Alscher *et al*., 2002). CAT (EC 1.11.1.6) are indispensable enzymes for ROS detoxification during stressed conditions. They directly reduce $H_2O_2$ to $H_2O$ and $O_2$ (Sharma & Ahmad, 2014). APX (EC 1.11.1.11) is thought to play the most essential role in ROS scavenging and protecting cells in higher plants. It is involved in

scavenging $H_2O_2$ in water-water and ASH-GSH cycles and utilizes ASH as a specific electron donor (Caverzan *et al*., 2012). GR (EC 1.6.4.2) is an enzyme of the ASH-GSH cycle and plays an essential role in defence system against ROS by sustaining the reduced status of GSH. It catalyses the reduction of GSH, a molecule involved in many metabolic regulatory and antioxidative processes (Yousuf *et al*., 2012). MDAR (EC 1.6.5.4) catalyzes the regeneration of AsA from the monodehydroascorbate radical using NAD(P)H as an electron donor, thus maintaining the AsA pool (Hossain *et al*., 1984). DHAR (EC 1.8.5.1) catalyses the reduction of dehydroascorbate to AsA using GSH as a reducing substrate, hence maintaining AsA in its reduced form (Ushimaru *et al.*, 2006). The ascorbate-glutathione cycle and a high GSH/GSSG ratio is essential for protection against oxidative stress (Anjum *et al*., 2010).

Ascorbate (AsA) is a crucial component of the ROS detoxification system, donating electrons in enzymatic and nonenzymatic reaction. It can directly eliminate $O_2^{\bullet-}$, $OH^{\bullet-}$ and $^1O_2$. It also reduces $H_2O_2$ to water via the ascorbate peroxidase reaction. It is usually maintained in its reduced state by a set of NAD(P)H-dependent enzymes, including MDAR, DHAR and GR (Akram *et al*., 2017). Glutathione (GSH) is oxidized by ROS to form oxidized glutathione (GSSG). GSH and GSSG maintains redox balance in cellular compartment (Hasanuzzaman *et al*., 2017).

### 2.2.4. Molecular response

Plant response to drought stress can be measured at many different levels from whole plant to the molecular level. Since responses are controlled by the plant genome, efforts are now focused on the molecular response of the plant to drought stress (Bray, 2004). During the process of plant responses to drought stress, a large number of genes

are up-regulated and can be classified into two major groups according to their putative functional modes (Figure 2.5) (Gong *et al*., 2010). The first group comprises the genes encoding structural proteins, which function in supporting cellular adaptation to drought stress. These include key enzymes for osmolyte biosynthesis, antioxidant proteins, aquaporins etc (Shinozaki & Yamaguchi-Shinozaki, 2007). The second group consists of the genes encoding regulatory proteins, which are protein factors involved in further regulation of signal transduction and stress-responsive gene expression. They include early response transcriptional activators such as transcription factors and protein kinases such as calmodulin dependent protein kinases (CDPKs), mitogen-activated protein kinases (MAPKs), receptor protein kinases (RPKs) and ribosomal protein kinases, which are involved in the signal cascade amplification in response to different environmental stress factors (Song *et al*., 2005). The expression of drought inducible genes can be governed by ABA-dependent or ABA-independent regulatory system (Yamaguchi-Shinozaki & Shinozaki, 2005). There are also extensive cross-talks between responses to drought and other environmental stresses such as light and biotic stresses (Huang *et al*., 2008).

Aquaporins (AQP) are water channel proteins that facilitate and regulate the passive movement of water down a water potential gradient (Kruse *et al*., 2006). These proteins belong to the large major intrinsic protein (MIP) family of transmembrane proteins, present in all kingdoms (Maurel, 2007). They are the most abundant transmembrane transporters of water and substrates like glycerol, urea, $CO_2$, $NH_3$, metalloids and ROS (Afzal *et al*., 2016). Because AMF can transfer water to the root of the host plants, it is thus expected that the plant must increase its permeability for water and that aquaporin genes should be upregulated in order to allow a higher rate

of transcellular water flow (Javot & Maurel, 2002). It has been demonstrated that the regulation of root hydraulic conductivity during AMF symbiosis is linked to regulation of plant aquaporins (Ruiz-Lozano & Aroca, 2010).

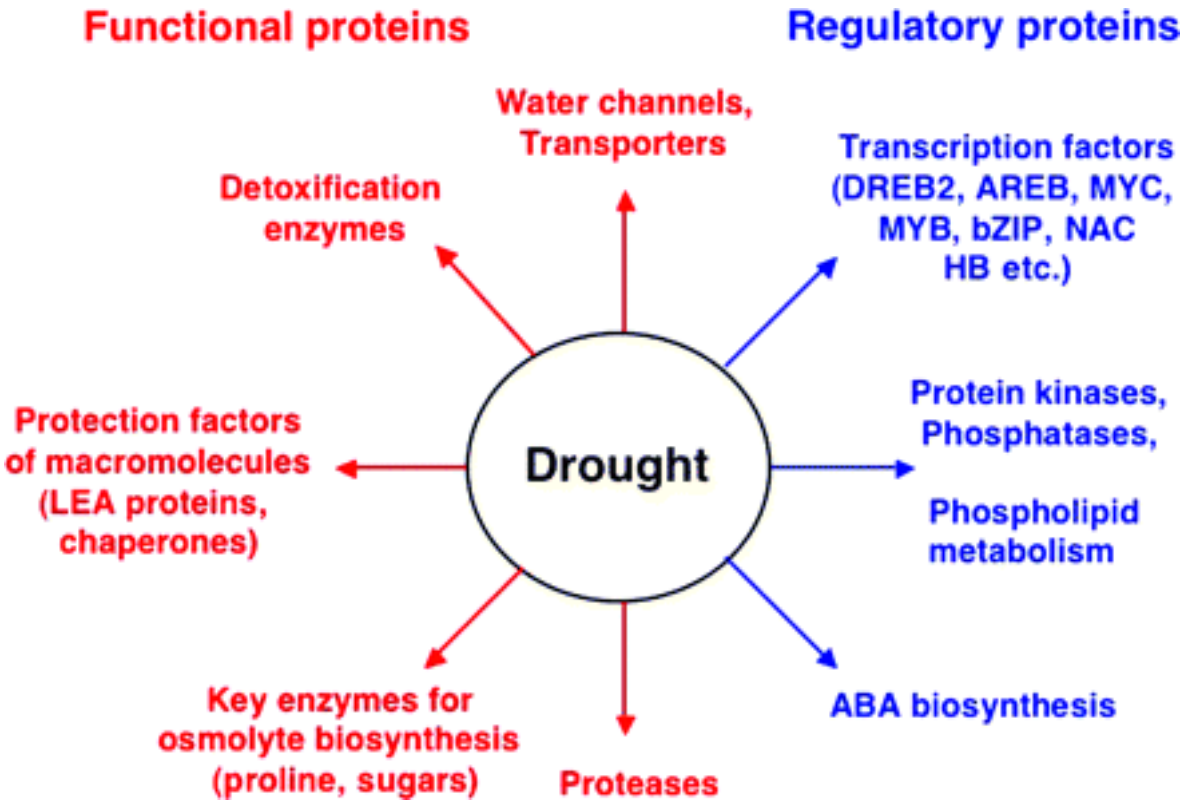


**Figure 2. 5: Functions of drought stress-inducible genes in stress tolerance and response**
**(Source: Shinozaki & Yamaguchi-Shinozaki (2007))**

The function of aquaporin in transporting water is crucial for plant survival in drought stress conditions (Li *et al*., 2016). A total of 47 aquaporin encoding genes (*AQPs*), belonging to five subfamilies (Plasma membrane intrinsic proteins, PIPs; tonoplast intrinsic proteins, TIPs; NOD26-like MIPs, NIPs; small basic intrinsic proteins, SIPs; and uncharacterized X intrinsic proteins, XIPs), have been identified in tomato

(Reuscher *et al.*, 2013). They differ in their tissue and developmental expression. Three *AQPs* (*SlPIP2;1*, *SlPIP2;7* and *SlPIP2;5*) were found to be highly expressed in the roots and their overexpression conferred drought tolerance and enhanced survival of tomato plants that were subjected to drought stress (Li *et al*., 2016). The expression of specific aquaporin genes are associated with plant tolerance to drought stress (Li *et al*., 2015). On the other hand, the ability of plants to conserve water during drought stress involves timely and sufficient down-regulation of gene expression of specific aquaporins (Zupin *et al.*, 2017).

### 2.2.5. Hormonal regulation

Major phytohormones, such as abscisic acid (ABA), cytokinin (CK), gibberellic acid (GA), auxin, and ethylene are key regulators of plant growth and development as well as mediators of environmental stress responses and adaptation. Among these phytohormones, ABA is the central regulator of abiotic stress resistance in plants (Peleg & Blumwald, 2011). ABA is considered a 'stress hormone' and its biosynthesis is rapidly promoted under drought stress (Osakabe *et al*., 2014). It is synthesized in the roots and translocated to leaves to initiate adaption of plants to drought stress though stomatal closure and reduced plant growth (Wilkinson & Davies, 2010). Besides it role in plant response under drought stress, it is also an important signalling molecule in regulation of plant growth and development, as well as promotion of plant defence responses. There are ABA-induced non stomatal adaptations of plants under drought stress that can be exploited to improve yield under reproductive drought (Basu *et al*., 2016).

Cytokinins are known to delay premature leaf senescence and death under drought stress, an adaptive trait very useful for increasing yield. An increase in the endogenous levels of CK leads to stress adaptation by delaying drought-induced senescence and increase in yield (Peleg *et al*., 2011). Auxins have been shown to negatively regulate drought adaptation in plants. Decrease in indole-3-acetic acid (IAA) content leads to drought adaptation in plants. GA is suggested to positively regulate plant adaptation to drought stress, as rapid decline in endogenous GA was observed in plants subjected to drought stress, resulting in growth inhibition (Wang *et al*., 2008). Ethylene promotes leaf senescence, inhibits root growth and development, shoot/leaf expansion, and photosynthesis (Sharp, 2002). It is thus a negative regulator of drought stress response. In addition to the major phytohormones, other hormones such as jasmonic acid (JA), salicylic acid (SA) and strigolactone also have important roles in plant growth and development. However, their function under drought stress is relatively less characterized. All hormones do not act in isolation, but may instead interact and modulate each other's biosynthesis and responses. Therefore, the net outcome of drought stress response is regulated by a balance between hormones that promote and those that inhibit the traits, rather than individual hormones (Basu *et al*., 2016b).

### 2.3. Mycorrhizal fungi

The term mycorrhiza is derived from the Greek words 'mükes' for 'fungus' and '*rhiza*' for 'root'. Mycorrhizal fungi are a heterogeneous group of species, spread over diverse fungal taxa (Bonfante & Genre, 2010). Although they can spend part of their life cycle as free-living organisms, mycorrhizal fungi always associate with the roots of higher plants. They are associated with about 90% of higher plants in nearly all terrestrial

ecosystem to form symbiotic associations called mycorrhizas (Bonfante, 2001). Both partners benefit from the relationship: mycorrhizal fungi improve the nutrient status of their host plants, influence mineral nutrition, water absorption, growth and disease resistance, whereas in exchange, the host plant is necessary for fungal growth and reproduction (Smith & Read, 2008). Mycorrhizal fungi play significant roles in nutrient cycling, as their mycelium absorbs soil nutrients and supply them to the plants. They also develop an extensive hyphal network in the soil, to connect whole plant communities, and enable horizontal transfer of nutrients. Mycorrhizas develop specialized areas, called symbiotic interfaces, to interact with host plants (Bonfante, 2001).

Mycorrhizal fungi can be divided into two broad categories: the ectomycorrhizal (EMs) and the endomycorrhizal fungi.

### 2.3.1. Ectomycorrhizal fungi (EM)

The ectomycorrhizal fungi, of the Basidiomycetes and Ascomycetes, associate majorly with temperate-zone trees, such as pine, poplar and willow. These fungi have therefore shaped the present forests (Smith & Read, 2008). EM fungi colonize lateral roots of these trees, forming sheaths around their host's root surfaces. The fungal mantle covers the root tip, while the Hartig net of the intercellular hyphae surround epidermal and outer cortical cell (Figure 2.6). EM fungi can live independently of plant roots, as they can be grown in pure culture (Smith & Read, 2008).

### 2.3.2. Endomycorrhizal fungi

Unlike EM Fungi which form a system of hyphae that grow around the cells of the root, the hyphae of endomycorrhizal fungi penetrate the root cell walls and become enclosed in the cell membrane as well. Thus, endomycorrhiza is a more invasive symbiotic relationship between the fungi and the plant (Bonfante & Genre, 2010). The penetrating hyphae creates a greater contact surface area between the hyphae of the fungi and the plant, facilitating greater transfer of nutrients between the two partners. Endomycorrhizae are further divided into five major groups: arbuscular, ericoid, arbutoid, monotropoid and orchid (Peterson *et al*., 2004). Arbuscular mycorrhizal fungi are the most common types of endomycorrhizal fungi (Smith & Read, 2008).

### 2.3.3. Arbuscular mycorrhizal fungi (AMF)

Arbuscular mycorrhizal fungi are the most widespread fungal symbionts of plants, occurring in a wide spectrum of temperate and tropical plant species, associating with more than 80% of land plants, and absent in less than 30 plant families (Smith & Read, 2008). They all belong to the monophyletic phylum *Glomeromycota*, which diverged from the same common ancestor as the *Ascomycota* and *Basidiomycota* (Schüßler *et al*., 2001). This group are among the oldest of fungi, as their spores and hyphae were found in the fossil roots of plants known to be as old as 450 million years (Arthur Schüßler & Walker, 2011).

Arbuscular mycorrhizal fungi are obligate symbionts, requiring the presence of actively growing plants during their reproduction. They strictly depend on their green hosts for growth and reproduction and are unable to absorb carbohydrates except from

inside a plant cell. They therefore cannot be cultured in the laboratory media (Bonfante & Genre, 2010).

Arbuscular mycorrhizal fungi invade cortical cells and form clusters of finely divided hyphae known as arbuscles, which are the sites of material exchange between the fungus and the host plant, in the cortex. They also form membrane-bound organelles of varying shapes known as vesicles, which serve as storage structures, inside and outside the cortical cells. Vesicles and arbuscules together with large spores constitute the diagnostic feature of AM association (figure 2.6) (Smith & Read, 2008).

Arbuscular mycorrhizal fungi are considered to be asexual, although the hyphae of genetically distinct strains can anastomose and exchange genetic material (Hijri & Sanders, 2005; Croll *et al*., 2009). There exist a high degree of genetic variability and functional diversity within this group. Despite their abundance and wide host range, only a few species of AMF have been described on the basis of their morphology (Kruger *et al*., 2012). Hence, AMF are thought to exhibit low species diversity compared to other fungal phyla. However, molecular evidences have suggested that there is a greater diversity in these fungi (Fitter, 2005). In addition, there exist high genetic variation within specie and even within a single spore (Croll *et al*., 2008). Their large asexual spores or aseptate hyphae contain hundreds or thousands of nuclei, making the genetic structure multi genomic or heterokaryotic (Kuhn *et al*., 2001). Each nucleus within a single spore has been shown to be genetically distinct, and the genetic variation is inherited in an individual nucleus, not shared by nuclei (Hijri & Sanders, 2005). Genetically different AMF, even within the same species, have different effects on their host plants (Munkvold *et al*., 2004).

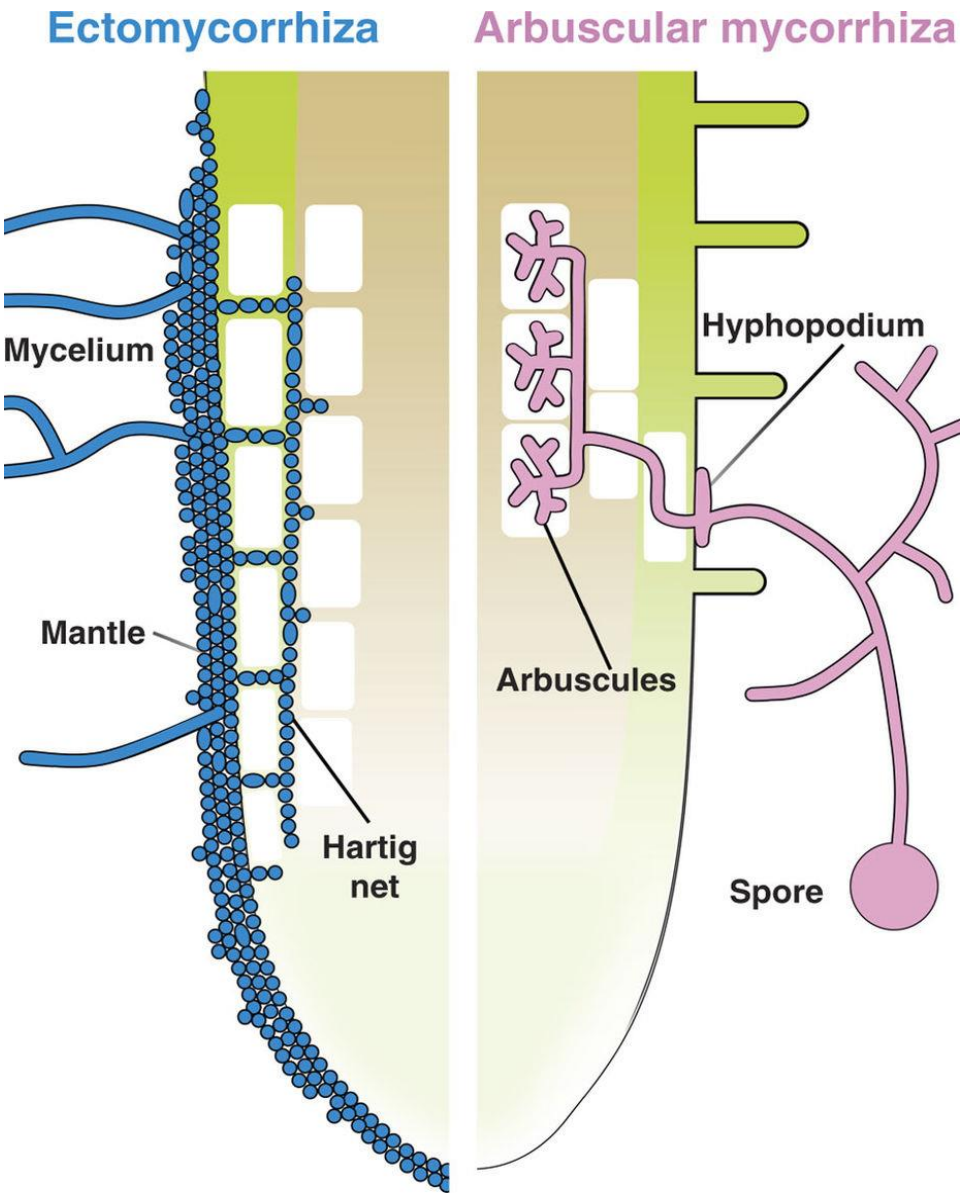


**Figure 2. 6: Illustration of root colonization in ectomycorhizal (blue) and arbuscular mycorrhizal (pink) interactions**
**(Source: Bonfante & Genre (2010))**

Functional diversity is the phenomenon in which different AMF specie have highly varying effects on plant growth, as well as physiological traits and nutritional benefits conferred to their host plants (Feddermann *et al.*, 2010). In cassava for example, field yields were highly variable following inoculation with AMF species, ranging from no effect, up to an approximately 20% yield increase (Ceballos *et al.*, 2013). In most

cases, AMF inoculation promotes growth and provide other benefits such as water and nutrient uptake, as well as stress tolerance. However, that is not always the case. Some isolates of AMF can even decrease the biomass of the host plant. The efficiency of AM symbiosis differs according to the genotype of the two partners (Burleigh *et al*., 2002; Munkvold *et al*., 2004). Hence, there is need to consider the AMF identity for inoculation of plants. Due to their wide array of functional traits, AMF species mixture may increase the multiple functionality of the system through complementarity when simultaneously colonizing a root system (Maherali & Klironomos, 2007). This complementarity phenomenon holds great promise for agriculture soil management.

### 2.3.4. Arbuscular mycorrhizal development

The dialogue between an AMF and plant roots begins before any physical contact. The establishment and functioning of AMF symbiosis requires a high degree of coordination between the two partners, which implies a signal exchange that leads to mutual recognition (Bucher *et al*., 2014). AMF spores in soil feed germinating hyphae though catabolism of storage lipids for a few days (Smith & Read, 2008). This so called asymbiotic stage does not require any plant factors. During this period, hyphae explore the soil in search of a host, but if they never meet one, they arrest their growth and retract their cytoplasm back into the spore, which may again become dormant and restart the germination process over and over (Bonfante & Genre, 2010).

#### 2.3.4.1. Presymbiosis

The pre-symbiotic stage starts with the production of strigolactones (SLs) by the host plants and its exudation into the rhizosphere. SLs are perceived by AM fungi,

stimulating intense hyphal growth and branching, increasing the chance of encountering the host root (Akiyama *et al*., 2005). SLs are mainly produced in the roots, and they have been detected in the root extracts and root exudates of monocot and dicot plants (Xie *et al*., 2010). SLs are classified as new class of hormones that control several processes in plants. They play pivotal role as modulators of the coordinated development of roots and shoots in response to nutrient deprivation, especially phosphorus shortage. They regulate above-ground and below-ground plant architecture, adventitious root formation, secondary growth, reproductive development, leaf senescence and defence responses (Ruyter-Spira *et al*., 2013).

In turn, the AMF releases signal molecules (collectively called Myc factors) that activate transcription of symbiosis related genes and induce symbiosis-specific organ responses in the host root prior to contact (Kosuta *et al*., 2003). Plant responses to Myc factors are part of a reprogramming under the control of the common symbiosis (SYM) pathway, the signal-transduction pathway that prepares the plant for successful association with both AMF and nitrogen-fixing rhizobia (Bonfante & Genre, 2010). Myc factors are diffusible compounds, containing a mixture of sulphated and non-sulphated simple lipochitooligosaccharides (LCOs) that have structural similarities with rhizobial Nod factors (Maillet *et al*., 2011).

#### 2.3.4.2. Symbiotic phase

Once a chemical acquaintance has been made between the fungus and the plant, the presymbiosis develops into a physical encounter between the symbionts, with the hyphal tip touching the surface of a root. The symbiotic phase begins with the formation of hyphopodium, which is the entry point structure for AMF hyphae into

the root, after contact of a fungal hypha with the host root surface (Genre *et al*., 2005). During hyphopodium formation, but preceding the first signs of penetration, the epidermal cell responds with a striking program of cellular reorganization to form the prepenetration apparatus (PPA) (Genre *et al*., 2005). Once the PPA is completed, the fungus start growing again, with a hyphal tip traversing the epidermal cell wall and along the track of the PPA. At this point, the perifungal membrane is assembled as PPA secretory vesicles fuse to produce an invagination of the plant plasma membrane. This marks the appearance of the symbiotic interface, the narrow intracellular compartment that allows AMF to grow inside the plant cell without breaking its integrity (Bonfante, 2001). This is accompanied by a tremendous structural reorganization in the plant cell and formation of arbuscules. All AM fungi are characterized by, and named after, arbuscules. These structures are formed in the inner root cortex by repeated branching of an intracellular hypha, and are the sites of nutrient exchange (Paszkowski, 2006).

Parallel to intraradical growth, AM fungi form a network of extraradical hyphae which explores far into the soil, giving the root system a much greater access to mineral nutrients and water by taking them up and transferring them to the plant (Neumann & George, 2005). The extraradical hyphae also establishes common mycorrhizal networks which connect plants of the same or of different species (Selosse *et al*., 2006), through which plants can exchange mineral elements (Meding & Zasoski, 2008) and communicate with each other (Song *et al*., 2010). The AM fungal life cycle is completed when the extraradical mycelium produces a new generation of spores which are major survival organs and able to tolerate adverse soil conditions for many years (Neumann & George, 2005).

### 2.3.5. Arbuscular mycorrhizal functions

#### 2.3.5.1. Exchange of nutrients

Arbuscular mycorrhizal symbiosis involves a bidirectional exchange of materials between the two partners: the fungus supplying the plants with essential mineral nutrients, in exchange for photosynthetically fixed carbohydrates (Smith & Read, 2008). The arbuscules represent the site of material exchange between the two symbionts. The symbiosis of plants with AMF often results in increased nutrient uptake (Bucking *et al*., 2012).

Arbuscular mycorrhizal fungi have been reported to improve the growth of plants under phosphate-limiting conditions (Elbon & Whalen, 2015). In mycorrhizal plants, the pathway of direct uptake of inorganic phosphate (Pi) from the soil at the root surface is suppressed and replaced entirely by the mycorrhizal pathway. The mycorrhizal pathway involves the import of Pi into fungal hyphae via Pi transporters, translocation of Pi to the arbuscule, and release to root cells where plant Pi transporters transfer the Pi into cortical cells (Bucher, 2007). AM fungi can also provide the host plant with N (Hawkins *et al*., 2000). The current model predicts that nitrate and ammonium are taken up by the extraradical mycelium, arginine is transported in the fungal hyphae and ammonium is finally transferred towards the plant (Guether *et al*., 2009; Bucking *et al*., 2012).

#### 2.3.5.2. Bioprotection against abiotic stress

Arbuscular mycorrhizal fungi are capable of improving plant growth and production under abiotic stress conditions (Nadeem *et al*., 2014; Latef *et al*., 2016) . They can play

important roles in improving plant health in metal contaminated soils. AMF produce a glycoprotein, known as glomalin, which strongly and irreversibly seizes metals like Cu, Cd, and Zn (Gonzalez-Chavez *et al.*, 2004). AMF thus lower the availability of the toxic metals and decrease their toxicity to their host plants, other soil microbes and plants growing in the immediate vicinity (Gamalero *et al.*, 2009). Under salinity stress, AM fungi can improve the growth and yield of their host plants (Porcel *et al.*, 2012; Aroca *et al.*, 2013). They help salinity exposed host plants to absorb more water through their hyphal networks, and also increase their nutrient uptake, gas exchange and photosynthesis (Ruiz-Lozano & Azcón, 1995a). AM symbiosis has also been reported to improve plant cold tolerance ( Liu *et al.*, 2011; Chen *et al.*, 2013; Liu *et al.*, 2013), and increase plant growth under heat stress conditions (Gavito *et al.*, 2005).

In the case of drought stress, plant symbiosis with AMF can improve overall plant growth by increasing root length, leaf area, plant biomass and nutrient uptake (Al-Karaki *et al.*, 2004; Augé, 2001, 2004). AM symbiosis is involved in several physiological and biochemical processes including (a) direct uptake and transfer of water and nutrients by AM fungi, (b) increased osmotic adjustment, (c) improved gas exchange and water use efficiency and (d) better protection against oxidative stress (Figure 2.7) (Rapparini & Peñuelas, 2014). AMF symbiosis resulted in greater leaf water potential, improved gas exchange, increase stomatal conductance and transpiration and photosynthetic rates of mycorrhizal plants under drought (Lee *et al.*, 2012; Gholamhoseini *et al.*, 2013). The higher water contents in mycorrhiza plants can be ascribed to increased water uptake in host roots by the extra-radical hyphae, increased effective root hydraulic conductivity and modification of root architecture (Ruiz-Lozano & Azcón, 1995). Improved water uptake and transport in roots translates

into enhanced hydration of the aerial tissues that in turn affects physiological and biochemical processes. AMF can also alter water regulation in the host plant through modulation in hormonal signalling (Ludwig-Müller, 2010).

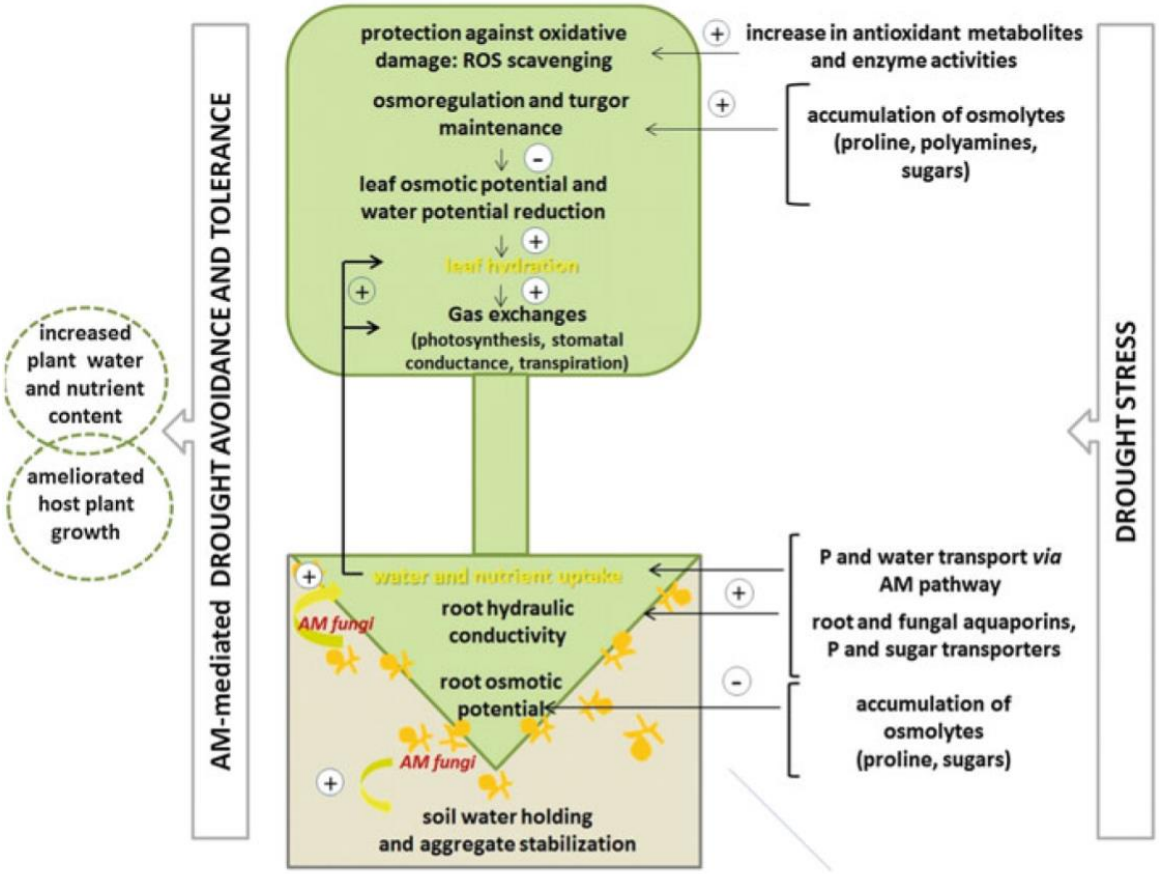


**Figure 2. 7: Arbuscular mycorrhizal fungi mediated drought tolerance mechanisms**
**(Source: Rapparini & Peñuelas (2014))**

The accumulation of compatible solutes (osmolytes and osmoprotectants), such as proline, glycine betaine and sugars, is another mechanism underlying AMF mediated drought stress tolerance in host plants. Osmoprotectants can lower the osmotic potential in drought tolerant mycorrhizal plants (Abbaspour *et al*., 2012). AMF

mediated accumulation of free proline, free polyamines and soluble nitrogenous compounds has been associated with drought tolerance in plants (Ruiz-Sánchez *et al*., 2010).

It has also been suggested that the protection against oxidative stress caused by drought through enrichment of antioxidant levels and antioxidant enzyme activities may be one the most important mechanisms by which the AMF symbiosis increases the tolerance of plants to drought stress. (Ruiz-Sánchez *et al*., 2010). Increased antioxidant levels is necessary to scavenge the excessive ROS generated by drought stress, which may cause oxidative stress, cellular damage and death if unchecked (Smirnoff, 1993). Molecular mechanisms activated by AM symbiosis to enhance drought tolerance include gene activation of functional proteins, such as the transmembrane water transporters, aquaporins, in both the host roots and the fungi (Rapparini & Peñuelas, 2014).

#### 2.3.5.3. Bioprotection against biotic stress

Arbuscular mycorrhizal fungi offer an alternate approach to controlling soil borne pathogens (Kamaruzaman & Othaman, 2010) as the symbiosis has been shown to reduce both the incidence and the severity of diseases (Caron, 1989). Disease reduction by AM fungi is as a result of complex interactions between pathogens, AM fungi and host plant. The bioprotection conferred by AM fungi is not effective for all plant pathogens, and the level of bioprotection conferred is plant species and AMF isolate specific (Harrier & Watson, 2004). AM fungi are not known to directly interact with pathogens, and therefore, the mechanisms proposed to explain the bioprotection are mostly indirect mechanisms. They include: (a) enhanced crop mineral nutritional

status; (b) alteration of root architecture and morphology; (c) competition for colonisation and infection sites; (d) alteration in the anatomical structure of the root; (e) competition for host photosynthates; (f) rhizosphere deposition; (g) damage compensation; (h) alteration of soil microbial populations; and (i) activation of plant defence responses (Harrier & Watson, 2004).

These bioprotective effects have been consistently demonstrated against different agronomically important pathogens, including: *Phytophthora species* (Cordier *et al*., 1996; Vigo *et al*., 2000), *Ganoderma boninense* (Rini, 2001), *Aphanomyces species* (Slezack *et al*., 1999), *Fusarium species* (Jaizme-Vega *et al*., 1997), *Phythium species* (Rosendahl & Rosendahl, 1990), *Rhizoctonia species* (Guillon *et al*., 2002), etc. The impact of AM fungi in biocontrol of nematodes has also been demonstrated (Forge *et al*., 2001; Talavera *et al*., 2001). Bioprotection of roots against such pathogens generally depends on a fully established mycorrhizal symbiosis (Slezack *et al*., 2000). However, there are reports suggesting pre-symbiotic effects of AM fungi (Gallou *et al*., 2011).

The effect of AMF symbiosis on leaf pathogens is variable and appears to depend on the pathogen lifestyle. The symbiosis mostly leads to higher susceptibility of host plants to leaf biotrophic pathogens, such as powdery mildew and rust fungi (Gernns *et al*., 2001). On the contrary, the symbiosis confers host resistance to phytoplasma or necrotrophic fungal pathogens (Lingua *et al*., 2002).

# CHAPTER THREE

# MATERIALS AND METHODS

## 3.1. Assessment of AMF root colonization and promotion of plant growth

### 3.1.1. Experimental site

Greenhouse experiments were carried out at Jomo Kenyatta University of Agriculture and Technology (JKUAT) (1537 MASL; Latitude: -1°5'47.04" Longitude: 37°0'46.08"). Daily average temperature (25-32 °C) and relative humidity (66-78%) during the experimental period (April, 2016 and November, 2017) were recorded using a data logger (MIC 98583, Linz, Austria).

### 3.1.2. Plant materials and growth conditions

Tomato seeds (var ANNA F1) were obtained from Amiran Ltd, Kenya and sowed in 6 L cylindrical plastic pots (of 21 cm height and 19 cm diameter). Cocopeat, which is low in nutrient (particularly phosphate) and free from AMF, was used as planting medium. The cocopeat was soaked overnight in water and thereafter repeatedly washed to remove excess salts before planting. The inoculum was added to each planting hole prior to direct seeding of the pots with tomato seeds, one seed per pot. All plants were irrigated daily and fertilised weekly with 200 ml Hoagland nutrient solution per plant (Hoagland and Arnon, 1950).

### 3.1.2. Arbuscular mycorrhizal fungal inocula

Arbuscular mycorrhizal fungi inoculum (*G. intraradices* and *G. mossea*) were purchased from Dudutech Division of Finlays Horticulture, Kenya. The AM fungi were supplied as crude inocula, containing spores, colonized root fragments and other propagules of AMF in a granular carrier, with an estimated concentration of 1600 propagules/1000 cc. The inocula were stored at room temperature, away from direct sunlight until use. The inocula were applied directly to the planting hole as supplied without any treatments.

The presence of AMF spores in the inocula was confirmed by wet sieving and sucrose density centrifugation to extract the spores (Pacioni, 1992), as well as staining of the root fragments with ink/vinegar solution (Vierheilig *et al*., 1998), followed my microscopic evaluation.

### 3.1.3. Experimental set-up and design

The experiment comprised of two factors (with and without phosphate) each at four levels of AMF inoculation treatments (A. Non-inoculated control plants; B. *G. intraradices* inoculated plants; C. *G. mossea* inoculated plants; and D. Mixed *G. intraradices* and *G. mossea* inoculated plants). This constitutes eight treatments which were laid out in a completely randomized design on the greenhouse benches (Table 3.1). Each treatment consisted of 8 independent plants, replicated three times each, totalling 192 plants (8 treatments x 8 plants x 3 replicates). 30 g of inoculum was applied to each plant. The mixed application consisted of 15 g of each AMF inoculum.

In the treatment without phosphate, $KH_2PO_4$ was omitted from the Hoagland solution used for fertilization.

For root colonization and phosphorus concentration, 5 plants per treatment were analysed, totalling 40 plants. As for dry shoot and root weights, 15 plants per treatment were analysed, totalling 120 plants. The plants were analysed at week 7 after inoculation with AMF and seeding of the pots.

**Table 3. 1: Description of treatments for determination of efficacy of *G. intraradices*, *G. mossea* and their combined application on plant growth with or without phosphate addition to the planting media**

| **Phosphate Treatments** | **AMF Treatments** | **Treatment Description** |
|---|---|---|
| **No Phosphate** | Control | Non inoculated plants;<br>Not receiving phosphate |
| | *G. intrararadices* | *G. intraradices* inoculated plants;<br>Not receiving phosphate |
| | *G. mossea* | *G. mossea* inoculated plants;<br>Not receiving phosphate |
| | G.I + G.M | *G. intraradices* + *G. mossea* inoculated plants;<br>Not receiving phosphate |
| **With phosphate** | Non AMF | Non inoculated plants;<br>Receiving phosphate |
| | *G. intrararadices* | *G. intraradices* inoculated plants;<br>Receiving phosphate |
| | *G. mossea* | *G. mossea* inoculated plants;<br>Receiving phosphate |
| | G.I + G.M | *G. intraradices* + *G. mossea* inoculated plants;<br>Receiving phosphate |

### 3.1.4. Estimation of root colonization

Estimation of root colonization was done by detecting the presence of AMF hyphae, arbuscules, vesicles and internal spores. After washing with tap water, root samples were cleared of the cytoplasmic contents by boiling in 10% (w/v) potassium hydroxide (KOH) solution for 10 min. The cleared roots were then washed in water and stained by boiling in ink/vinegar (10% ink in 25% acetic acid) solution for 10 min. The roots were destained in vinegar (25% acetic acid) before microscopic examination for root colonization (Vierheilig *et al.*, 1998). Quantification of root colonization was done by counting the number of root segments colonized and expressed as a percentage of total root segments examined (Giovannetti and Mosse, 1980).

$$\% \text{ colonization } = \frac{\text{number of colonized segments}}{\text{total number of segments examined}} \times 100 \qquad 3.1$$

### 3.1.5. Determination of plant biomass

The plants were harvested seven weeks after inoculation and seeding and each plant was separated into shoots and roots. The roots were thoroughly washed with tap water to remove the cocopeat. The root and shoot were placed in paper bags and oven dried for 48 hrs at 70 ºC. The dry shoot weight (DSW) and dry root weight (DRW) of each plant was then determined by weighing the oven dried shoots and roots.

### 3.1.6. Phosphorus concentration

Total Phosphorus concentration in leaves was determined by colorimetric method using the ammonium-molybdate-vanadate method (Anderson and Ingram, 1989) and a spectrophotometer (UV mini 1240, Shimadzu, Japan) at 400 nm wavelength. 0.3 g

of oven dried and homogenized leaf tissues from different plants were placed in separate digestion tubes. To each tube, 2.5 ml of digestion mixture containing 3.2 g salicylic acid in 100 ml of sulphuric acid-selenium mixture (3.5 g of selenium in 1 litre of sulphuric acid) was added. The samples were digested by heating the mixture at 110 ºC for 1 h, followed by addition of 3 ml of 2% hydrogen peroxide, after which the temperature was increased to 330 ºC until the solution turned colourless. The contents of the digestion flask were cooled and water was added up to a final volume of 50 ml. The samples were then filtered and pH of the filtrate was adjusted (using p-nitrophenol, 6N $NH_3$, 1N $HNO_3$) and yellow colour was developed by adding Ammonium molybdate/ammonium vanadate mixed reagent. The absorbance of the solution was measured using a colorimeter at 400 nm wavelength. The amount of phosphorus present was determined from a calibration curve of standard phosphorus.

### 3.1.6. Statistical analysis

Data on percentage root colonization, dry shoot weight, dry root weight and phosphorus concentration were subjected to analysis of variance (ANOVA) using general linear model (GLM) procedure of SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). Where there was significant treatment effect, the means were separated using Tukey's test (P = 0.05). All statistical tests were compared at 5% level of significance.

## 3.2. Determination of plant water status, pigment content and proline concentration

Two experiments were conducted to determine the effects of inoculation of tomato with AMF and application of drought stress on the growth, plant water status, pigment content and proline concentration

### 3.2.1. Experimental design

The first experiment comprised of two factors (i. watered; and ii. droughted) each at four levels of AMF inoculation treatments (A. Non-inoculated control plants; B. *G. intraradices* inoculated plants; C. *G. mossea* inoculated plants; and D. Mixed *G. intraradices* and *G. mossea* inoculated plants). This constitutes eight treatments which were laid out in a completely randomized design on the greenhouse benches. Each treatment consisted of 5 independent plants, replicated three times each, totalling 120 plants (8 treatments x 5 plants x 3 replicates). 20 g AMF inoculum was applied to each plant (10 g of each in the mixed AMF treatments). For the watered treatments, the plants were irrigated daily and the substrate moisture content was maintained between 40 to 60%. For the droughted treatment, the substrate moisture content was maintained between 20 to 40 % from week 4. The percentage of substrate moisture for each plant pot was determined using a MiniTrase time domain reflectometer (TDR) (Soilmoisture Equipment Corp, California, USA) and recorded as volumetric water content (%). The plants were harvested 8 weeks after inoculation with AMF and sowing of seeds for determination of dry shoot weights and dry root weights. All plants were fertilised weekly by adding 200 ml Hoagland nutrient solution (Hoagland and Anon, 1950),

modified by using 50% of normal $KH_2PO_4$ concentration (i.e. 0.5 mM final concentration).

The second experiment consisted of three factors (i. watered; ii. drought week 1; and drought week 2) each at four AMF inoculation treatments as outlined earlier (A. Non-inoculated control plants; B. *G. intraradices* inoculated plants; C. *G. mossea* inoculated plants; and D. Mixed *G. intraradices* and *G. mossea* inoculated plants). This constitutes 12 treatments which were laid out in a completely randomized design on the greenhouse benches. Each treatment consisted of 4 independent plants, replicated 3 times each, totalling 144 plants (12 treatments x 4 plants x 3 replicates). The watered plants were irrigated daily, while the plants subjected to drought stress had water withheld from them for one week (from week 6 to week 7) and two weeks (from week 5 to week 7). The plant tissues were harvested seven weeks after planting for determination of leaf relative water content, pigment content and proline concentrations.

In the first experiment, a total of 80 plants were analysed for SDW and RDW (10 plants per treatment), while 24 plants were analysed for root colonization (3 per treatment; excluding control plants) eight weeks after sowing the seeds. In the second experiment, a total of 60 plants were analysed (5 per treatment) seven weeks after inoculation and seeding.

### 3.2.2. Plant water status

To determine leaf relative water content (LRWC), fresh leaves were collected from each plant. Individual leaves were weighed on the balance to determine their fresh

weight, and immediately placed in 5 mM $CaCl_2$ solution for 24 h to regain full turgor, then weighed to determine their turgid weight. These leaves were dried in oven for 72 h at 70°C and weighed to determine the dry weight. LRWC was then calculated using the following formula as described by Sade *et al*. (2014):

$$\text{LRWC} = [(\text{fresh weight} - \text{dry weight})/ (\text{turgid weight} - \text{dry weight})] \times \frac{}{100} \quad 3.2$$

### 3.2.3. Pigment content

#### 3.2.3.1. Chlorophyll

The total chlorophyll content of the leaves was estimated according to Arnon (1949). 100 mg of leaf tissue was frozen in liquid nitrogen and macerated in a mortar with 2 mL cold ethanol. The homogenate was transferred to a microcentrifuge tube and to 200 µL of homogenate, 1 mL cold ethanol was added and the mixture was incubated 1 h at 4 ºC. The mixture was thereafter centrifuged at maximum speed for 5 min at 4 ºC. The supernatant was collected and used to determine chlorophyll content by measuring the optical density at a wavelength of 645 nm and 663 nm in a spectrophotometer against ethanol as a blank. The chlorophyll content was calculated as follows:

$$A_{663} = A_{663}\,\text{ChlA} + A_{663}\,\text{ChlB} \quad 3.3$$

$$A_{645} = A_{645}\,\text{ChlA} + A_{645}\,\text{ChlB}$$

For each chlorophyll, the absorbance $A = \varepsilon lC$, where $\varepsilon$ is the specific absorption coefficient of the chlorophyll considered, $l$ is th light path, 1 cm, and $C$ is the chlorophyll concentration.

For chlorophyll A, $\varepsilon$ values are 82.04 at 663 nm and 17.75 at 645 nm. For Chlorophyll B, $\varepsilon$ values are 9.27 at 663 nm and 45.6 at 645 nm (with $\varepsilon$ expressed in L/g cm)

$$C_a = 12.7A_{663} - 2.63A_{645} \quad (3.4)$$

$$C_b = 22.9A_{645} - 4.68A_{663}$$

Where $C_a$ is the chlorophyll A concentration and $C_b$ is the chlorophyll B concentration, both expressed in mg/L

### 3.2.3.2. Total carotenoid

Total carotenoid content of the leaves was estimated by the method described by Zakaria *et al*. (1979). 0.5 g of samples was homogenized in liquid nitrogen and saponified with 2.5 ml of 12% (w/v) alcoholic potassium hydroxide in a water bath at 60°C for 30 minutes (Lee *et al*., 2001; Kurilich *et al*., 2003). The saponified extract was transferred to a separating funnel containing 10 ml of petroleum ether and mixed well. The lower aqueous layer was then transferred to another separating funnel and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colourless. A small amount of anhydrous sodium sulphate was added to the petroleum ether extract to remove excess moisture and the final volume of the petroleum ether extract was noted. The sample absorbance was read in a spectrophotometer at 450nm with petroleum ether as a blank. The amount of total carotenoids (expressed as mg/g of the sample) was calculated using the formula:

$$\text{Amount of total carotenoids} = \frac{\text{A450 x Volume of sample x 100 x 4}}{\text{Weight of the sample}} \quad (3.5)$$

### 3.2.4. Proline concentration

The proline content was measured in both leaf and root tissues using a colorimetric assay as described by Bates *et al*. (1973). 100 mg of fresh plant tissue was homogenized in liquid nitrogen. 3% sulfosalicylic acid (5 µL/mg fresh weight) was added to the plant material, followed by homogenization and centrifugation at maximum speed in a Heraeus Megafuge 8R benchtop centrifuge (ThermoFisher Scientific, Germany). To 100 µL of the supernatant, 500 µL of reaction mixture containing 100 µL of 3% sulfosalicylic acid, 200 µl glacial acetic acid, 200 µL acidic ninhydrin (prepared by dissolving 1.25 g of Ninhydrin in a warm mixture of 30 mL glacial acetic acid and 20 mL of 6 M phosphoric acid) was added and incubated at 96 $^{o}$C for 60 min. The reaction was terminated on ice. Proline was extracted by adding 1 mL toluene to the reaction mixture and vortexing. The mixture was left on the bench for 5 min to allow the separation of the organic and water phases. The chromophore containing toluene was removed and the absorbance was measured at 520 nm wavelength using a 6800 Double Beam spectrophotometer (Jenway, UK) with toluene as a blank. The proline concentration was determined using a standard concentration curve and calculated on fresh weight basis as follows:

$$[(\mu g \text{ proline/ml} \times \text{ml toluene}) / 115.5\ \mu g/\mu mole]/[(g \text{ sample})/5] = \mu moles \text{ proline/g of fresh weight material.} \quad (3.6)$$

### 3.2.6. Statistical analysis

Data on percentage colonization and percentage leaf relative water content were subjected to angular transformation, and these as well as data on total chlorophyll, total carotenoids and proline concentration were subjected to analysis of variance

(ANOVA) using the general linear model procedure of SPSS (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA) with AMF inoculation and drought treatments being the sources of variation. Whenever there is a significant treatment effect, the means were separated using a Tukey test ($P = 0.05$). Statistical comparisons were considered significant at $P < 0.05$.

### 3.3. Assessment of reactive oxygen species and antioxidant activities

To determine the effect of tomato inoculation with AMF and application of drought stress on biochemical responses, hydrogen peroxide concentration and lipid peroxidation were assayed as indicators of oxidative stress, while catalase activity was measured as antioxidant response in the tissues of the plants. The level of lipid peroxidation in leaf and roots was measured by estimation of malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acid as described by Heath & Packer (1968). The experimental design and set up have been outlined earlier (section 3.2.1). A total of 60 plants were analysed (5 per treatment) seven weeks after inoculation and seeding.

#### 3.3.1. Hydrogen peroxide extraction and assay

Hydrogen peroxide ($H_2O_2$) was extracted from tomato leaf and root tissues by grinding 100 mg of plant tissue into fine powder in liquid nitrogen. The powder was homogenised in 450 µL of ice-cold sodium phosphate buffer (100 mM phosphate buffer, pH 7.0) containing the catalase inhibitor, hydroxylamine (1 mM), and then centrifuged at 12,000 x *g* for 15 min at 4 ºC. $H_2O_2$ levels were determined by spectrophotometry, using a modified ferrous ammonium sulphate/xylenol orange

method as described by Cheeseman (2006). About 60 µL of the supernatant was added to 600 µL of the assay mixture (or *eFox* reagent) containing 250 µM ferrous ammonium sulphate, 100 µM sorbitol, 100 µM xylenol orange in 25 µM $H_2SO_4$ and 1% ethanol. The absorbance was measured at 550 and 800 nm in a spectrophotometer, and the difference in absorbance between the two values was calculated. A standard curve was generated with standards prepared from 30% $H_2O_2$. The concentration of $H_2O_2$ in all standards was determined by measuring the absorbance at 240 nm and by calculating the actual $H_2O_2$ concentration using an extinction coefficient of 43.6 $M^{-1}$ $cm^{-1}$.

### 3.3.2. Lipid peroxidation assay

About 200 mg of plant tissues was finely ground in liquid nitrogen and homogenized in 4 mL of 0.1% trichloroacetic acid (TCA). The mixture was centrifuged for 15 min at 10,000 g. One mL of supernatant was mixed with 2 mL of 20% TCA and 2 mL of 0.5% TBA. The mixture was heated at 95 ˚C for 30 min and later cooled on ice. The absorbance of the coloured supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of MDA was calculated using Beer-Lambert's equation (extinction coefficient of MDA is 155 $mM^{-1}$ $cm^{-1}$).

$$\text{MDA (nmol g}^{-1}\text{ FW)} = [(A_{532} - A_{600}) \times V \times 1000/ \varepsilon] \quad 3.7$$

Where ε is the specific extinction coefficient, V is the volume of the homogenizing medium, W is the fresh weight of tissue, $A_{600}$ and $A_{532}$ are the absorbance at 600 nm and 532 nm respectively.

### 3.3.3. Antioxidant enzyme extraction and catalase activity

Crude plant tissue extracts for antioxidant enzyme assays was prepared as described by Elavarthi & Martin (2010). Two hundred (200) mg of plant tissue was ground to fine powder in liquid nitrogen using a precooled mortar and pestle. The powdered samples were then thoroughly homogenized in 1.2 mL of 0.2 M potassium phosphate buffer (pH 7.0 with 0.1 mM EDTA). The samples were centrifuged at 15,000 g for 20 min at 4˚C. The supernatant was removed, the pellet resuspended in 0.8 mL of the same buffer, and the suspension centrifuged for another 15 min at 15,000 x *g*. The combined supernatants were stored on ice and used to determine catalase (CAT) activity as described by Aebi & Lester (1984). The decomposition of $H_2O_2$ was followed as a decrease in absorbance at 240 nm in a UV/Vis spectrophotometer. The 3 mL assay mixture contained 2 mL leaf extract (diluted 200 times in 50 mM potassium phosphate buffer, pH 7.0) and 10 mM $H_2O_2$. The extinction coefficient of $H_2O_2$ (40 $mM^{-1}$ $cm^{-1}$ at 240 nm) was used to calculate the enzyme activity that was expressed in terms of millimoles of $H_2O_2$ per minute per gram fresh weight.

### 3.3.4. Statistical analysis

Data on hydrogen peroxide and MDA concentrations, as well as catalase activities, were subjected to analysis of variance (ANOVA) using the general linear model procedure of SPSS (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA) with AMF inoculation and drought treatments being the sources of variation. Whenever there is a significant treatment effect, the means were separated using a Tukey test ($P = 0.05$). Statistical comparisons were considered significant at $P < 0.05$.

## 3.4. Assessment of gene expression

The expression of ribosomal RNA genes of AMF (*ITS1 + 18S* rRNA of *G. intraradices* and *28S rRNA* of *G. mossea*), abscisic acid related genes (*LeNCED1*, encoding 9-cis-epoxycarotenoid dioxygenase and *Le4*, encoding dehydrin), tomato aquaporin genes (*SlPIP2;1*, *SlPIP2;5*, and *SlPIP2;7*) and AMF aquaporin genes (*GintAQPF1* and *GintAQPF2*) was assessed using real-time quantitative polymerase chain reaction.

The experimental design and set up have been outlined earlier (section 3.2.1). The experiment consisted of two factors (i. watered and ii. droughted). For the droughted plants, water was withheld from the plants until the substrate moisture content was 0%. A total of 32 plants were analysed (4 per treatment) eight weeks after inoculation and seeding.

### 3.4.1. RNA extraction and cDNA synthesis

Total RNA was extracted from the root of the plant using the ZR Plant RNA MiniPrep™ from Zymo Research (USA), according to manufacturer's protocol, and immediately stored at -70 $^{o}$C pending use. The integrity and purity of the extracted RNA was determined by resolution on a 1% agarose gel and nanodrop spectrophotometry on PCR max Lambda Spectrophotometer (UK) ($A_{260}/A_{280} > 1.8$) respectively. The concentration of RNA in all the samples was adjusted to 50 ng/µl to increase accuracy and enable comparison of gene expression measurements. 60 ng of RNA was used for cDNA synthesis using OneTaq® RT-PCR kit (New England Biolabs® Inc.) and following the manufacturer's instructions.

### 3.4.2. Drought related genes

All primer pairs used in this study were synthesized at Inqaba Biotechnical Industries (Pty) Ltd, South Africa (Table 3.2).

### 3.4.3. Quantitative real-time-polymerase chain reaction (qRT-PCR)

The qRT-PCR mixtures were set up with Luna® Universal qPCR Master Mix (New England Biolabs® Inc.) following the manufacturer's instructions. A 20 µl reaction contained 10 µl Luna Universal qPCR Mix, 0.5 µl each of forward and reverse primers (10 µM), 4 µl cDNA and 5 µl nuclease-free water. Each reaction was done in triplicates. The qRT-PCR was carried out in a LightCycler® 96 instrument (Roche Diagnostics GmbH, Germany), with the following cycling program: 300 s at 95 ºC, followed by 45 cycles of 95 ºC for 15 s, 60 ºC for 30 s, and 72 ºC for 30 s. This program was used for all the primers except *G. mossea*-specific and EF1-α primers, for which the annealing temperature was kept at 62 ºC. The threshold cycle (Ct) was calculated by the LightCycler® 96 application software to indicate significant fluorescence signals rising above background during the early cycles of exponential amplification phase of the PCR amplification process. The melting curve of the amplified product was examined for each reaction to prove that only the desired PCR product has been amplified and to rule out the possibility of primer-dimers contributing to the amplification signal. A 2% agarose gel electrophoresis was also used to confirm that only a single PCR amplicon of expected size was produced.

**Table 3. 2: Genes selected for expression profiling and their primers sequences**

| Target Organism | Primer Sequence | Target Gene | Reference |
|---|---|---|---|
| **Reference Housekeeping Genes** | | | |
| Tomato | TTGCTTGCTTTCACCCTTGG (F)<br>TTGGCACCAGTTGGGTCCTT (R) | Elongation factor-1α | Ruiz-Lozano *et al*. (2015) |
| **AMF Genes** | | | |
| *G. intraradices* | GAGACCATGATCAGAGGTCAGGT (F)<br>GGTCATTTAGAGGAAGTAAAAGTCGTAAC (R) | ITS1 + 18S rRNA | Alkan *et al*. (2006) |
| *G. mossea* | GAAGTCAGTCATACCAACGGGAA (F)<br>CTCGCGAATCCGAAGGC (R) | 28S rRNA gene | Alkan *et al*. (2006) |
| **Abscisic Acid Related Genes** | | | |
| Tomato | ACCCACGAGTCCAGATTTC (F)<br>GGTTCAAAAAGAGGGTTAGC (R) | *LeNCED1* | Ruiz-Lozano *et al*. (2015) |
| Tomato | ACTCAAGGCATGGGTACTGG (F)<br>CCTTCTTTCTCCTCCCACCT (R) | *Le4* | Ruiz-Lozano *et al*. (2015) |
| **Plant Aquaporin Genes** | | | |
| Tomato | ACGTACCCGTGTTGGCACCTCTTCC (F)<br>ATGTTCGTCCCACGCCTTGTCACC (R) | *SlPIP2;1* | Li *et al*. (2016) |
| Tomato | ATTCCCATATCCCTGTGTTGGCTCC (F)<br>AGCTGCAGCTCTCAAAATGTATTGG (R) | *SIPIP2;7* | Li *et al*. (2016) |
| Tomato | GTCCTCTTCCAGCCATCCA (F)<br>ACCACTGAGCACAATGTTACCG (R) | *SIPIP2;5* | Li *et al*. (2016) |
| **Fungal Aquaporin Genes** | | | |
| *G. intraradices* | CATTTGGGCTCCAATCTCTGGAGG (F)<br>CTCCATCTGCAAGTAAGGTTGCTG (R) | *GintAQPF1* | Li *et al*. (2013) |
| *G. intraradices* | GAACAAGAGGAGCACCAGCCACTG (F)<br>CCACTAACTGCAATACCCAAAGCG (R) | *GintAQPF2* | Li *et al*. (2013) |

F = Forward primer R = Reverse primer

### 3.4.4. Relative gene expression

Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001). Expression values were normalized using the reference genes for tomato elongation factor-1α.

### 3.4.5. Statistical analysis

The ΔCt values were log transformed to meet the assumptions of a normal distribution, and the values were subjected to analysis of variance (ANOVA) using the general linear model procedure of SPSS (SPSS 16.0 for Windows, SPSS Inc., Chicago, IL, USA) with AMF inoculation and drought treatments being the sources of variation. Whenever there is a significant treatment effect, the means were separated using a least significant difference (LSD) ($P = 0.05$). Statistical comparisons were considered significant at $P < 0.05$.

# CHAPTER FOUR

# RESULTS

## 4.1. Root colonization and plant biomass

The effect of tomato inoculation with AMF on plant growth was assessed. Both *G. intraradices* and *G. mossea* were able to colonize the root of the tomato plants (Figure 4.1) and colonization was significantly enhanced by the elimination of phosphate from the nutrient solution. Additionally, there was a significant effect of inoculation with *G. intraradices* and *G. mossea* on growth of tomato plants (Figure 4.2) with and without the addition of phosphate to the nutrient solution.

### 4.1.1. Root colonization

The effect of inoculation with AMF was dependent on addition of phosphate in the media for root colonization, as indicated by significant interaction (AMF*Phosphate) [$F(7, 32) = 39.779$, $P \leq 0.001$]. Root colonization was significantly higher in all the three AMF treatments where phosphate was absent in the nutrient solution (Table 4.1). The highest percentage colonization (76.67%) was obtained in the mixed AMF inoculated plants without phosphate addition, and the lowest percentage colonization (42.67%) was obtained in the mixed AMF treated plants with phosphate addition to the nutrient solution. Although, colonization by *G. mossea* was significantly higher when phosphate was added to the nutrient solution, there was no significant difference

between colonization by either AMF species when phosphate was absent in the nutrient solution.

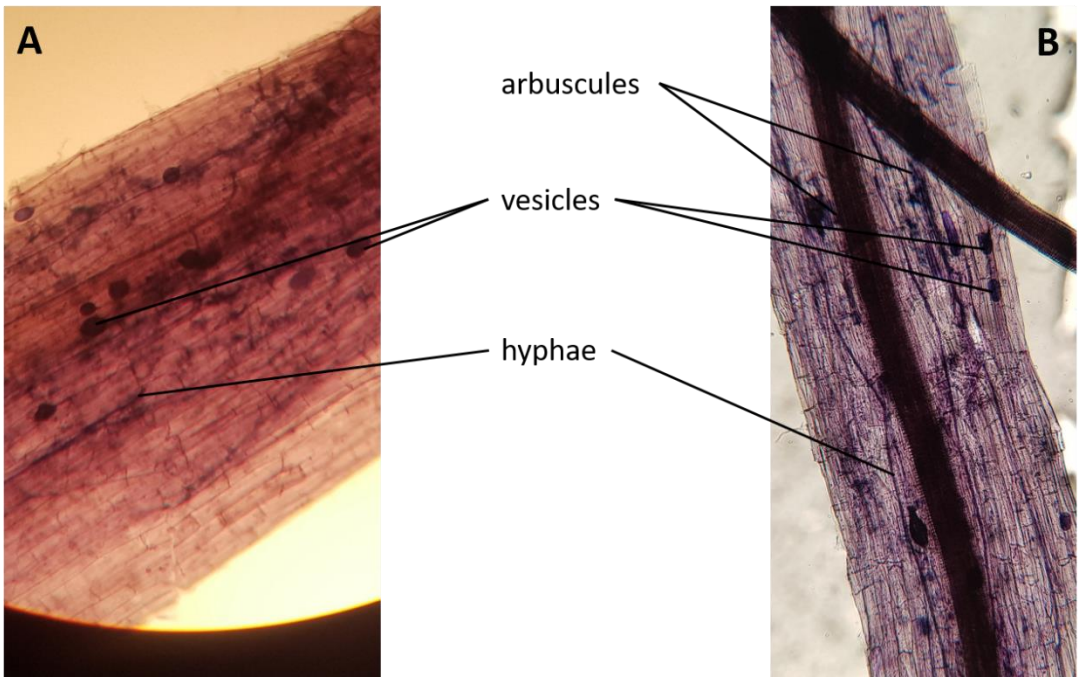


**Figure 4. 1: Colonization of tomato root by *G. intraradices* (A) and *G. mossea* (B) at week 7 after inoculation and seeding**

**Table 4. 1: Root colonization (%) by *G. intraradices* and *G. mossea* with and without phosphate in the nutrient solution at week seven after inoculation and seeding**

| Mycorrhiza treatments | Root Colonization (%)* | |
|---|---|---|
| | +P | -P |
| Control | 0a | 0a |
| *G. intraradices* | 47.33b | 63.33d |
| *G. mossea* | 56.0c | 68.67d |
| Mixed | 42.67b | 76.67e |

* presented as % of total root fragments. +P indicates addition of 0.5mM $KH_2P0_4$ per pot, -P indicates no $KH_2PO_4$ addition. Means with different letters are significantly different at P = 0.05 (n = 5)

### 4.1.2. Effect of AMF inoculation on growth

To determine the effect of AMF inoculation on growth, the plants were grown with AMF inoculation and all required mineral nutrients were supplied including phosphate at the first instance of fertilization, i.e. one week after plant germination. Subsequently, phosphate was omitted from the nutrient solution and plant biomass was determined after 5 weeks of cultivation. Inoculation with either *G. intraradices* or *G. mossea* significantly increased dry shoot and root weights. However, co-inoculation with both AMF resulted in a significantly reduced dry shoot and root weights (Figure 4.2 and 4.3).

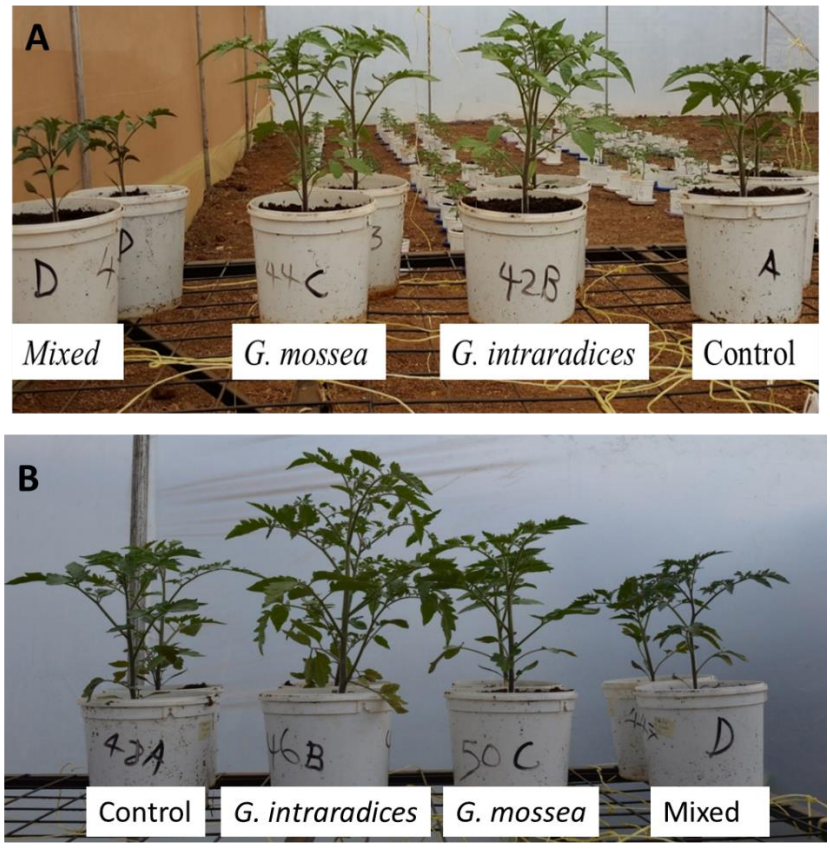


**Figure 4. 2: Effect of inoculation with AMF on growth of tomato (var ANNA F1) at weeks 5 and 6**

A two-way ANOVA was conducted to examine the effect of mycorrhiza inoculation and phosphate addition on growth enhancement in terms of dry root and shoot weights (figure 4.4). There was a statistically significant interaction between the effect of mycorrhiza inoculation and phosphate addition on dry shoot [$F(7, 112) = 34.555$, $P \leq 0.001$] and root [$F(7, 112) = 10.433$, $P \leq 0.001$] weights.

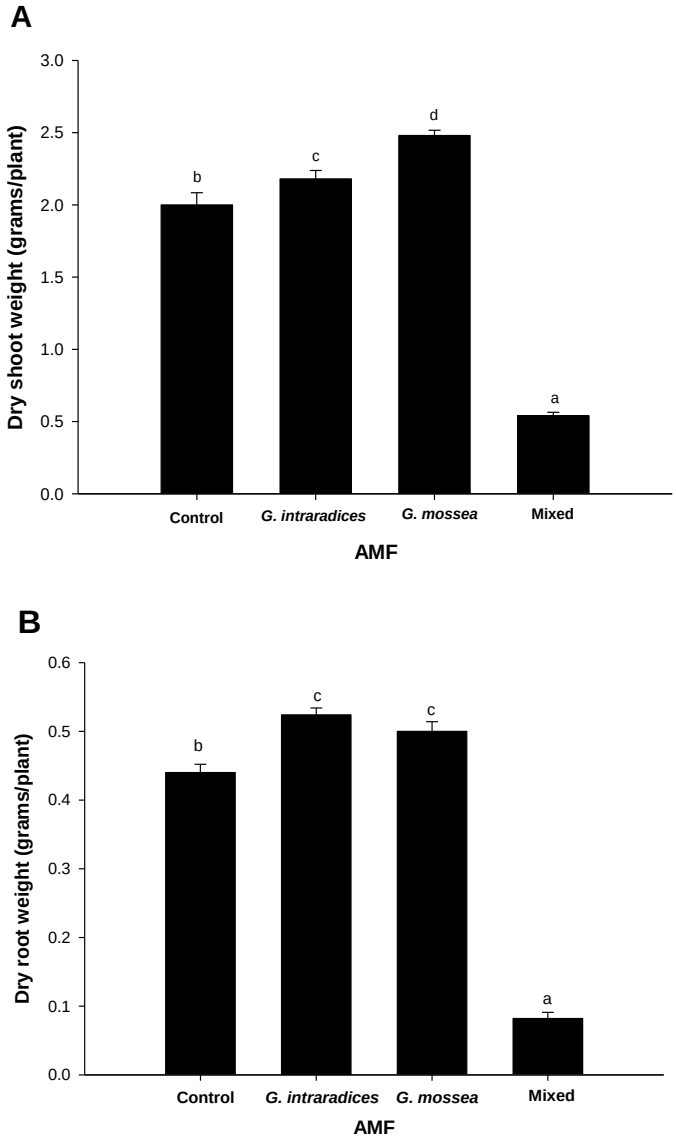


**Figure 4. 3: Effects of inoculating tomato with AMF on dry shoot weight (A) and dry root weight (B) at week 5 after seeding.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different ($P = 0.05$, $n = 5$)

Regardless of phosphate addition, inoculation with either *G. intraradices* or *G. mossea* increased plant tissue dry weights. The highest dry weights were obtained when inoculation with either of the AMFs was combined with addition of phosphate. Interestingly, inoculation with AMF without phosphate resulted in higher dry shoot and root weights than non-inoculated plants with phosphate addition. Co-inoculation with both AMFs resulted in significantly lower dry shoot and root weights compared to the control and inoculation with either AMFs in the absence of phosphate. With the addition of phosphate however, co-inoculation resulted in higher dry weights (Figure 4.4).

### 4.1.3. Phosphorus concentration

Analysis of leaf phosphorus showed that *G. intraradices* and *G. mossea* inoculated plants had the highest phosphorus concentrations (0.69 and 0.63 %, respectively), while the non-inoculated plants (0.54 %) and plants that were co-inoculated with both AMFs (0.52 %) had the lowest phosphorus concentration, when phosphate was omitted from the nutrient solution (Table 4.2). Plants inoculated with *G. intraradices* or *G. mossea* but without phosphate had higher phosphorus concentration compared to non-inoculated plants with added phosphate.

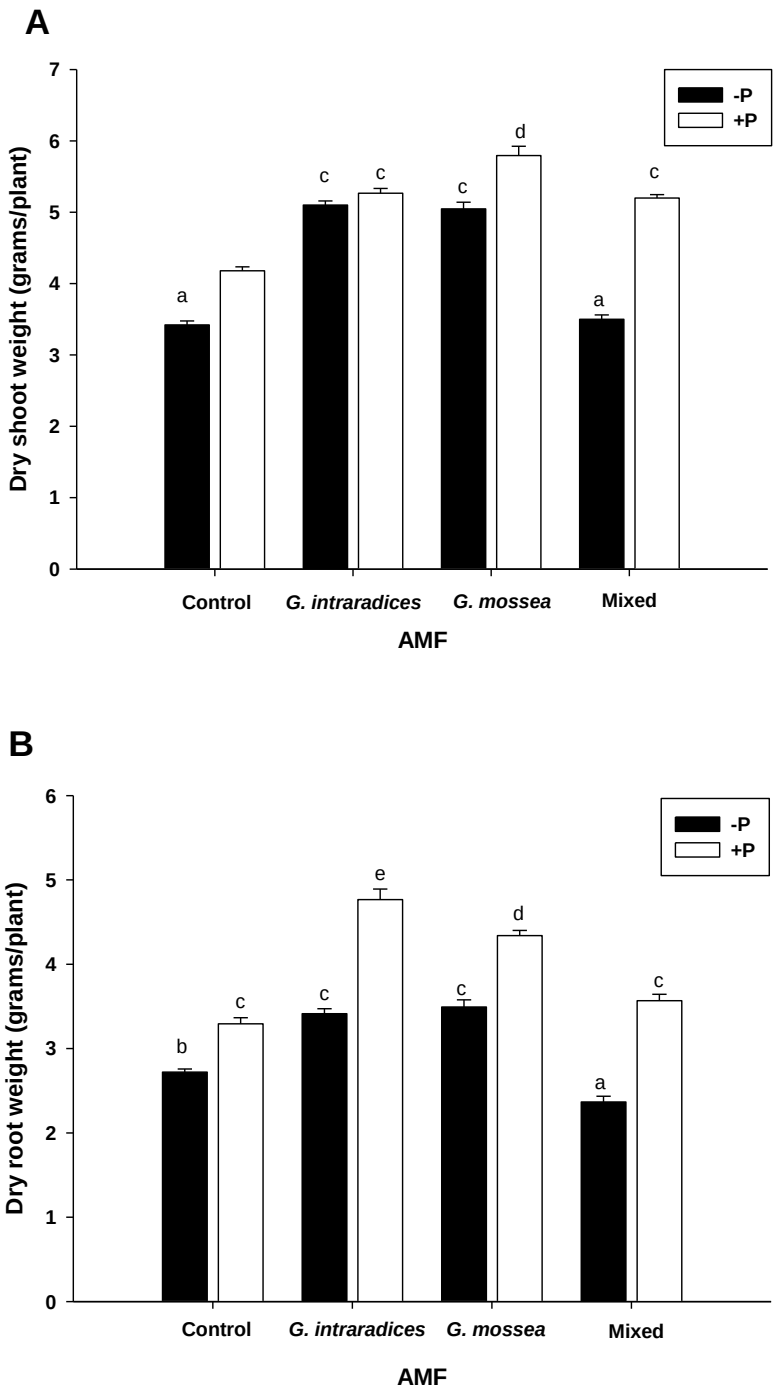


**Figure 4. 4: Effects of inoculating tomato with AMF and addition of phosphate on dry shoot weight (A) and dry root weight (B) at week 7 after seeding.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 15)

**Table 4. 2: Effect of inoculating tomato with *G. intraradices* and *G. mossea* and addition of phosphate on leaf phosphorus concentration (%) at 7 weeks after seeding**

| Mycorrhiza treatments | P concentration (%) | |
|---|---|---|
| | +P | -P |
| Control | 0.64b | 0.54a |
| *G. intraradices* | 0.84bc | 0.69b |
| *G. mossea* | 0.66b | 0.63c |
| Mixed | 0.87c | 0.52a |

+P indicates addition of 0.5mM $KH_2P0_4$ per pot, -P indicates no $KH_2PO_4$ addition.
Means with different letters indicate significant difference at P = 0.05 (n = 5)

## 4.2. Leaf relative water content (LRWC), pigment content and proline concentration

In the first study, tomato plants were cultivated under well-watered (40-60% substrate volumetric moisture content) and drought stress conditions (20-40%). The percentage root colonization, as well as the dry root weight (DRW) and dry shoot weight (DSW), were determined eight weeks after inoculation and sowing.

In the second study, seven weeks old tomato plants, were either maintained under well-watered conditions, or subjected to drought for one week (weeks 6-7) or subjected to drought for 2 weeks (weeks 5-7). Root colonization, leaf relative water content, chlorophyll and carotenoids concentration and proline concentration were determined at the seventh week after inoculation and sowing.

### 4.2.1. Root colonization and plant biomass

Root colonization by AMF was independent of drought stress application as indicated by two-way interaction between AMF and drought (AMF*Drought) [F (5, 18) = 0.827, P= 0.453] and [F (8, 27) = 0.519, P = 0.723] consistently in studies one and two, respectively. There was a significant increase in percentage root colonization by AMF due to drought stress in study one ($P \leq 0.001$) (Table 4.3) but no significant difference in percentage root colonization due to drought stress was observed in study two (P= 0.253) (Table 4.4). In both studies, there was no significant difference between root colonization by *G. mossea* and *G. intraradices*. The combined application of the two AMF species however appears to have synergistic effect as indicated by significantly increased root colonization.

**Table 4. 3: Root colonization (%) by *G. intraradice*s and *G. mossea* under watered and drought stress conditions at the eight week after inoculation**

| Water stress | % moisture content | AMF inoculation | Root colonization (%)* |
|---|---|---|---|
| **Watered** | 40-60 | *G. intraradices* | 50.0a |
| | | *G. mossea* | 52.25a |
| | | Mixed | 62.5b |
| **Droughted** | 20-40 | *G. Intraradices* | 64.25b |
| | | *G. mossea* | 65.75b |
| | | Mixed | 79.75c |

* presented as % of total root fragments. Values with different letters indicate significant difference at P = 0.05 (n = 5)

**Table 4. 4: Root colonization (%) by *G. intraradices* and *G. mossea* under watered and continuous drought stress conditions at the seventh week after inoculation**

| Water stress | % moisture content | AMF inoculation | Root colonization (%)* |
|---|---|---|---|
| **Watered** | 40-60 | *G. intraradices* | 47.25a |
| | | *G. mossea* | 51.75a |
| | | Mixed | 62.5b |
| **Drought week 1** | 20-40 | *G. Intraradices* | 51.75a |
| | | *G. mossea* | 55.0a |
| | | Mixed | 62.5b |
| **Drought week 2** | 0-20 | *G. intraradices* | 50.75a |
| | | *G. mossea* | 53.25a |
| | | Mixed | 61.5b |

* presented as % of total root fragments. Values with different letters indicate significant difference at P = 0.05 (n = 5)

The effect of inoculation with AMF on dry shoot weight (DSW) was dependent on drought stress application as indicated by a significant interaction (AMF*Drought) ($F(7, 72) = 23.113$, $P \leq 0.001$). SDW in the AMF inoculated plants was significantly ($P \leq 0.001$) higher than in the non-inoculated plants under well-watered and drought stress conditions. In all treatments, except the mixed AMF inoculation, drought stress significantly ($P \leq 0.001$) reduced DSW. Under watered conditions, *G. intraradices* inoculated plants had the highest DSW (12.28g), followed by plants inoculated with *G. mossea* (11.77g), non-inoculated plants (9.84g), and plants inoculated with

combination of both AMF (9.41g). Under drought stress, plants inoculated with *G. mossea* had the highest DSW (9.85g), followed by those inoculated with *G. intraradices* (9.66g), mixed AMF (8.78) and non-inoculated plants (6.63g) (Figure 4.5A).

The effect of inoculation with AMF on dry root weight was independent of drought stress application as indicated by the interaction (AMF*Drought) [F (7, 72) = 1.783, P = 0.158]. AMF inoculation had a similar effect on root dry weight (DRW) as in DSW (P = 0.017). Under watered and drought conditions, *G. mossea* inoculated plants had the highest DRW (6.48g and 6.04g, respectively), followed by *G. intrarardices* (5.90g and 5.71g, respectively), mixed AMF (4.27g and 4.13g respectively) and non-inoculated plants (4.13g and 4.16g respectively) (Figure 4.5B).

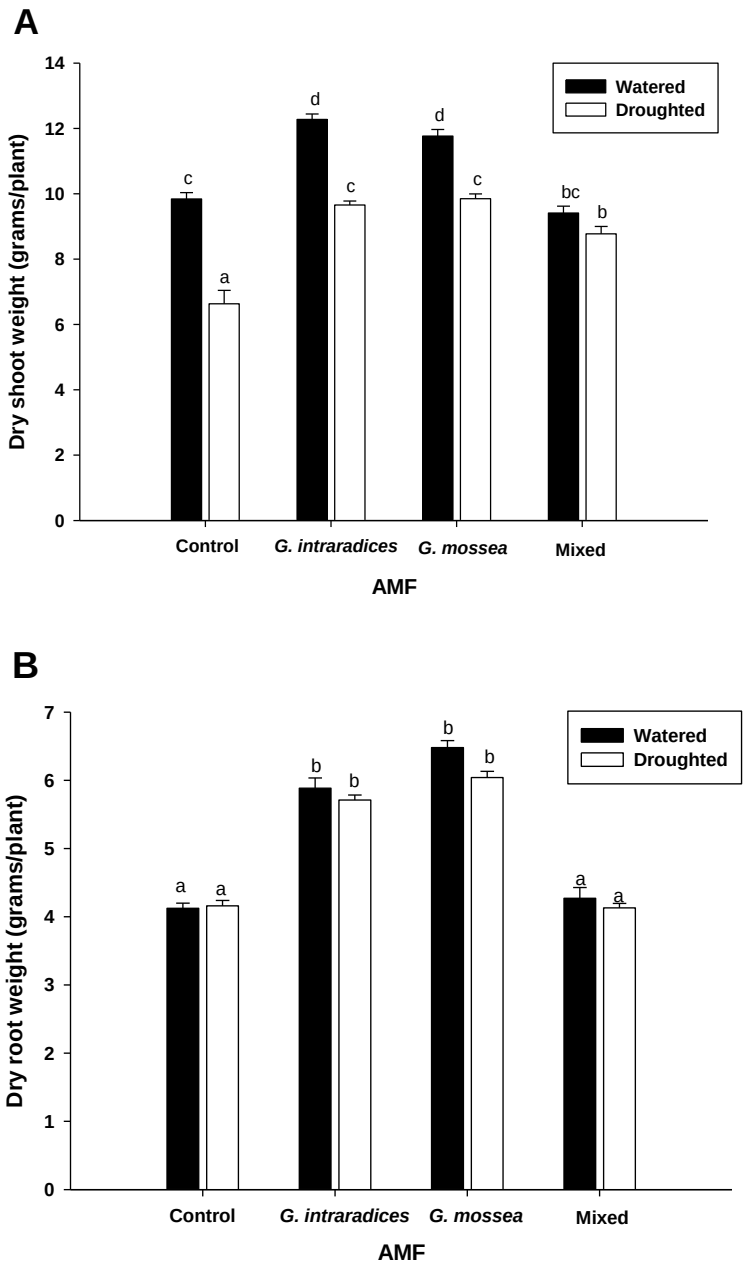


**Figure 4. 5: Effect of AMF inoculation and drought on dry shoot weight (A) and dry root weight (B) in eight weeks old tomato plants.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 10)

### 4.2.2. Leaf relative water content (LRWC)

The effect of inoculation of tomato with AMF on LRWC was dependent on drought stress application as indicated by a significant interaction (AMF*Drought) ($F(11, 48) = 9.059$, $P \leq 0.001$). Drought stress significantly reduced LRWC regardless of AMF inoculation ($P \leq 0.001$). Plants inoculated with AMF had significantly higher LRWC compared to control plants after two weeks of drought stress exposure ($P \leq 0.001$) (Figure 4.6).

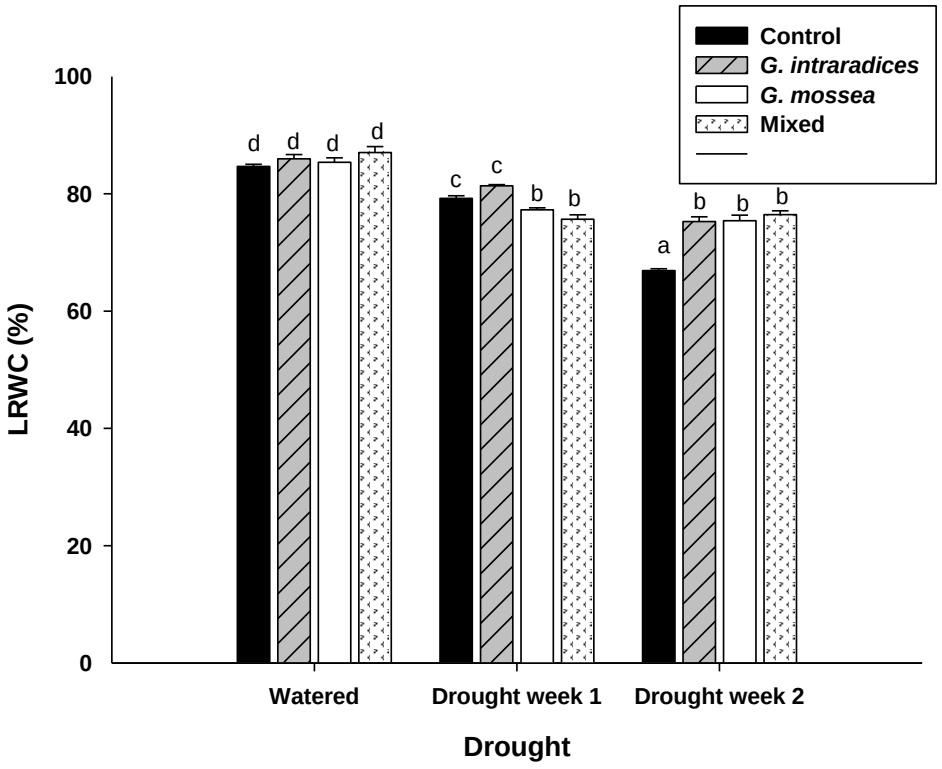


**Figure 4. 6: Effect of AMF on leaf relative water content (LRWC) of seven weeks old tomato grown under watered and drought stress conditions for one and two weeks.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different ($P = 0.05$, $n = 5$)

### 4.2.3. Leaf pigment content

The effect of inoculation with AMF on total chlorophyll [F (11, 48) = 0.246, P= 0.959] and total carotenoids [F (11, 48) = 0.183, P= 0.980] was independent on drought stress application as indicated by an insignificant interaction (AMF*Drought). Drought stress significantly reduced total chlorophyll concentration ($P \leq 0.001$) and total carotenoid concentration ($P = 0.004$). Plants inoculated with AMF had significantly higher chlorophyll concentration (Figure 4.7A) and total carotenoid concentration (Figure 4.7B) compared to control plants after two weeks of drought stress.

### 4.2.4. Proline concentration

The effect of inoculation with AMF on leaf proline [F (11, 48) = 8.359, $P \leq 0.001$] and root proline [F (7, 24) = 3.974, P = 0.020] was dependent on drought stress as indicated by a significant interaction (AMF*Drought). Drought stress significantly increased proline concentration in both leaf and root tissues ($P \leq 0.001$). However, proline concentration was significantly higher in leaves (Figure 4.8A) and roots (Figure 4.8B) of plants inoculated with AMF after two weeks of drought stress compared to non-inoculated control plants.

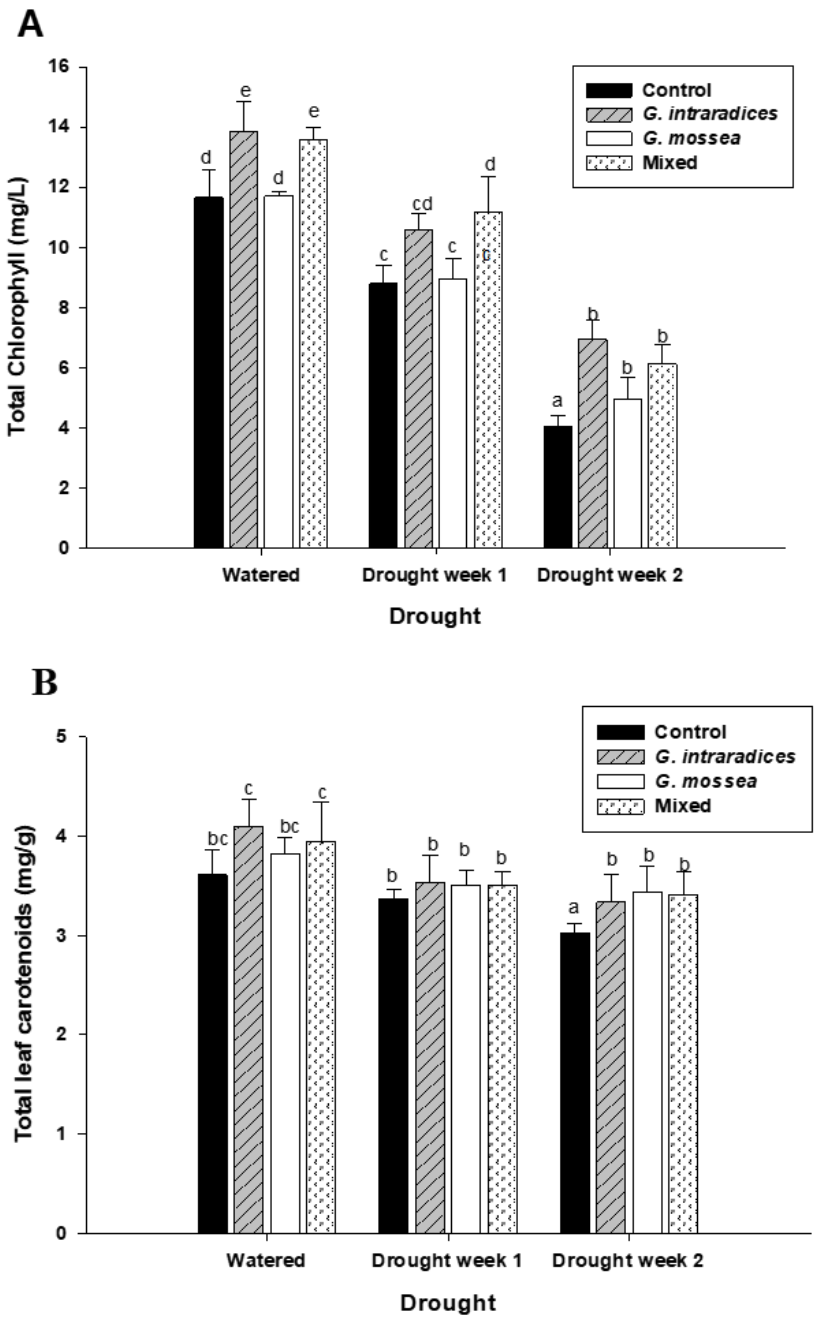


**Figure 4. 7: Effect of AMF on leaf total chlorophyll (A) and total carotenoids (B) concentration of seven weeks old tomato plants grown under watered and drought stress for one and two weeks.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 5)

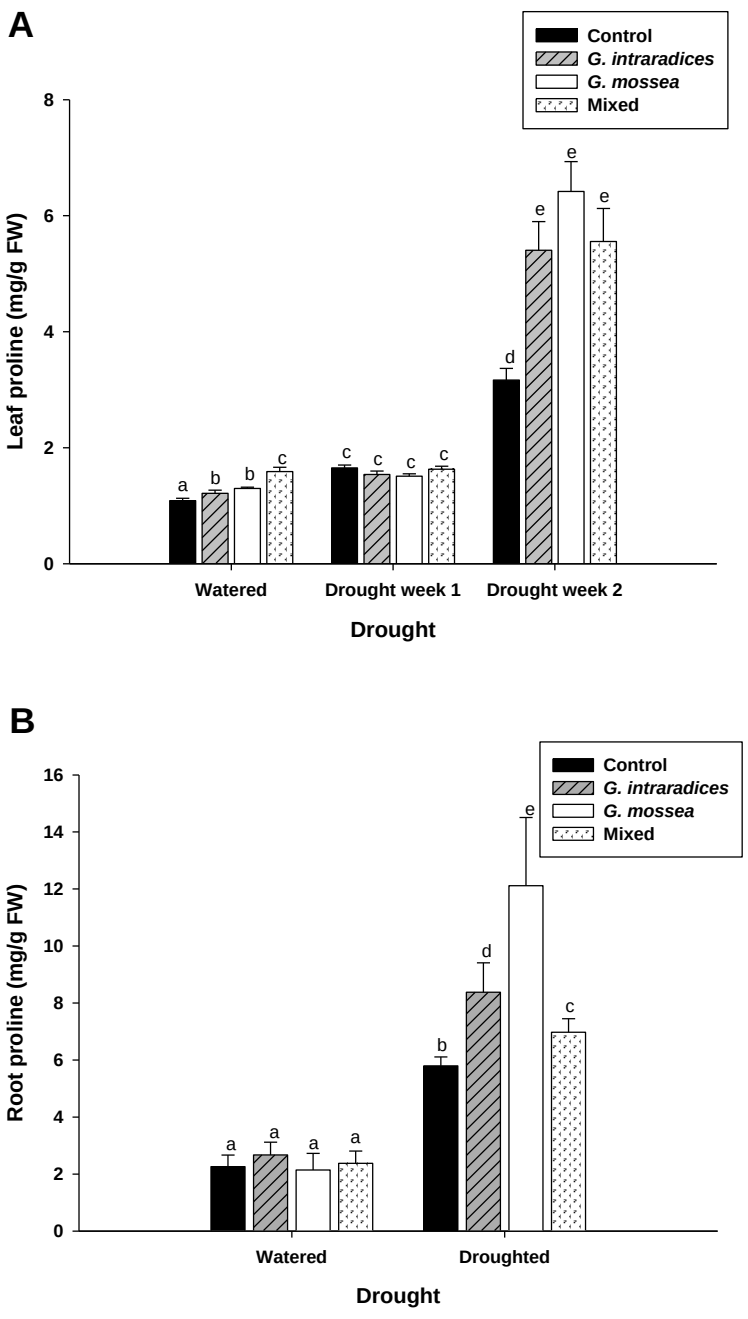


**Figure 4. 8: Effect of AMF on leaf (A) and root proline (B) concentration of seven weeks old tomato plants grown under watered and drought stress for one and two weeks.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 5)

## 4.3. Reactive oxygen species and antioxidant activities

### 4.3.1. Hydrogen peroxide

The effect of inoculation with AMF on leaf $H_2O_2$ was dependent on drought stress [$F(11, 48) = 16.224$, $P \leq 0.001$], but drought stress did not influence the effects of AMF on root $H_2O_2$ [$F(7, 24) = 2.340$, $P = 0.099$]. The accumulation of $H_2O_2$ in leaf and root tissues was significantly increased by drought stress at weeks 1 and 2 ($P \leq 0.001$). Under watered and drought stress conditions, the level of $H_2O_2$ in the leaves was significantly higher in plants inoculated with *G. mossea* and mixed AMF, while plants inoculated with *G. intraradices* had lower leaf $H_2O_2$ concentration at week 2 of drought stress (Figure 4.9A). In the roots, *G. intraradices* inoculated plants had the lowest $H_2O_2$ concentration under drought stress (Figure 4.9B).

### 4.3.2. Lipid peroxidation

Oxidative damage to lipids was measured by estimation of malondialdehyde (MDA), a by-product of lipid peroxidation. The effect of inoculation with AMF on leaf MDA concentration did not depend on drought stress [$F(11, 48) = 0.527$, $P = 0.785$]. Drought stress significantly increased leaf MDA concentration regardless of AMF inoculation ($P \leq 0.001$). Inoculation with AMF also significantly affected MDA concentration under watered and drought stress ($P = 0.008$). Compared to the control, *G. intraradices* inoculated plants had lower MDA concentration under watered and drought stress conditions, while plants inoculated with *G. mossea* and mixed AMF had higher MDA concentrations (Figure 4.10A).

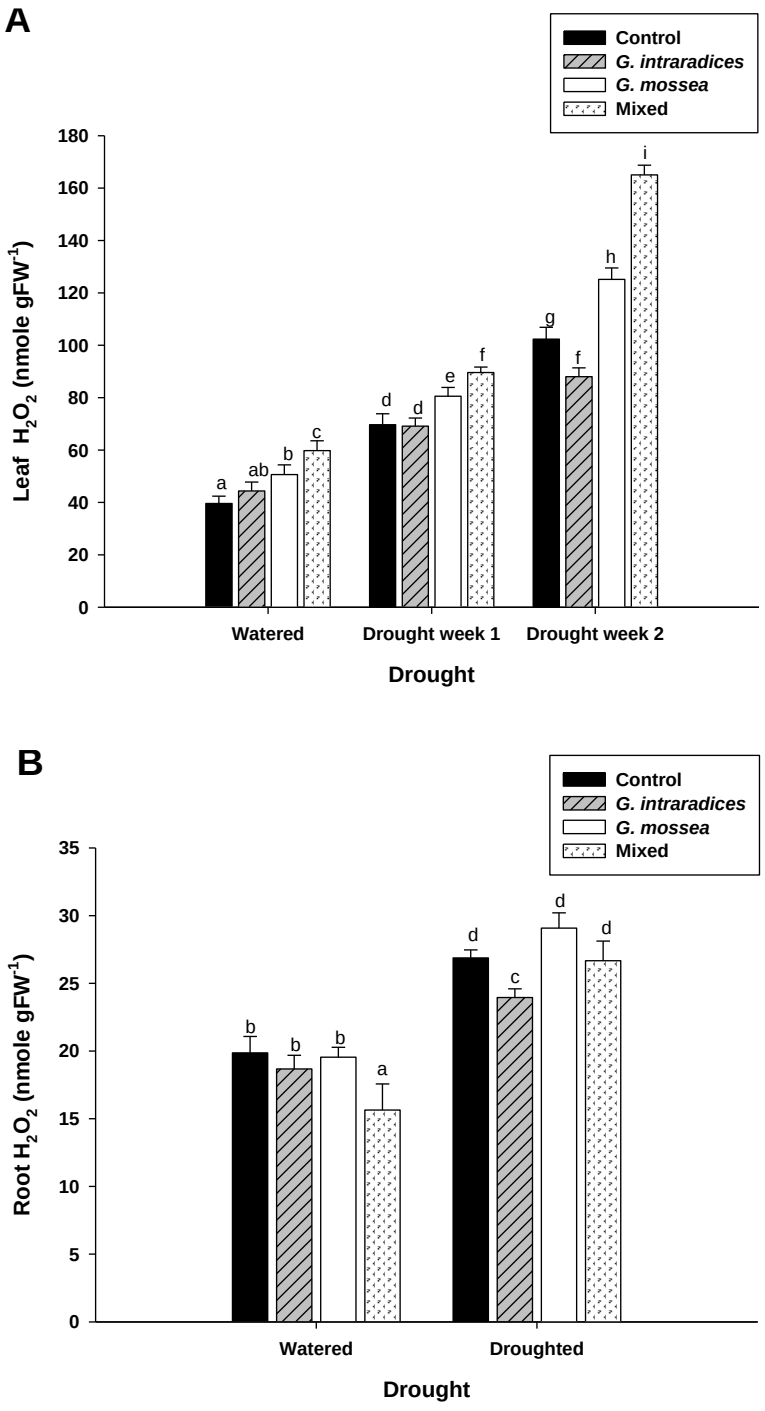


**Figure 4. 9: Effect of AMF on leaf (A) and root (B) $H_2O_2$ concentration of seven weeks old tomato plants grown under watered and drought stress for one and two weeks.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 5)

In the roots, the effect of inoculation with AMF on MDA concentration was dependent on drought stress [F (7, 24) = 2.975, P = 0.052]. Drought stress increased MDA concentration in all treatments ($P \leq 0.001$). Inoculation with AMF also had a significant effect on root MDA concentrations (P = 0.004). Both *G. intraradices* and *G. mossea* inoculated plants had significantly higher MDA concentrations under drought stress compared to the control (Figure 4.10B).

### 4.3.3. Antioxidant enzyme activities

The effect of inoculation with AMF on leaf catalase activity was dependent on drought stress [F (11, 24) = 4.647, P = 0.003]. Leaf catalase activity was significantly enhanced by drought stress in AMF inoculated plants compared to non-AMF inoculated plants (Figure 4.11A).

In the roots, the effect of inoculation with AMF was also dependent on drought stress as indicated by a significant (AMF*Drought) interaction (F (7,24) = 3.188, P = 0.042). Root catalase activity was significantly higher in *G. intraradices* and *G. mossea* inoculated plants compared to control plants (Figure 4.11B).

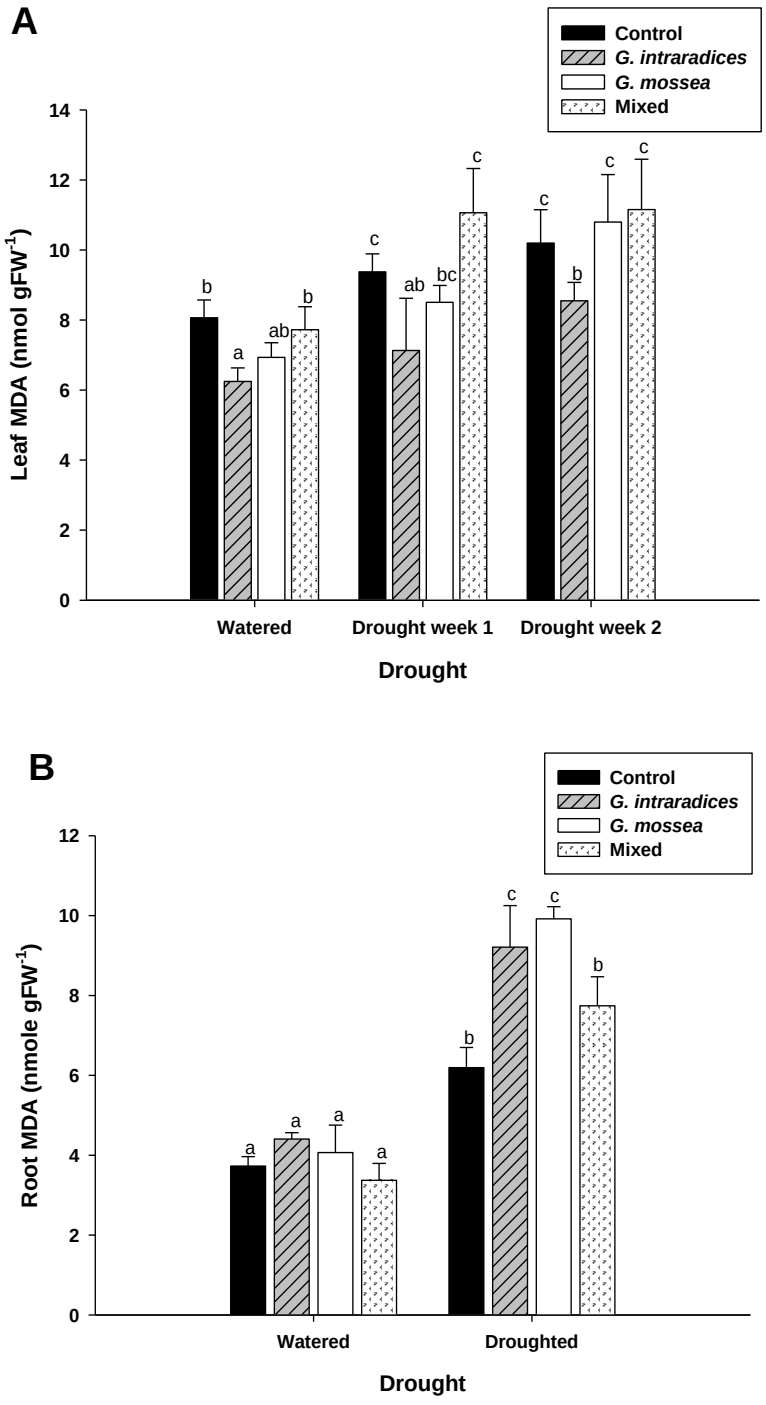


**Figure 4. 10: Effect of AMF on leaf (A) and root (B) MDA concentration of tomato plants grown under watered and drought stress for one and two weeks.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 5)

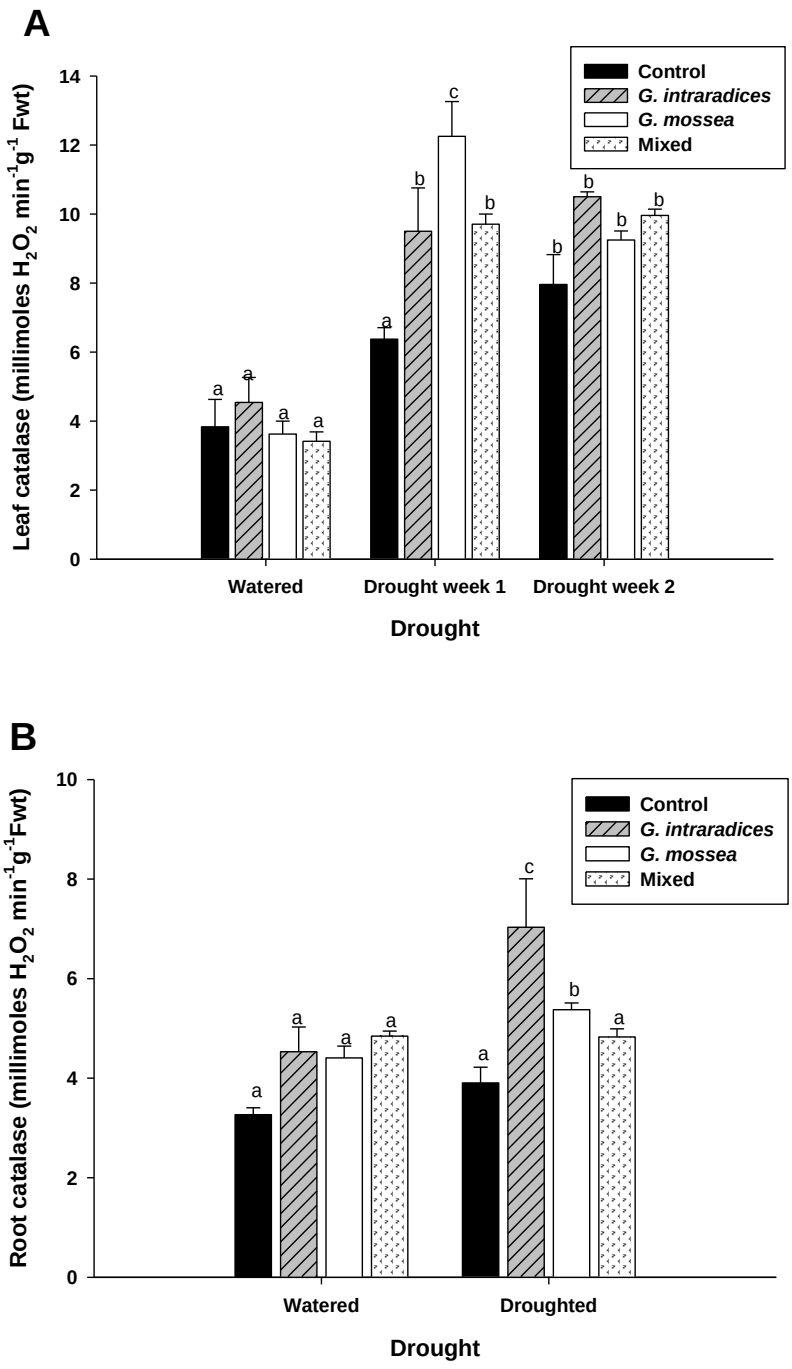


**Figure 4. 11: Effect of AMF on catalase activity in leaf (A) and root (B) of seven weeks old tomato plants grown under watered and drought stress for one and two weeks.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 5)

## 4.4. Gene expression

### 4.4.1. Expression of arbuscular mycorrhizal fungi ribosomal RNA genes

There was a significant effect of drought stress and inoculation with *G. intraradices* on expression of ribosomal RNA gene (*ITS1+18SrRNA*) in single and mixed inoculation [F (3, 28) = 4.982, P = 0.007). Post hoc comparison using LSD indicates that, expression of *G. intraradices* ribosomal gene was enhanced in the mixed inoculation (P = 0.004). While the gene expression was unaffected by drought stress in single inoculation, drought stress significantly downregulated the gene expression in mixed inoculation (P = 0.004) (Figure 4.12A). Conversely, drought and inoculation with *G. mossea* had no effect on expression of the ribosomal RNA gene (*28SrRNA*) in single and mixed inoculation [F (3, 28) = 0.658, P = 0.709] (Figure 4.12B).

### 4.4.2. Expression of tomato abscisic acid related genes

There was a significant effect of drought stress and AMF inoculation on expression of the tomato ABA-biosynthesis gene, *LeNCED1*, encoding 9-cis-epoxycarotenoid dioxygenase (Thompson *et al.*, 2000) [F (7, 56) = 3.838, P = 0.002]. Post hoc comparison using LSD test indicated that the gene expression was significantly upregulated by drought stress in non-AMF plants. In plants inoculated with *G. intraradices* and mixed inoculation, there was no significant difference in gene expression. In plants inoculated with *G. mossea*, the gene expression was significantly reduced (Figure 4.13A).

The expression of ABA-responsive marker gene, *Le4*, encoding dehydrin (Kahn *et al.*, 1993), was significantly affected by drought stress and AMF inoculation [F (7, 56) =

4.497, P ≤ 0.001]. The expression of the gene was significantly upregulated by drought stress in non-AMF plants and, but not significantly affected by drought stress in AMF plants (Figure 4.13B).

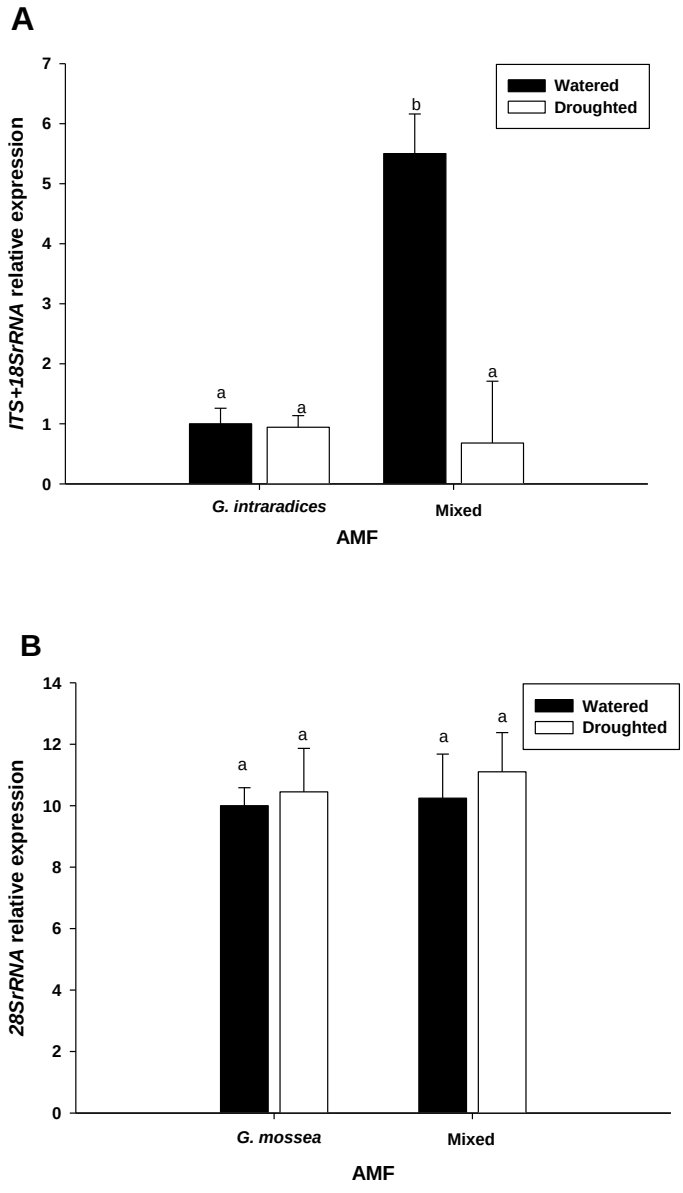


**Figure 4. 12: Effect of drought on expression of ribosomal RNA genes of *G. intraradices* (*ITS+18SrRNA*) (A) and *G. mossea* (*28SrRNA*) (B) in single and mixed inoculations.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 4)

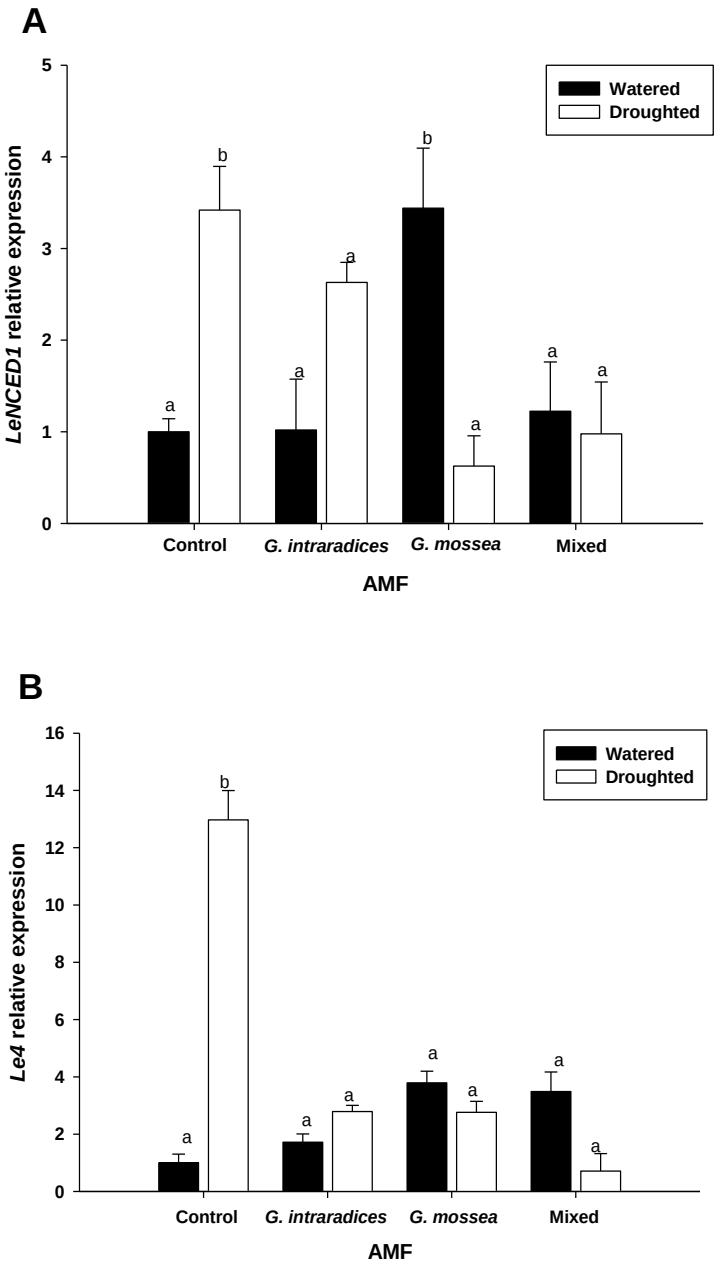


**Figure 4. 13: Effect of AMF on expression of abscisic acid-biosynthesis (*LeNCED1*) (A) and responsive marker gene (*Le4*) (B) in the root of seven weeks old tomato plants grown under watered and drought stress conditions.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 4)

### 4.4.3. Expression of tomato aquaporin genes

Drought stress and inoculation with AMF significantly affected the expression of all three tomato aquaporin genes, *SlPIP2;1* [F (7; 56) = 2.387, P = 0.33] (Figure 4.14A), *SIPIP2;5* [F (7; 56) = 2.292, P = 0.40] (Figure 4.14B) and *SlPIP2;7* [F (7; 56) = 5.166, P ≤ 0.001] (Figure 4.14C).

Expression of the each of the aquaporin genes was significantly enhanced by drought stress in non-AMF plants. In plants inoculated with AMF however, expression of the aquaporin genes was either not significantly affected, or downregulated by drought stress. Under watered conditions, expression of *SlPIP2;7* was significantly enhanced in plants inoculated with *G. mossea* or mixed AMF, while the expression of *SlPIP2;5* was significantly increased in plants inoculated with mixed AMF (Figure 4.14).

### 4.4.4. Expression of AMF aquaporin genes

There was a significant effect of drought and inoculation with AMF on expression of both AMF aquaporin genes *GintAQPF1* [F (5; 42) = 9.983, P ≤ 0.001) (Figure 4.15A) and *GintAQPF2* [F (5; 42) = 6.848, P ≤ 0.001] (Figure 4.15B).

Drought stress significantly downregulated the expression of both AMF aquaporin genes in all AMF treatments, except *G. mossea* inoculated plants, where the expression of *GintAQPF2* was not significantly affected. Under watered condition, expression of *GintAQPF1* was significantly higher in *G. intraradices* inoculated plants than *G. mossea* inoculated plants, while the expression of *GintAQPF2* was highest in mixed inoculation treatments (Figure 4.15).

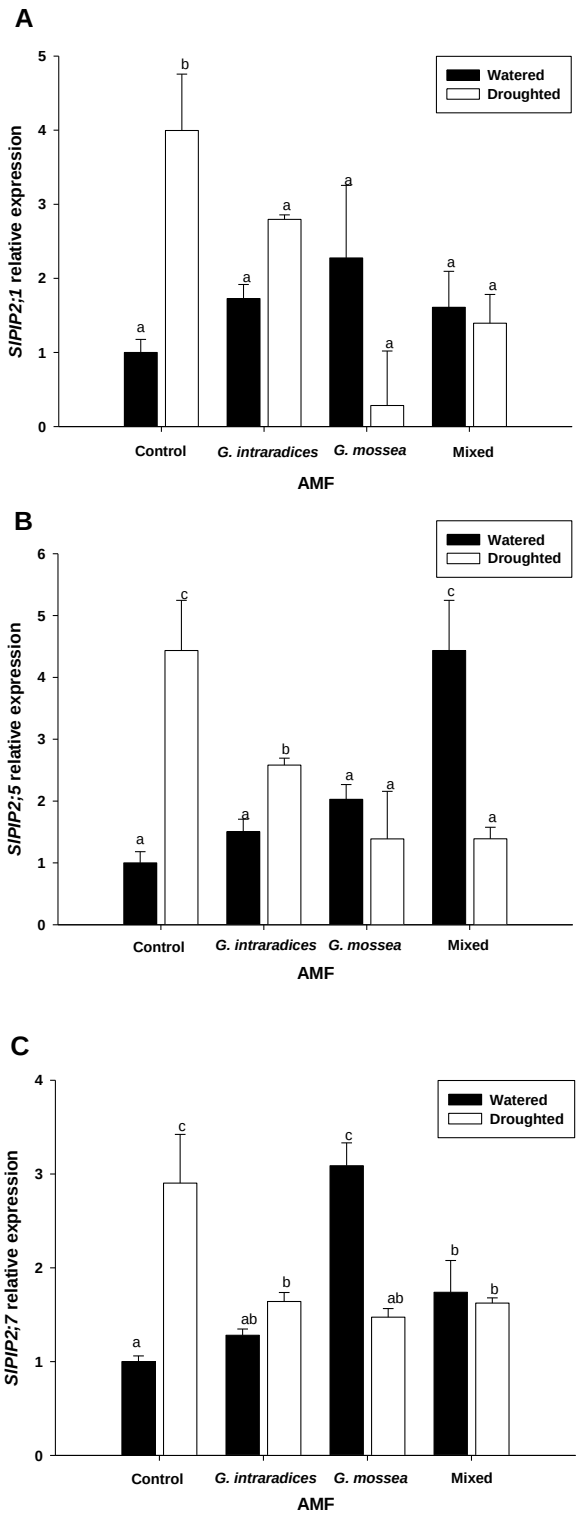


**Figure 4. 14: Effect of AMF on expression of aquaporin genes, *SlPIP2;1* (A), *SlPIP2;5* (B) and *SlPIP2;7* (C) in the root of seven weeks old tomato plants grown under watered and drought stress conditions.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 4)

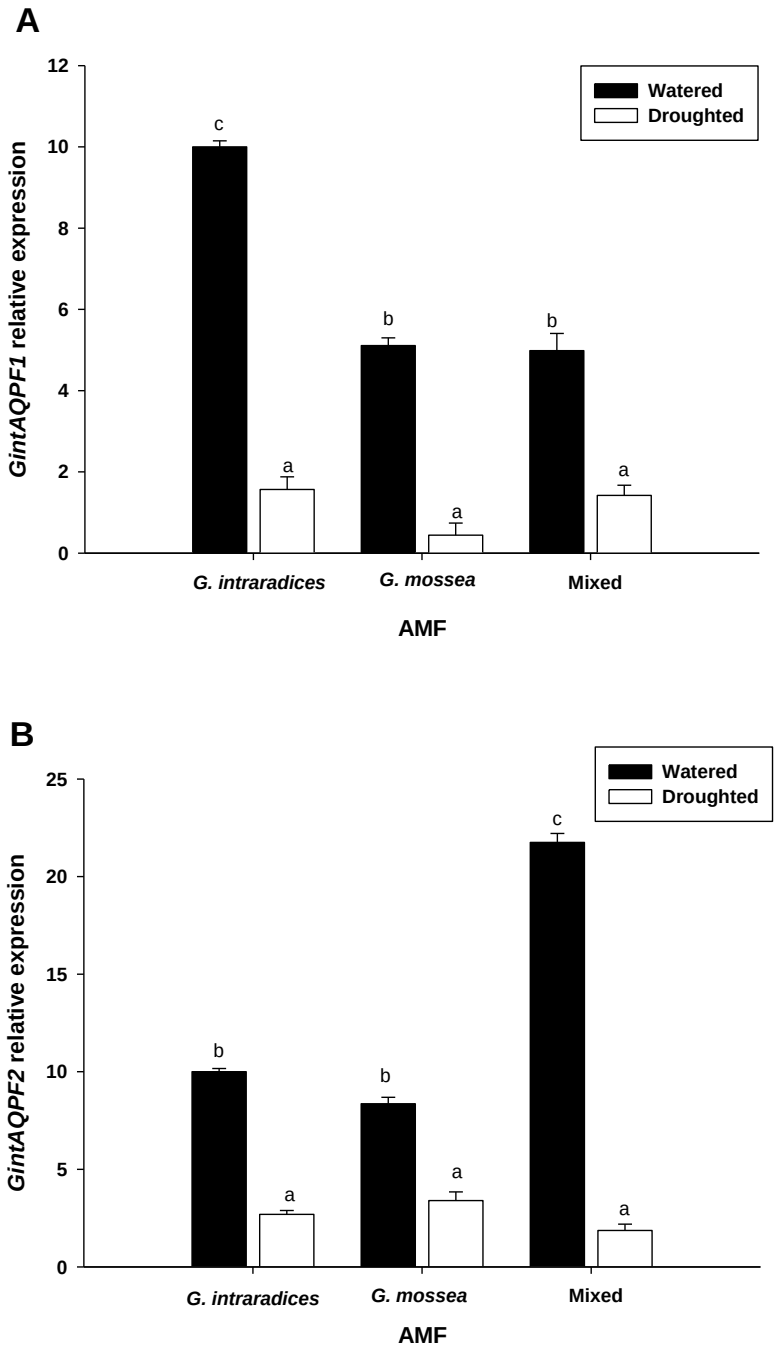


**Figure 4. 15: Effect of drought on expression of AMF aquaporin genes, *GintAQPF1* (A) and *GintAQPF2* (B) in the root of seven weeks old tomato plants grown under watered and drought stress conditions.**

Error bars represent S.E. Vertical bars followed by the same letter are not significantly different (P = 0.05, n = 4)

# CHAPTER FIVE

# DISCUSSION

## 5.1. Root colonization and promotion of tomato growth under phosphate-limiting condition by AMF

Phosphate limitation is a major constraint to crop production, affecting the growth, yield and quality of crops such as tomato (Di Candilo and Silvestri, 1995). In soils with low available phosphorus, it is a common practice to apply phosphate fertilizers to achieve increased tomato fruit yields. One approach towards achieving improved phosphate use efficiency is through the use of AM fungal symbioses for efficient phosphorus mining and uptake. There is increasing interest in the cultivation of tomato with AMF. They have been shown to improve tomato seedling growth, fruit yield and nutrient uptake under low levels of fertilization (Ortas *et al*., 2013). The possibility of using AMF in soilless media such as cocopeat is an open question. Studies on mycorrhiza in soilless media have reported varying results ranging from enhanced growth (Dasgan *et al*., 2008) to no effects (Mueller *et al*., 2009) on growth, fruits yield or nutrient uptake of tomato. As AMF increases nutrient uptake, it is possible that low level of phosphate in the nutrient solution would be beneficial to the AMF themselves, while still supplying enough for the plant.

The results of this study showed that inoculation of tomato with *G. intraradices* or *G. mossea* enhanced root and shoot growth. Although, the most effective treatment was to add phosphate and AMF, application of AMF without phosphate gave similar or

even higher dry plant weights than non-AMF inoculated plants with phosphate addition. This indicates that AMF is capable of making use of limited phosphorus available in the cocopeat.

Inoculation with AMF has been observed to increase growth in many plants species (Smith and Read 2008). Comparable differences in dry shoot and root weight between the control and inoculated plants showed a clear contribution of AMF to the growth of the plant. AMFs are well known to have the ability to improve growth of plants under phosphate-limiting conditions (Elbon and Whalen, 2014) due to their abilities to enhance phosphate uptake from the soil, thereby increasing phosphate nutrient supply to the plant (Bücking *et al*., 2012). In tomato, AMFs have been shown to improve tomato seedling growth and nutrient uptake under low levels of fertilization (Ortas *et al*., 2013).

The phosphate content in soil or planting media is a major factor affecting root colonization by AMF and it is widely recognised that phosphate fertilization often negatively affects root colonization of many host plants by AMF (Smith and Read 1997). In this experiment, the addition of phosphate significantly reduced root colonization compared to treatments without phosphate addition. Increased colonization, due to phosphate omission however, did not translate to better growth, as the treatments with the highest root and shoot weights were the ones inoculated with either of the AMFs and supplied with phosphate.

In many agricultural systems, application of phosphate to soil is necessary to ensure plant productivity. Phosphorus deficiency has been identified as the most frequently occurring essential element deficiency limiting crop yields, hence, its recommended

addition in substantial quantities to the growing medium. Due to its immobile nature, adsorption, precipitation or conversion to organic form, phosphate recovery by crops is usually very low (Holford, 1997). Symbiosis with AMF can increase phosphate uptake in phosphate-limited growth media, thereby improving plant growth (Smith and Read, 2008). The fungi play critical role in phosphate uptake through the activity of their hyphae which extends from the roots, enabling the plant to explore a greater volume of soil, thereby overcoming the limitations imposed by the slow diffusion of phosphate in the soil (Smith & Read, 2008). In this study, all four treatments under phosphate addition showed higher phosphate concentration in the leaves compared to plants without phosphate addition. When phosphate was omitted from the nutrient solution, *G. mossea* and *G. intraradices* inoculated plants had the highest phosphate concentration in the plant leaves. This is an indication of the role of AM fungi in enhancing uptake of phosphorus which in turn leads to increased plant growth.

The beneficial effect when phosphate was omitted from the nutrient solution was only observed with inoculation of the plants with either *G. intraradices* or *G. mossea*. Combined application however resulted in a significantly lower plant growth and phosphate uptake under phosphate limiting condition. In an ecological context, it is normal to observe multiple AMF colonizing a single host plant, as a single root system is capable of accommodating more than one AMF species (van Tuinen *et al.*, 1998). The phenomenon of co-colonization is poorly understood, and it remains unclear whether such colonization results in competitive, synergistic, or antagonistic interaction (Alkan *et al.*, 2006).

AMF are completely dependent on host plants for organic carbon (C). The outcome of the symbiosis for host plants often depend on the balance between net costs (C loss to the fungus) and net benefits (additional P supply via the fungus). Where net costs exceed net benefits, and plant growth depressions follow, it is then conventionally assumed that the fungus is a parasite that exploits its host by obtaining C but providing little or no P. This conventional explanation is tenable when AMF colonize the root extensively (Smith *et al.*, 2010) as observed in this study. It appears that this is the case in this study, as root colonization was highest in mixed inoculation without phosphate addition. It was also observed that this treatment combination had the lowest leaf phosphorus concentration.

However, other studies have shown that growth depressions are not necessarily associated with high AM fungal colonization, but also occur when there is very low internal root colonization, and in some cases also low external mycelium in soil (Li *et al*., 2008; Facelli *et al.,* 2009). It is also possible that growth depressions in the absence of high fungal biomass are the result of P deficiency, induced by reduced activity of the direct P uptake pathway and inadequate contribution of the AM pathway because of low root colonization or hyphal development in soil (Li *et al*., 2008; Smith *et al*., 2009).

## 5.2. Improvement of growth and physiological responses of tomato under drought stress by AMF

Drought stress adversely affects plant growth, physiology and productivity (Golldack *et al*., 2014; Osakabe *et al*., 2014). Plants have evolved several mechanisms to flexibly adapt to and tolerate drought stress (Basu *et al*., 2016). One of such mechanisms is the

establishment of symbiosis with AMF, which is a key component in helping plants to cope with drought stress (Augé, 2001, 2004). The results of this study showed that inoculation of tomato with *G. intraradices* or *G. mossea* enhanced growth under watered and drought conditions. Also inoculation with either AMF improved plant water status, reduced pigment damage and increased proline accumulation under drought stress.

It has also been shown that AMF inoculated tomato plants performed better than non-AMF control plants under drought stress (Aroca *et al*., 2008). Although in this study, as in most studies, drought stress was applied after establishment of colonization, Ruiz-Lozano *et al*. (2015) showed that the beneficial effect of the symbiosis on tomato performance also takes place when the stress is applied from the beginning.

Drought has been shown to steadily increase root colonization by AMF (Ruiz-Lozano *et al*., 2015). In this study, root colonization was observed to significantly increase in AMF tomato plants subjected to drought stress (20 - 40% volumetric moisture content) for 4 weeks but not in tomato plants subjected to continuous drought for one and two weeks. It is possible that no significant increase in colonization was detected as a result of the short time period of drought stress (one and two weeks) or because the plants were not maintained at a constant volumetric moisture content as water was completely withheld from these plants.

This study also showed that after two weeks of drought stress, AMF tomato had a significantly higher leaf relative water content (LRWC) than non AMF controls indicating increased water uptake by AMF under low substrate water content or reduced water loss as a result of inoculation with AMF. AMF plants generally show

improved water status due to the increased absorbing surface caused by AMF hyphae combined with the fungal capability to take up water from soils with low water potential (Augé, 2001). Because of their lengths and diameter, AMF hyphae may be able to penetrate a much higher proportion of soil pores than is accessible to host roots (Smith *et al.*, 2010). In this study, no significant difference in LRWC was observed under watered conditions. This is probably because, increased water uptake by AMF hyphae is less important when the growing medium or substrate is near saturation and large pores are filled with water as the root surfaces are also in contact with water. However, as the substrate dries up and water is retained only in smaller pores where fungal hyphae can grow, but roots cannot, the water uptake function of hyphae becomes more critical for survival (Allen, 2007).

As the substrate dries out and soil water potential becomes more negative, plants must decrease their water potential to avoid cell dehydration and to maintain a favourable gradient for water flow from soil into roots (Ruiz-Lozano *et al.*, 2012). The most important mechanism to achieve such an effect, known as osmotic adjustment (OA) or osmoregulation, is to decrease the plant osmotic potential by active accumulation of solutes such as inorganic ions, uncharged organic compounds, amino acids and sugars (Hoekstra *et al.*, 2001). This allows cells to maintain turgor, keeping a gradient of water potential favourable to water entrance into the plants. Proline is an amino acid that accumulates in most tissues subjected to water stress and it is readily metabolized upon recovery from drought (Singh *et al.*, 2000). In the present study, proline was observed to accumulate in the leaf and root of both AMF and control tomato plants after 2 weeks of drought stress. However, proline accumulation was significantly higher in AMF plants under drought stress in both below ground and aerial tissues,

indicating that AMF significantly contribute to osmotic adjustment during drought stress through promotion of plant accumulation of free proline.

Studies on osmoregulation by AMF in plants reveal complex and contradictory results. While some studies have shown an increase in proline accumulation in AMF plants subjected to drought (Goicoechea *et al*., 1998; Bheemareddy & Lakshman, 2011), the increase is variable and depends on the AMF involved. For instance, plants colonized by *G. deserticola* accumulated three times the amount of proline compared to the plants colonized by *G. intraradices* (Ruiz Lozano *et al*., 1995). In contrast, other studies have found lower proline accumulation in AMF inoculated plants under drought stress than in non-AMF controls (He *et al*., 2011). In another study by Ruíz-Lozano *et al*. (2011), it was found that drought stress non-AMF lettuce plants under drought stress accumulated more proline in the shoots than AMF plants. In contrast, AMF plants subjected to drought stress accumulated more proline than non-AMF plants in the roots, as was observed in this study. This suggests that AMF plants accumulate more proline in their roots in order to cope with low water potential of drying soil and to keep a water potential gradient in favour of water entrance into the roots (Porcel & Ruiz-Lozano, 2004). In this way, AMF plants would have a better water status than non-AMF plants and their shoots would be less strained by drought stress, thus the shoots of AMF plants would need to accumulate less proline (Ruíz-Sánchez *et al*., 2011).

Chlorophyll loss is a negative consequence of drought stress. In this study, regardless of AMF treatments, drought stress significantly reduced the levels of the photosynthetic pigments, total chlorophyll and total carotenoids. AMF tomato plants

however had higher levels of both pigments after two weeks of drought stress indicating that the symbiosis is potentially capable of preventing, reducing or delaying photosynthetic pigment damage. Carotenoids are part of plant antioxidant defence system and may play important roles in plant tolerance to drought stress (Mittler, 2002). The reduction in chlorophyll content under drought stress could be as a result of reduction in Mg and K concentrations (Augé, 2001). Interestingly, Abdel-Salam *et al*., (2017), found that the contents of those elements were higher in AMF plants and may be responsible for the higher levels of chlorophyll in AMF plants under drought stress.

### 5.3. Protection against the oxidative stress caused by drought stress in tomato by AMF

Drought stress induces oxidative stress in plants as a result of excessive production of reactive oxygen species (ROS) which are toxic molecules capable of causing oxidative damage to proteins, DNA and lipids (Miller *et al*., 2010). Increased antioxidant levels is necessary to scavenge the excessive ROS generated by drought stress (Smirnoff, 1993). It has been proposed that protection against oxidative stress through enrichment of antioxidant levels may be a mechanism by which AMF symbiosis increases plant tolerance to drought stress (Ruiz-Sánchez *et al*., 2010)

In this study, $H_2O_2$ levels and lipid peroxidation were assessed as indicators of oxidative stress and damage. Malondialdehyde (MDA) formed from the breakdown of polyunsaturated fatty acids serves as a convenient index for the determination of the extent lipid peroxidation. Both oxidative stress markers were observed to be significantly increased in the leaf and root by drought stress. It was observed that while

*G. intraradices* inoculated plants showed lower levels of both $H_2O_2$ and MDA compared to the control ones under drought stress, *G. mossea* and the mixed inoculation increased the levels of both stress markers. This indicates that inoculation of tomato with *G. intraradices* is better at protecting the plants against oxidative stress compared to *G. mossea* or mixed inoculation. Studies have shown a substantial reduction in oxidative damage to lipids in AMF plants subjected to drought stress (Ruiz-Lozano *et al*., 2001).

Plants have evolved antioxidant defence mechanisms to avoid oxidative damage linked to drought stress conditions, and this includes the enzyme catalase (CAT) (Scheibe & Beck, 2011) which converts $H_2O_2$ to $H_2O$ and $O_2$ in the peroxisomes (Noctor & Foyer, 1998;Miller *et al*., 2010). In the present study, catalase activity was assayed in the leaf and root of tomato plants subjected to drought stress and it was observed that AMF inoculated plants showed significantly higher catalase activities compared to non-AMF plants. Catalase activity was highest in plants inoculated with *G. intraradices*, and it was the same treatment with the lowest levels of oxidative stress markers. This indicates that *G. intraradices* protects plants from drought related oxidative stress through enhancement of catalase activity.

It was previously found that AMF lettuce plants subjected to drought have increased antioxidant enzyme (SOD) activity compared to non-AMF controls (Ruiz-Lozano *et al*., 1996), and molecular analyses have confirmed this response at the transcriptional level (Ruiz-Lozano *et al.*, 2001). Under drought stress, *G. mossea* and *G. intraradices* increased SOD activity by 50% and 138% respectively relative to non-AMF plants (Ruiz-Lozano *et al*., 2001). The increase in SOD activity and gene expression were

related to enhanced tolerance to drought. AMF symbiosis has also been shown to increase the glutathione reductase (GR) activity both in root and nodules of soybean plants subjected to drought stress (Porcel *et al*., 2003). An increase in the nonenzymatic antioxidant, glutathione (GSH), content in AMF plants has also been found concomitantly with a reduced oxidative damage to lipids (Wu *et al*., 2006; Wu & Zou, 2009). Subramanian *et al*. (2006) showed an increase in ascorbic acid (AsA) content in tomato fruits of AMF plants under well-watered, moderate drought and severe drought stress. On the other hand, Marulanda *et al*. (2007) found no difference between antioxidant enzyme activities of GR, CAT and SOD in AMF plants grown under drought conditions, although there was a decline in $H_2O_2$ accumulation.

### 5.4. Modulation of drought related genes in tomato by AMF

Previously, it was generally considered that multiple AMF species are incapable of colonizing a single host root system, as it is believed that AMF competed for a root zone occupancy (Hepper *et al*., 1988; Pearson *et al*., 1993). With the development of qRT-PCR technology, and its application in AMF biology, the occurrence of multiple occupancy of a single root segment by AMF of diverse genera and/or species is now commonly acceptable (Alkan *et al*., 2006). AMF exhibit a high degree of functional diversity, and they can have varying effects on host plant growth and performance under normal and stressed conditions (Feddermann *et al*., 2010). Moreover, multiple occupancy seems to be more beneficial to the host plants, since it enables it to harness a wider array of benefits compared to colonization by a single AMF isolate. In the present study, both *G. intraradices* and *G. mossea* specific genes were detected in the root of tomato plants inoculated with mixed AMF under well-watered as well as under

drought condition indicating that there was indeed co-colonization when the plant was inoculated with mixed AMF.

Research findings have emerged showing the ability of AMF to ameliorate the effect of drought stress on plant growth (Amiri *et al*., 2015; Yooyongwech *et al*., 2016). However, very little information is available on the direct effect of drought stress on the growth of the AMF in single and mixed inoculation. In the present study, drought stress did not affect either AMF in single inoculation, but during co-inoculation, drought stress negatively affected the growth of *G. intraradices* as indicated by the down regulation of the *G. intraradices* specific ribosomal RNA gene. It is thus possible that in the mixed AMF, *G. intraradices* plays more significant role under watered condition, while *G. mossea* plays more significant role under drought conditions, protecting the plants from adverse effect of drought stress.

Abscisic acid is a critical hormone in plant responses to abiotic stresses such as drought (Christmann *et al*., 2006) and its biosynthesis is rapidly promoted under drought stress (Hong *et al*., 2013; Osakabe *et al*., 2014). It is synthesized in the roots and translocated to leaves where it initiates plant adaptation to drought stress through stomatal closure (Wilkinson & Davies, 2010). The enhanced tolerance of AMF plants to drought stress has been associated with an alteration in ABA balance and several studies have reported that the levels of plant ABA actually changes upon the establishment of AM symbiosis (Ruiz-Lozano *et al*., 2012). ABA modulates plant water status through regulation of root hydraulic conductivity, transpiration rate, and induction of genes that encode enzymes and other proteins involved in dehydration tolerance (Zhang *et al*., 2006; Hirayama & Shinozaki, 2007). In this study, while the expression of the

ABA biosynthesis gene *LeNCED1* was significantly enhanced by drought stress in non AMF plants, the gene expression was either down regulated or not significantly affected by drought stress in AMF plants. In similar fashion, the expression of ABA-responsive marker gene, *Le4*, was significantly enhanced by drought stress in non-AMF plants, but unaffected in AMF plants. This could indicate that AMF response to drought stress is not ABA dependent. It is also possible that the down-regulation of ABA biosynthesis gene is a mechanism through which AMF symbiosis maintains plant growth under drought stress, by preventing ABA-mediated plant response to drought, which involves stomatal closure, prevention of $CO_2$ uptake, reduced photosynthesis, and reduced growth rate. Ruiz-Lozano *et al*. (2012) suggested alteration of ABA levels in by AMF symbiosis in host plants could be highly dependent on the AMF species used, as contradicting results were obtained. It has been suggested that ABA is necessary for sustained root colonization by AMF (Fester & Hause, 2007) and to improve the symbiotic efficiency under drought stress condition (Aroca *et al*., 2008a; Aroca *et al*., 2008b; Ruiz-Lozano *et al*., 2009).

Other studies have also found that when plants were subjected to drought stress, the levels of ABA are lower in AMF plants than in non-AMF controls (Goicoechea *et al*., 1997; Estrada-Luna & Davies, 2003). In this study however, the transcript level of the ABA biosynthesis gene was measured and not the level of hormone itself. Ruiz-Lozano *et al*., (2015) reported a correlation between *LeNCED1* gene expression and ABA levels in lettuce, but not in tomato, indicating that reduced transcript level does not necessarily translate to lower hormone levels. The lack of correlation between transcript levels and hormone level could be as a result of feedback inhibition, a

regulatory mechanism to prevent further accumulation of ABA when it has reached saturation or a peak level.

During symbiosis, AMF can transfer water to the host plant, and it is expected that the host increase its permeability for water and that aquaporin genes should be upregulated. The upregulation of aquaporin genes under well-watered conditions is thus expected as observed in this study. Under drought or water-deficit conditions however, contradictory results were obtained. Porcel *et al*. (2006) showed that aquaporin genes studied were downregulated under drought stress, and the downregulation was even more severe in AMF plants than in non-AMF plants. In the present study, the expression of each of the aquaporin genes studied was increased in non-AMF plants, while in the AMF inoculated plants, there was less increase in gene expression as in the case of *G. intraradices*, or no significant change and even decrease in gene expression as in the case of *G. mossea* and mixed AMF.

The effect of AMF symbiosis downregulating aquaporin gene may have a physiological importance to help AMF plants to cope with drought stress. It could be that the decreased expression of plasma membrane aquaporin genes during drought stress in AMF plants can be a regulatory mechanism to limit the water lost from the cells (Porcel *et al*., 2006). In a study by Aroca *et al*. (2007), the expression of four aquaporin genes was analysed in AMF and non-AMF plants subjected to drought stress, and three of these genes showed differential regulation by AMF. *PIP1;1* was slightly inhibited by AMF under drought stress, while non-AMF plants did not change its expression pattern. *PIP1;2* was inhibited by drought stress in the same way in AMF and non-AM plants. In contrast, *PIP1;3* was induced in non-AM plants under drought

stress but inhibited in AMF plants. Thus, the effect of AMF symbiosis on regulation of aquaporin expression under drought stress varies and depends on the AMF and the specific aquaporin genes. In any case, the up or downregulation of particular aquaporins by AMF symbiosis should result in a better regulation of plant water status and contribute to the global plant tolerance to drought stress, as evidenced by their better growth and water status under conditions of water deficit (Jang *et al*., 2004). In summary, AMF symbiosis inhibition of the expression of aquaporins during drought stress is as a strategy of water conservation in the host plant, allowing for such plants to maintain a higher shoot and leaf relative water content (Ruiz-Lozano *et al*., 2009).

There have been very few reports on functional aquaporin in AMF. Aroca *et al*. (2009) cloned the first aquaporin gene from an AM fungus (*GintAQP1*), and suggested that fungal aquaporins could compensate for the downregulation of host plant aquaporins caused by drought stress. Li *et al*., (2013) reported cloning and characterization of two functional aquaporin genes (*GintAQPF1* and *GintAQPF2*) from *G. intraradices* and provided a strong support to the direct involvement of AMF in plant drought tolerance. They observed that the expression of both AMF aquaporins was significantly enhanced during drought stress. In this study however, both AMF aquaporin genes (*GintAQPF1* and *GintAQPF2*) were downregulated by drought stress indicating that the downregulation may also be crucial for preventing water loss from the host through the AMF. Thus, the aquaporins could play important roles in delivering water via AMF hyphal structures to the host plant only under watered condition or under low soil water potential (Li *et al*., 2013).

# CHAPTER SIX

# GENERAL CONCLUSIONS AND RECOMMENDATIONS

## 6.1. General conclusions

In conclusion, the study shows that AMF symbiosis promotes tomato plant growth under normal conditions as well as under phosphate limitation and water deficit or drought stress. Under phosphate limitation, inoculation of tomato plants with either *G. intraradices or G. mossea* improved tomato plant growth and phosphate uptake. Mixed inoculation of both AMF however resulted in poor plant growth and phosphate uptake. The study also provides clear evidences that the symbiosis enhances tomato plant tolerance to drought stress through alteration of several physiological, biochemical and molecular processes. Under drought conditions, inoculation with either *G. intraradices* or *G. mossea*, in single or mixed application improved plant growth, maintained higher leaf water content, reduced damage to photosynthetic pigments (chlorophyll and carotenoids), and improved osmotic adjustment by accumulation of proline. AMF symbiosis improved the activity of the antioxidant enzyme, catalase, under drought stress. However, reduction in oxidative stress marker, $H_2O_2$ and MDA, in this study was only observed in plants inoculated with *G. intraradices*. In contrast, treatment with *G. mossea* and mixed AMF resulted in higher oxidative stress marker indices. Finally, the differential regulation of tomato abscisic acid biosynthesis gene and aquaporin genes may play very important roles in tomato plant drought tolerance enhancement

by preventing ABA-mediated plant response to drought stress, which may reduce plant growth, and by prevention of water loss from the plant.

## 6.2. Recommendations and Future Perspectives

Based on the findings of this study, it can be recommended that:

a) In greenhouse production of tomato, AMF inoculum should be applied to the media prior to seeding to improve growth and yield.
b) AMF inoculum should be used to improve nutrient status of tomato while reducing nutrient input, thereby saving cost.
c) Under low substrate phosphate level, tomato plants should be inoculated with AMF to improve growth.
d) AMF should be used in drought stress condition to improve tomato plants water use as well as adaptation.

For future studies,

a) Field studies should be carried out, as it resembles more the natural conditions that tomato plants will be exposed to under commercial production. In this way, other parameters like interaction with other rhizosphere microorganisms and climatic conditions can be assessed.
b) Reports on the effect of AMF on plant physiological and biochemical parameters have been contrasting. This is probably due to lack of standard and accurate reporting of the levels of water stress. Therefore, there is need for accurate measurement and standardized reporting of substrate moisture content

and plant water status at the time of measuring the drought related physiological and biochemical parameters.

c) The participation of other antioxidant compounds such as carotenoids in the reduction of oxidative damage should be investigated with emphasis on the role of AMF in accumulation of these compounds in the fruits. Such antioxidants are important for the improvement of nutritional quality of tomato fruits which is of interest for human consumption.

d) The possible role of the fungal aquaporins should be further investigated. There have been very few reports on functional aquaporin genes from AMF. The study of more fungal aquaporins is needed to completely understand the role of AMF aquaporins under drought stress.

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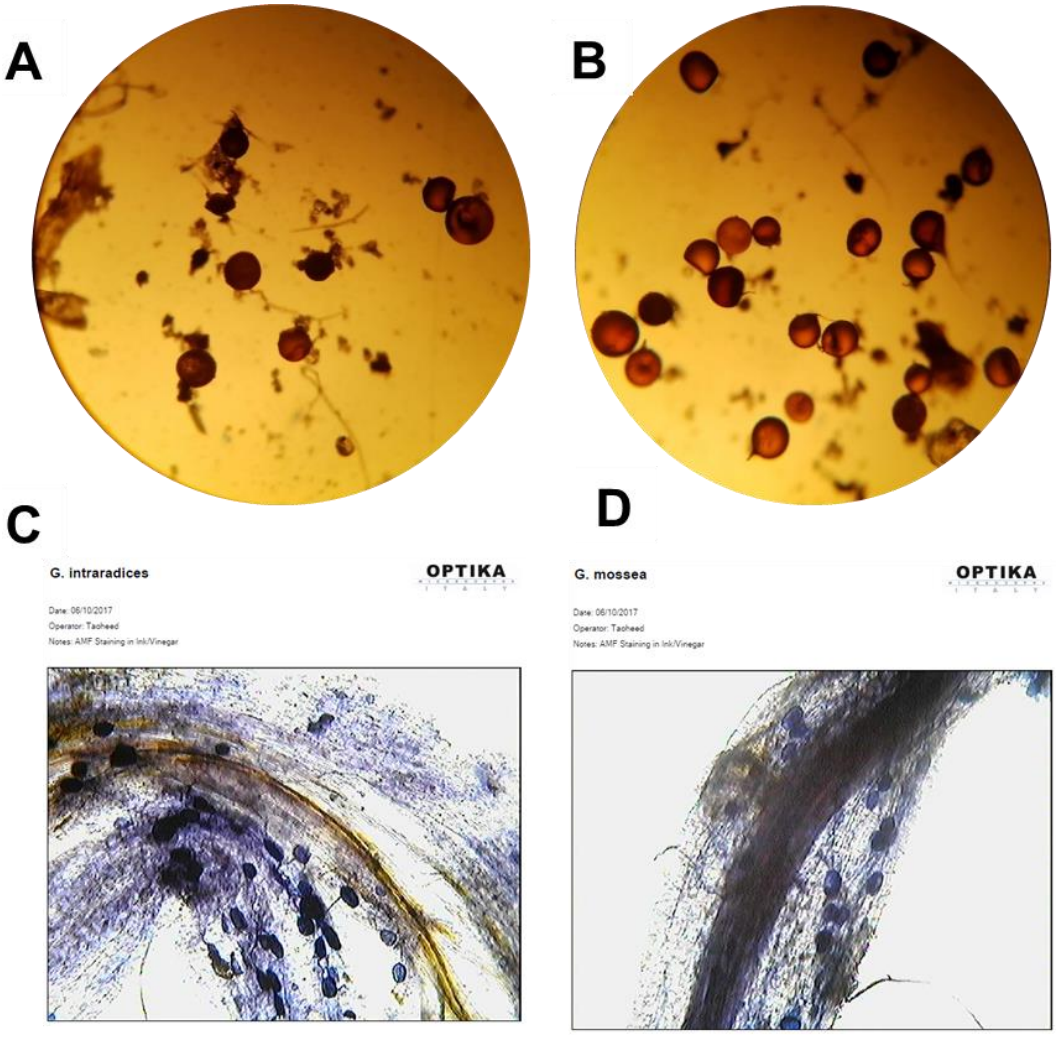
# APPENDICES

**APPENDIX A:** Supplementary Information for Section 4.1




**Appendix 1. 1: Confirmation of spore presence in the commercial crude inocula of *G. intraradices* (A) and *G. mossea* (B) by sieving and sucrose density centrifugation and by staining of root fragments present in the *G. intraradices* (C) and *G. mossea* (D) inocula**

**Appendix 1. 2: ANOVA for effects of inoculation with AMF on dry shoot weight**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 11.172[a] | 3 | 3.724 | 240.258 | .000 |
| Intercept | 64.800 | 1 | 64.800 | 4.181E3 | .000 |
| Mycorrhiza | 11.172 | 3 | 3.724 | 240.258 | .000 |
| Error | .248 | 16 | .016 | | |
| Total | 76.220 | 20 | | | |
| Corrected Total | 11.420 | 19 | | | |

a. R Squared = .978 (Adjusted R Squared = .974)

**Appendix 1. 3: ANOVA for effects of inoculation with AMF on dry root weight**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .637[a] | 3 | .212 | 314.496 | .000 |
| Intercept | 2.988 | 1 | 2.988 | 4.426E3 | .000 |
| Mycorrhiza | .637 | 3 | .212 | 314.496 | .000 |
| Error | .011 | 16 | .001 | | |
| Total | 3.635 | 20 | | | |
| Corrected Total | .648 | 19 | | | |

a. R Squared = .983 (Adjusted R Squared = .980)

**Appendix 1. 4: ANOVA for root colonization with and without phosphate in the nutrient solution**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 20561.107[a] | 7 | 2937.301 | 660.976 | .000 |
| Intercept | 57226.756 | 1 | 57226.756 | 1.288E4 | .000 |
| Mycorrhiza | 19164.836 | 3 | 6388.279 | 1.438E3 | .000 |
| Phosphate | 865.956 | 1 | 865.956 | 194.864 | .000 |
| Mycorrhiza * Phosphate | 530.316 | 3 | 176.772 | 39.779 | .000 |
| Error | 142.204 | 32 | 4.444 | | |
| Total | 77930.068 | 40 | | | |
| Corrected Total | 20703.312 | 39 | | | |

a. R Squared = .993 (Adjusted R Squared = .992)

**Appendix 1. 5: ANOVA for effect of inoculation with AMF and phosphate addition on dry shoot weight**

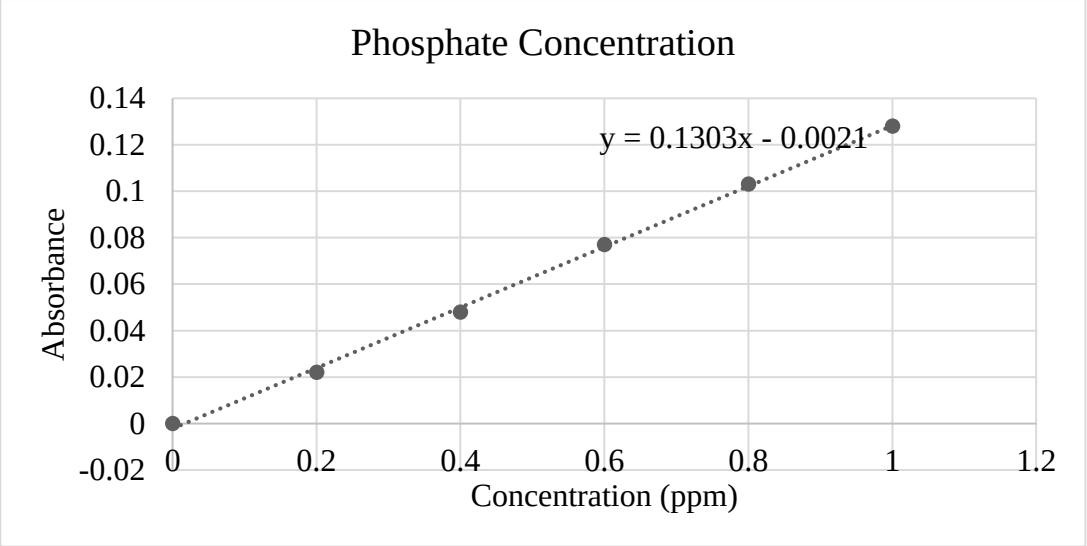
| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 27.448[a] | 7 | 3.921 | 28.938 | .000 |
| Intercept | 876.096 | 1 | 876.096 | 6.466E3 | .000 |
| Mycorrhiza | 16.826 | 3 | 5.609 | 41.392 | .000 |
| Phosphate | 7.396 | 1 | 7.396 | 54.583 | .000 |
| Mycorrhiza * Phosphate | 3.226 | 3 | 1.075 | 7.936 | .000 |
| Error | 4.336 | 32 | .136 | | |
| Total | 907.880 | 40 | | | |
| Corrected Total | 31.784 | 39 | | | |

a. R Squared = .864 (Adjusted R Squared = .834)

**Appendix 1. 6: ANOVA for effect of inoculation with AMF and phosphate addition on dry root weight**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 21.456[a] | 7 | 3.065 | 32.826 | .000 |
| Intercept | 470.596 | 1 | 470.596 | 5.040E3 | .000 |
| Mycorrhiza | 8.914 | 3 | 2.971 | 31.822 | .000 |
| Phosphate | 11.664 | 1 | 11.664 | 124.916 | .000 |
| Mycorrhiza * Phosphate | .878 | 3 | .293 | 3.134 | .039 |
| Error | 2.988 | 32 | .093 | | |
| Total | 495.040 | 40 | | | |
| Corrected Total | 24.444 | 39 | | | |

a. R Squared = .878 (Adjusted R Squared = .851)




**Appendix 1. 7: Standard curve of absorbance against phosphate concentration**

**APPENDIX B:** Supplementary Information for Section 4.2

**Appendix 2. 1: ANOVA for root colonization by AMF under watered and drought stress**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 859.957[a] | 5 | 171.991 | 24.774 | .000 |
| Intercept | 65895.600 | 1 | 65895.600 | 9.492E3 | .000 |
| AMF | 351.645 | 2 | 175.823 | 25.326 | .000 |
| Drought | 496.833 | 1 | 496.833 | 71.566 | .000 |
| AMF * Drought | 11.479 | 2 | 5.739 | .827 | .453 |
| Error | 124.961 | 18 | 6.942 | | |
| Total | 66880.519 | 24 | | | |
| Corrected Total | 984.919 | 23 | | | |

a. R Squared = .873 (Adjusted R Squared = .838)

**Appendix 2. 2: ANOVA for root colonization by AMF under watered and continuous drought**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 345.404[a] | 8 | 43.176 | 9.433 | .000 |
| Intercept | 82852.224 | 1 | 82852.224 | 1.810E4 | .000 |
| AMF | 322.647 | 2 | 161.323 | 35.247 | .000 |
| Drought | 13.263 | 2 | 6.631 | 1.449 | .253 |
| AMF * Drought | 9.495 | 4 | 2.374 | .519 | .723 |
| Error | 123.578 | 27 | 4.577 | | |
| Total | 83321.206 | 36 | | | |
| Corrected Total | 468.982 | 35 | | | |

a. R Squared = .736 (Adjusted R Squared = .658)

**Appendix 2. 3: ANOVA for effect of AMF and drought on dry shoot weight**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 236.364[a] | 7 | 33.766 | 102.176 | .000 |
| Intercept | 7573.832 | 1 | 7573.832 | 2.292E4 | .000 |
| AMF | 117.965 | 3 | 39.322 | 118.987 | .000 |
| Drought | 95.484 | 1 | 95.484 | 288.934 | .000 |
| AMF * Drought | 22.914 | 3 | 7.638 | 23.113 | .000 |
| Error | 23.794 | 72 | .330 | | |
| Total | 7833.990 | 80 | | | |
| Corrected Total | 260.158 | 79 | | | |

a. R Squared = .909 (Adjusted R Squared = .900)

**Appendix 2. 4: ANOVA for effect of AMF and drought on dry root weight**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 72.404[a] | 7 | 10.343 | 95.827 | .000 |
| Intercept | 2080.800 | 1 | 2080.800 | 1.928E4 | .000 |
| AMF | 71.178 | 3 | 23.726 | 219.813 | .000 |
| Drought | .648 | 1 | .648 | 6.003 | .017 |
| AMF * Drought | .577 | 3 | .192 | 1.783 | .158 |
| Error | 7.771 | 72 | .108 | | |
| Total | 2160.975 | 80 | | | |
| Corrected Total | 80.175 | 79 | | | |

a. R Squared = .903 (Adjusted R Squared = .894)

**Appendix 2. 5: ANOVA for the effect of AMF and drought on leaf relative water content**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 965.257[a] | 11 | 87.751 | 39.997 | .000 |
| Intercept | 238896.516 | 1 | 238896.516 | 1.089E5 | .000 |
| Mycorrhiza | 56.067 | 3 | 18.689 | 8.519 | .000 |
| Drought | 789.944 | 2 | 394.972 | 180.029 | .000 |
| Mycorrhiza * Drought | 119.246 | 6 | 19.874 | 9.059 | .000 |
| Error | 105.309 | 48 | 2.194 | | |
| Total | 239967.082 | 60 | | | |
| Corrected Total | 1070.566 | 59 | | | |

a. R Squared = .902 (Adjusted R Squared = .879)

**Appendix 2. 6: ANOVA for the effect of AMF and drought on total chlorophyll**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 591.323[a] | 11 | 53.757 | 21.281 | .000 |
| Intercept | 5251.022 | 1 | 5251.022 | 2.079E3 | .000 |
| Mychorriza | 62.723 | 3 | 20.908 | 8.277 | .000 |
| Drought | 524.878 | 2 | 262.439 | 103.893 | .000 |
| Mychorriza * Drought | 3.721 | 6 | .620 | .246 | .959 |
| Error | 121.250 | 48 | 2.526 | | |
| Total | 5963.594 | 60 | | | |
| Corrected Total | 712.573 | 59 | | | |

a. R Squared = .830 (Adjusted R Squared = .791)

**Appendix 2. 7: ANOVA for the effect of AMF and drought on total carotenoids**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 4.676[a] | 11 | .425 | 1.549 | .145 |
| Intercept | 756.502 | 1 | 756.502 | 2.757E3 | .000 |
| AMF | .985 | 3 | .328 | 1.197 | .321 |
| Drought | 3.390 | 2 | 1.695 | 6.177 | .004 |
| AMF * Drought | .301 | 6 | .050 | .183 | .980 |
| Error | 13.171 | 48 | .274 | | |
| Total | 774.350 | 60 | | | |
| Corrected Total | 17.847 | 59 | | | |

a. R Squared = .262 (Adjusted R Squared = .093)

**Appendix 2. 8: ANOVA for the effect of AMF and drought on leaf proline**

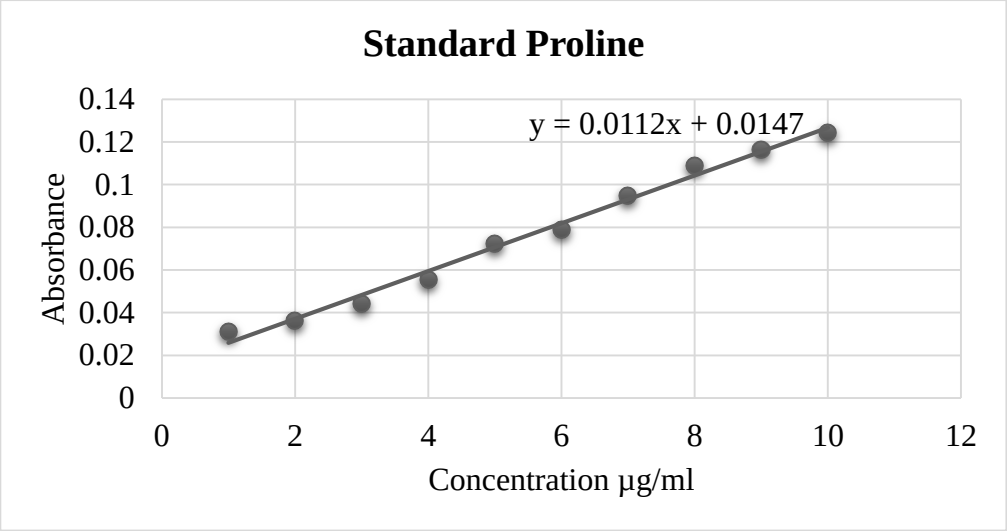
| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 212.609[a] | 11 | 19.328 | 51.731 | .000 |
| Intercept | 428.247 | 1 | 428.247 | 1.146E3 | .000 |
| AMF | 10.851 | 3 | 3.617 | 9.681 | .000 |
| Drought | 183.018 | 2 | 91.509 | 244.919 | .000 |
| AMF * Drought | 18.740 | 6 | 3.123 | 8.359 | .000 |
| Error | 17.934 | 48 | .374 | | |
| Total | 658.790 | 60 | | | |
| Corrected Total | 230.543 | 59 | | | |

a. R Squared = .922 (Adjusted R Squared = .904)

**Appendix 2. 9: ANOVA for effect of AMF and drought on root proline**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 374.309[a] | 7 | 53.473 | 13.346 | .000 |
| Intercept | 912.271 | 1 | 912.271 | 227.696 | .000 |
| AMF | 43.141 | 3 | 14.380 | 3.589 | .028 |
| Drought | 283.404 | 1 | 283.404 | 70.736 | .000 |
| AMF * Drought | 47.764 | 3 | 15.921 | 3.974 | .020 |
| Error | 96.157 | 24 | 4.007 | | |
| Total | 1382.736 | 32 | | | |
| Corrected Total | 470.465 | 31 | | | |

a. R Squared = .796 (Adjusted R Squared = .736)




**Appendix 2. 10: Standard curve of proline concentration**

**APPENDIX C:** Supplementary Information for Section 4.3

**Appendix 3. 1: ANOVA for effect of AMF and drought on leaf hydrogen peroxide concentration**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 71376.382[a] | 11 | 6488.762 | 100.739 | .000 |
| Intercept | 403315.881 | 1 | 403315.881 | 6.262E3 | .000 |
| AMF | 13242.038 | 3 | 4414.013 | 68.528 | .000 |
| Drought | 51864.347 | 2 | 25932.174 | 402.601 | .000 |
| AMF * Drought | 6269.997 | 6 | 1044.999 | 16.224 | .000 |
| Error | 3091.760 | 48 | 64.412 | | |
| Total | 477784.023 | 60 | | | |
| Corrected Total | 74468.142 | 59 | | | |

a. R Squared = .958 (Adjusted R Squared = .949)

**Appendix 3. 2: ANOVA for effect of AMF and drought on root hydrogen peroxide concentration**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 636.913[a] | 7 | 90.988 | 16.167 | .000 |
| Intercept | 16253.714 | 1 | 16253.714 | 2.888E3 | .000 |
| AMF | 57.854 | 3 | 19.285 | 3.427 | .033 |
| Drought | 539.554 | 1 | 539.554 | 95.872 | .000 |
| AMF * Drought | 39.506 | 3 | 13.169 | 2.340 | .099 |
| Error | 135.068 | 24 | 5.628 | | |
| Total | 17025.696 | 32 | | | |
| Corrected Total | 771.982 | 31 | | | |

a. R Squared = .825 (Adjusted R Squared = .774)

**Appendix 3. 3: ANOVA for effect of AMF and drought on leaf lipid peroxidation**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 158.012[a] | 11 | 14.365 | 3.307 | .002 |
| Intercept | 4658.103 | 1 | 4658.103 | 1.072E3 | .000 |
| AMF | 56.940 | 3 | 18.980 | 4.369 | .008 |
| Drought | 87.323 | 2 | 43.662 | 10.050 | .000 |
| AMF * Drought | 13.748 | 6 | 2.291 | .527 | .785 |
| Error | 208.525 | 48 | 4.344 | | |
| Total | 5024.640 | 60 | | | |
| Corrected Total | 366.537 | 59 | | | |

a. R Squared = .431 (Adjusted R Squared = .301)

**Appendix 3. 4: ANOVA for effect of AMF and drought on root lipid peroxidation**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 188.261[a] | 7 | 26.894 | 19.959 | .000 |
| Intercept | 1182.391 | 1 | 1182.391 | 877.463 | .000 |
| AMF | 23.109 | 3 | 7.703 | 5.716 | .004 |
| Drought | 153.125 | 1 | 153.125 | 113.635 | .000 |
| AMF * Drought | 12.027 | 3 | 4.009 | 2.975 | .052 |
| Error | 32.340 | 24 | 1.348 | | |
| Total | 1402.993 | 32 | | | |
| Corrected Total | 220.601 | 31 | | | |

a. R Squared = .853 (Adjusted R Squared = .811)

**Appendix 3. 5: ANOVA for effect of AMF and drought on leaf catalase activity**

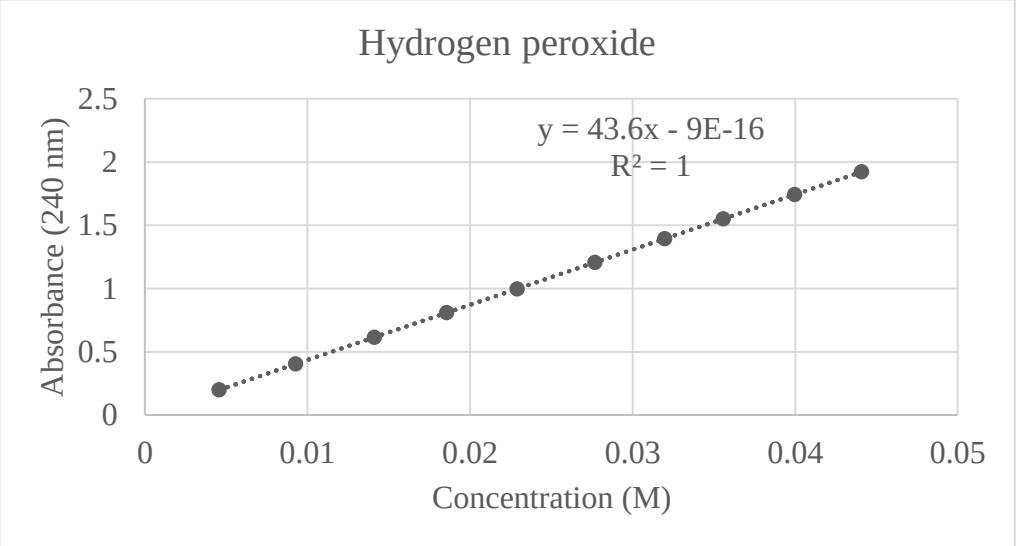
| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 314.509[a] | 11 | 28.592 | 22.684 | .000 |
| Intercept | 2066.460 | 1 | 2066.460 | 1.640E3 | .000 |
| AMF | 29.967 | 3 | 9.989 | 7.925 | .001 |
| Drought | 249.399 | 2 | 124.700 | 98.935 | .000 |
| AMF * Drought | 35.142 | 6 | 5.857 | 4.647 | .003 |
| Error | 30.250 | 24 | 1.260 | | |
| Total | 2411.219 | 36 | | | |
| Corrected Total | 344.759 | 35 | | | |

a. R Squared = .912 (Adjusted R Squared = .872)

**Appendix 3. 6: ANOVA for effect of AMF and drought on root catalase activity**

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 34.746[a] | 7 | 4.964 | 6.962 | .000 |
| Intercept | 729.143 | 1 | 729.143 | 1.023E3 | .000 |
| AMF | 19.548 | 3 | 6.516 | 9.139 | .000 |
| Drought | 8.379 | 1 | 8.379 | 11.753 | .002 |
| AMF * Drought | 6.819 | 3 | 2.273 | 3.188 | .042 |
| Error | 17.111 | 24 | .713 | | |
| Total | 781.000 | 32 | | | |
| Corrected Total | 51.857 | 31 | | | |

a. R Squared = .670 (Adjusted R Squared = .574)




**Appendix 3. 7: Standard Curve of Hydrogen Peroxide**

**APPENDIX D:** Supplementary Information for Section 4.4

**Appendix 4. 1: ANOVA for effect of drought on *G. intraradices ITS+18rRNA***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 5.106[a] | 3 | 1.702 | 4.982 | .007 |
| Intercept | 188.202 | 1 | 188.202 | 550.898 | .000 |
| Treatment | 5.106 | 3 | 1.702 | 4.982 | .007 |
| Error | 9.566 | 28 | .342 | | |
| Total | 202.873 | 32 | | | |
| Corrected Total | 14.672 | 31 | | | |

a. R Squared = .348 (Adjusted R Squared = .278)

**Appendix 4. 2: ANOVA for effect of drought stress on *G. mossea 28SrRNA***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | .917[a] | 3 | .306 | .465 | .709 |
| Intercept | 256.521 | 1 | 256.521 | 389.863 | .000 |
| Treatment | .917 | 3 | .306 | .465 | .709 |
| Error | 18.423 | 28 | .658 | | |
| Total | 275.862 | 32 | | | |
| Corrected Total | 19.341 | 31 | | | |

a. R Squared = .047 (Adjusted R Squared = -.055)

**Appendix 4. 3: ANOVA for effect of AMF and drought on *LeNCED1***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 6.729[a] | 7 | .961 | 3.838 | .002 |
| Intercept | 299.936 | 1 | 299.936 | 1.198E3 | .000 |
| Treatments | 6.729 | 7 | .961 | 3.838 | .002 |
| Error | 14.025 | 56 | .250 | | |
| Total | 320.691 | 64 | | | |
| Corrected Total | 20.754 | 63 | | | |

a. R Squared = .324 (Adjusted R Squared = .240)

**Appendix 4. 4: ANOVA for effect of AMF and drought on *Le4***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 7.899[a] | 7 | 1.128 | 4.497 | .000 |
| Intercept | 499.674 | 1 | 499.674 | 1.991E3 | .000 |
| Treatments | 7.899 | 7 | 1.128 | 4.497 | .000 |
| Error | 14.052 | 56 | .251 | | |
| Total | 521.624 | 64 | | | |
| Corrected Total | 21.950 | 63 | | | |

a. R Squared = .360 (Adjusted R Squared = .280)

**Appendix 4. 5: ANOVA for effect of AMF and drought on *SlPIP2;1***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 3.422[a] | 7 | .489 | 2.387 | .033 |
| Intercept | 381.982 | 1 | 381.982 | 1.865E3 | .000 |
| Treatments | 3.422 | 7 | .489 | 2.387 | .033 |
| Error | 11.469 | 56 | .205 | | |
| Total | 396.873 | 64 | | | |
| Corrected Total | 14.891 | 63 | | | |

a. R Squared = .230 (Adjusted R Squared = .134)

**Appendix 4. 6: ANOVA for effect of AMF and drought on *SlPIP2;5***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 24.447[a] | 7 | 3.492 | 2.292 | .040 |
| Intercept | 2325.892 | 1 | 2325.892 | 1.527E3 | .000 |
| Treatments | 24.447 | 7 | 3.492 | 2.292 | .040 |
| Error | 85.311 | 56 | 1.523 | | |
| Total | 2435.650 | 64 | | | |
| Corrected Total | 109.758 | 63 | | | |

a. R Squared = .223 (Adjusted R Squared = .126)

**Appendix 4. 7: ANOVA for effect of AMF and drought on *SlPIP2;7***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 17.099[a] | 7 | 2.443 | 5.166 | .000 |
| Intercept | 543.446 | 1 | 543.446 | 1.149E3 | .000 |
| Treatments | 17.099 | 7 | 2.443 | 5.166 | .000 |
| Error | 26.478 | 56 | .473 | | |
| Total | 587.024 | 64 | | | |
| Corrected Total | 43.577 | 63 | | | |

a. R Squared = .392 (Adjusted R Squared = .316)

**Appendix 4. 8: ANOVA for effect of drought on *GintAQPF1***

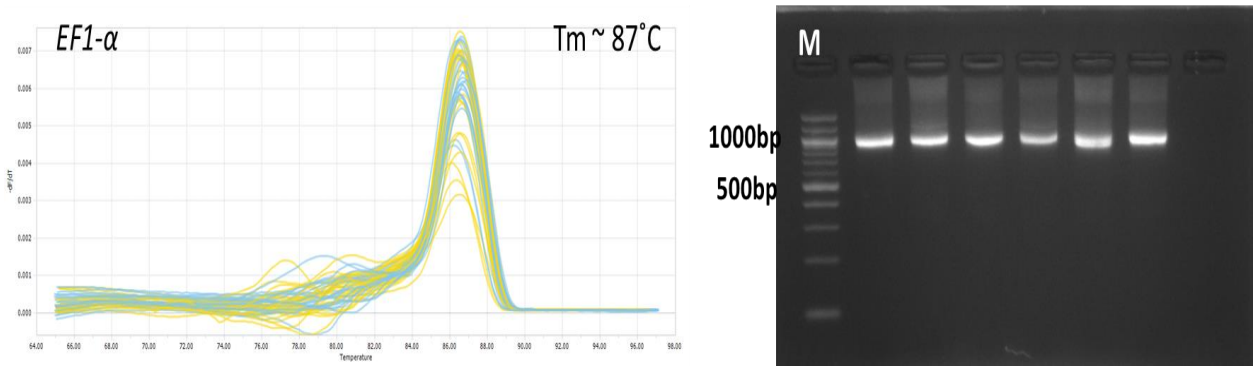
| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 16.257[a] | 5 | 3.251 | 9.983 | .000 |
| Intercept | 152.013 | 1 | 152.013 | 466.738 | .000 |
| Treatment | 16.257 | 5 | 3.251 | 9.983 | .000 |
| Error | 13.679 | 42 | .326 | | |
| Total | 181.949 | 48 | | | |
| Corrected Total | 29.936 | 47 | | | |

a. R Squared = .543 (Adjusted R Squared = .489)

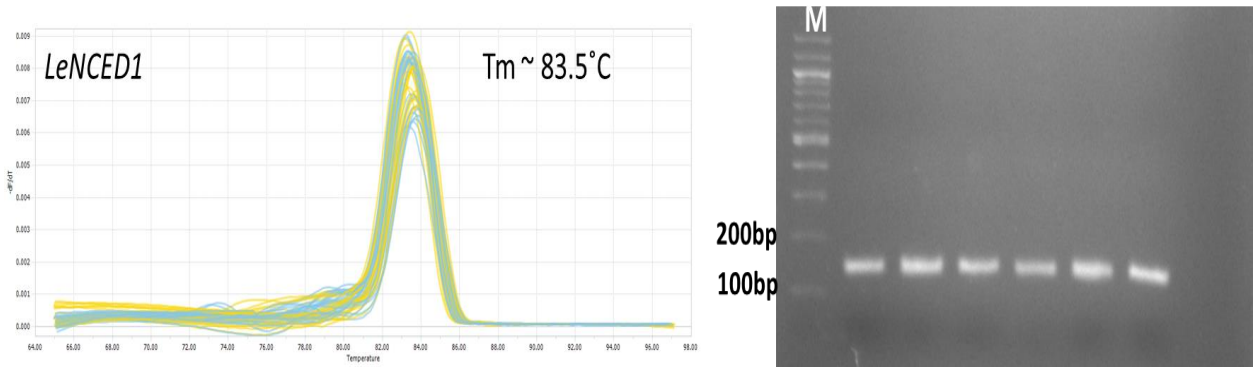
**Appendix 4. 9: ANOVA for effect of drought on *GintAQPF2***

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 7.350[a] | 5 | 1.470 | 6.848 | .000 |
| Intercept | 257.428 | 1 | 257.428 | 1.199E3 | .000 |
| Treatment | 7.350 | 5 | 1.470 | 6.848 | .000 |
| Error | 9.016 | 42 | .215 | | |
| Total | 273.794 | 48 | | | |
| Corrected Total | 16.366 | 47 | | | |

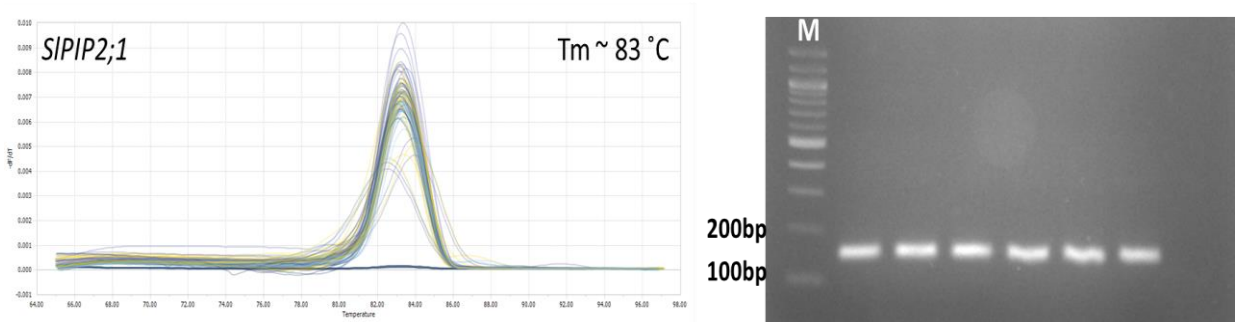
a. R Squared = .449 (Adjusted R Squared = .384)



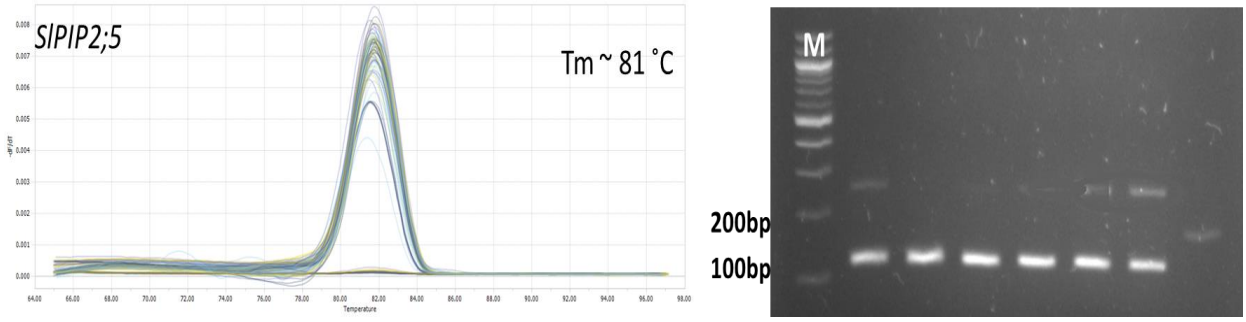

**Appendix 4. 10: Melting curves and gel image for *EF1-α***



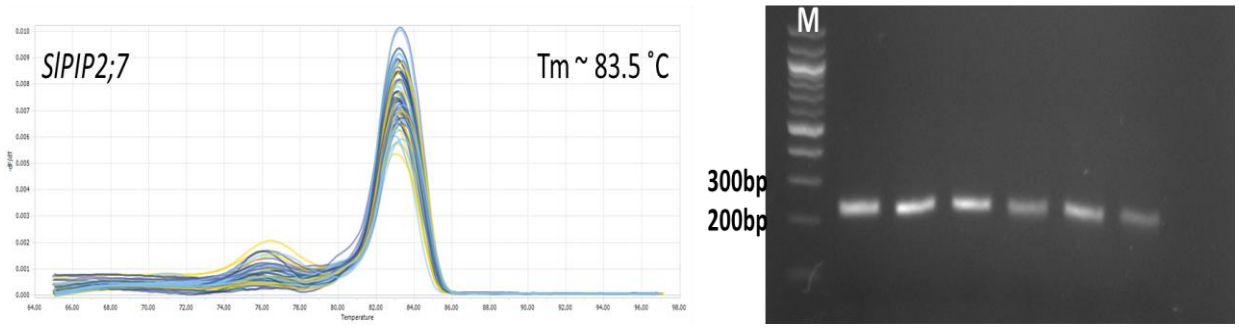

**Appendix 4. 11: Melting curves and gel image for *LeNCED1***



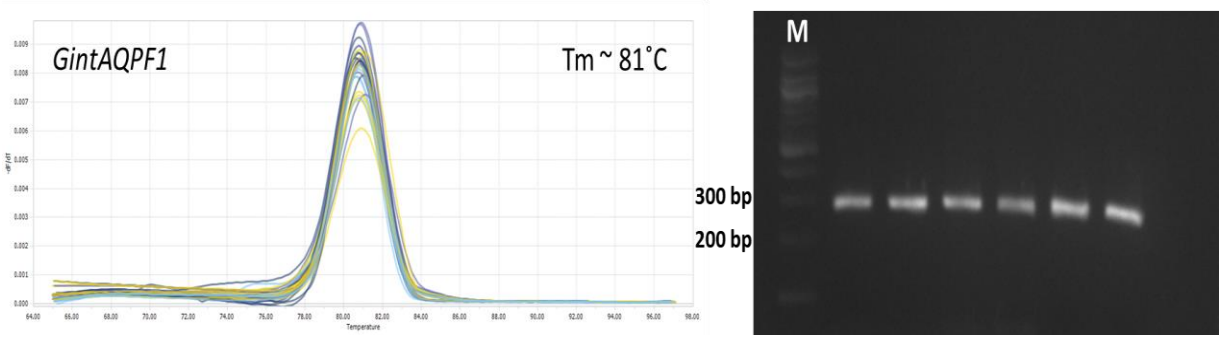

**Appendix 4. 12: Melting curves and gel image for *SlPIP2;1***



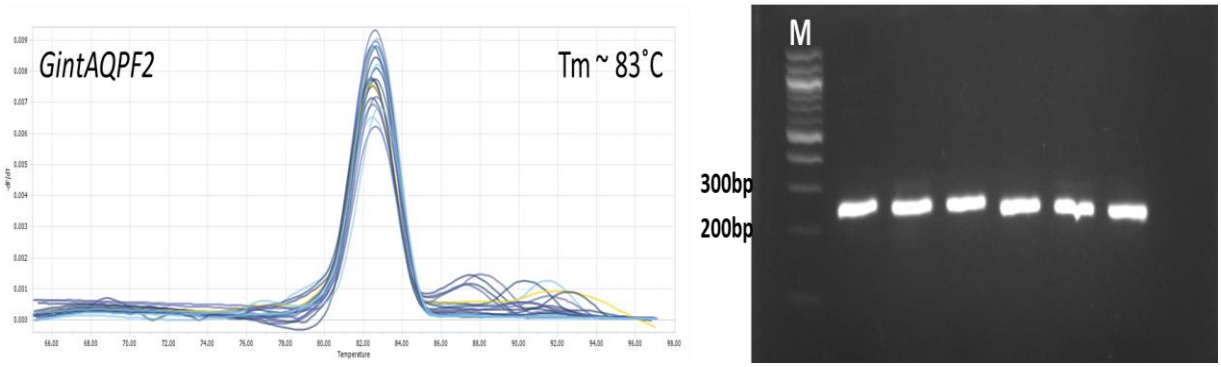

**Appendix 4. 13: Melting curves and gel image for *SlPIP2;5***




**Appendix 4. 14: Melting curves and gel image for *SlPIP2;7***




**Appendix 4. 15: Melting curve and gel image for *GintAQPF1***




**Appendix 4. 16: Melting curve and gel image for *GintAQPF2***