THE DEVELOPMENT OF AN IN VITRO SYSTEM TO ASSESS THE EFFECT OF ARUSCULAR MYCORRHIZAL FUNGI ON CEREAL CROPS IN KWAZULU-NATAL, SOUTH AFRICA

By

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Abstract

Cereal crops such as maize and sorghum are economically important in South Africa (SA) as a staple food diet. In order to meet the needs of South Africa's growing population, higher yields in crop production need to be attained. However, the two major stress factors that affect yield production and require primary attention are nutrient deficiencies and pest infestations. Research is now being focused on certain endophytes that have become a valuable tool for agriculture as they protect crops against the above-mentioned stresses. The endophyte focused on in this study was Arbuscular Mycorrhizal fungi (AMF). This research was aimed at developing an *in vitro* culture system for SA cereal crops to enable interaction studies of endophytes. This dissertation is divided into two parts; the first part focused on the development of an in vitro culture system, the assessment of sorghum plant growth and exudate production in the presence of the *Glomus intraradices* strain. The results indicated that sorghum produces the required root exudates in the second stage of growth. Using high pressure liquid chromatography with mass spectrometry (HPLC/MS), it was noted that sorghum produced phytochemicals as chemoattractants for the respective endophytes. However, it was documented that when the plant underwent certain stresses they produced exudates, which acted as phytotoxic compounds that destroyed symbiotic organisms around sorghum rhizophere. The second part focused on optimization of the surface sterilization of maize seeds. The results indicated that maize contained unidentified endophytes, which negatively affected plant development. Surface sterilization of maize seeds was accomplished. The successful *in vitro* development can be used for future use to study plant development. Understanding plant development and interaction with symbiotic endophytes would not only be of great benefit but would also make it easier to create a biocontrol agent *in vitro*, which would bring about high crop yields at cost-effective prices and would be less labour intensive.

Preface

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, South Africa, from January 2008 to December 2009, under the supervision of Mrs Sumaiya Jamal-Ally.

These studies represent original work by the author (candidate) and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it was duly acknowledged in the text.

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        Ms Avrashka Govender

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Table of Contents

List of Tables:

List of Figures:

List of Abbreviations

Arbuscular Mycorrhizal fungi... AMF

Arbuscular Mycorrhizal – Plant system... AM-P

Electrospray ionization.. ES

Growth stage 0 – coleoptile... GS-0

Growth stage 1 – Three leaf stage.. GS-1

Growth stage 2 – Five leaf stage... GS-2

Growth stage 3 – Growth point differentiation................................... GS-3

Half arbuscular mycorrhizal – plant system....................................... HAM-P

High pressure liquid chromatography.. HPLC

High pressure liquid chromatography/ mass spectrometry............. HPLC/MS

Modified Strullu Romand medium... MSR

Modified Strullu Romand medium without sugar and vitamins....... MSR-S-V

Nitrogen (N), Phosphorous (P) and Potassium (K)........................... NPK

South Africa... SA

Foreword

The research presented in this thesis was carried out over a period of two years (2008-2009). Training for the *in vitro* culture system set up was conducted at the Universite Catholique de Louvain, Belgium, over a three-month period. Thereafter, the remainder of the research was conducted at the Department of Microbiology at the University of KwaZulu-Natal, Pietermaritzburg.

Our main aim was to develop an *in vitro* culture system for two major crops in South Africa – maize and sorghum. As a result, this thesis was divided into two parts. Part A focused on sorghum crops and Part B focused on maize.

Part A consisted of two chapters:

Chapter two looked at the development of an *in vitro* culture system and investigated nutritional requirements at the different stages of sorghum development.

Chapter Three investigated the effects of Arbuscular mycorrhizal fungi (AMF) associated with sorghum. High pressure liquid chromatography with mass spectrum (HPLC/MS) was used for the detection of root exudates, which were produced by sorghum as a stimulant and/or phytotoxic compound on AMF development.

Part B consisted of the following:

Chapter Four focused on developing a maize kernel sterilization protocol. This was done in order to produce "clean" plantlets, meaning free of contaminants and culturable endophytes. Kernels were subjected to various sterilization methods, followed by germination under *in vitro* systems.

Chapter One
General Literature Review

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The biological control of pests and diseases affecting plants has been the main concern for microbiologist in the past decade (Alves, 1998). The use of agrochemicals may decrease attacks by pests and pathogens, yet they present a risk to consumers. Products such as fungicide or pesticide control fungi and pests that are harmful to plants, whilst being detrimental to indigenous microorganisms, which perform a crucial role in the environment (Azevedo, 1998). Once below-ground ecosystems containing microorganisms are destroyed, the nutrient supplies to the plants are disturbed. Apart from chemicals being hazardous, farmers in Africa cannot afford high agrochemical and fertilizer costs, thus resulting in poor crop yields (Azevedo, 1998). We now aim to use biological control agents, such as endophytes, to control pests of plants indigenous to South Africa.

Farmers in South Africa are faced with major losses in crop yields and income as a result of challenges such as pests and the cost of fertilizers. Research is now focused on creating affordable biofertilizers and biocontrol agents in an effort to decrease the loss farmers currently experience. Therefore, in this project we developed an *in vitro* culture system for two agricultural crops indigenous to SA: *Zea mays L* (maize) and *Sorghum moench L* (sorghum). In addition, the effects of Arbuscular mycorrhizal fungi on the sorghum crop were investigated.

Endophytes

Any type of microorganism, either bacterial or fungal, that resides within a plant for at least one period of its life cycle and remains symptomless is considered an endophyte (Azevedo, 1998). It may be transmitted either vertically or horizontally. In terms of fungal endophytes, vertical transmission occurs asexually via fungal hyphae penetrating the host seeds. These fungi are mutualistic as their reproductive fitness depends on their host plants. Conversely, horizontal transmission occurs sexually via spores being dispersed by wind and/or insect vectors. Fungi exhibiting this type of transmission are pathogenic in nature (Azevedo *et al.* 2000).

This research focused on a partial endophyte, as it requires a host to complete its life cycle (Paszkowski, 2006). Partial endophytes infect plants to produce larger numbers of roots and tillers, making them more drought-tolerant and able to recovery rapidly from injury. An example is Arbuscular Mycorrhizal Fungi (Declerck *et al.* 2000). This type of endophyte allows the host plant to have a higher performance rate under stressful conditions, such as nutrient and water deficiency.

Arbuscular Mycohhrizal Fungi (AMF)

Endomycorrhizae characteristically grow within the root cortical cells of plants, mainly grasses and vegetables (Ingham, 2000). AMF are the most common type of endomycorrhizae fungi. They are classified by their soil-borne spores, which are used for identification of these fungi into various genera (Figure 1.1). AMF are classified according to two suborders based on the presence/absence of vesicles (Figure 1.1). These are further divided into genus and species according to morphological characteristics, such as colour, size and shape of spores, stalk attachment, cytoplasmic structure, wall thickness, structure and ornamentation, as well as mode of spore germination and presence of secondary spores and sporocarps (Fortin *et al.* 2002 and Morton, 1988).
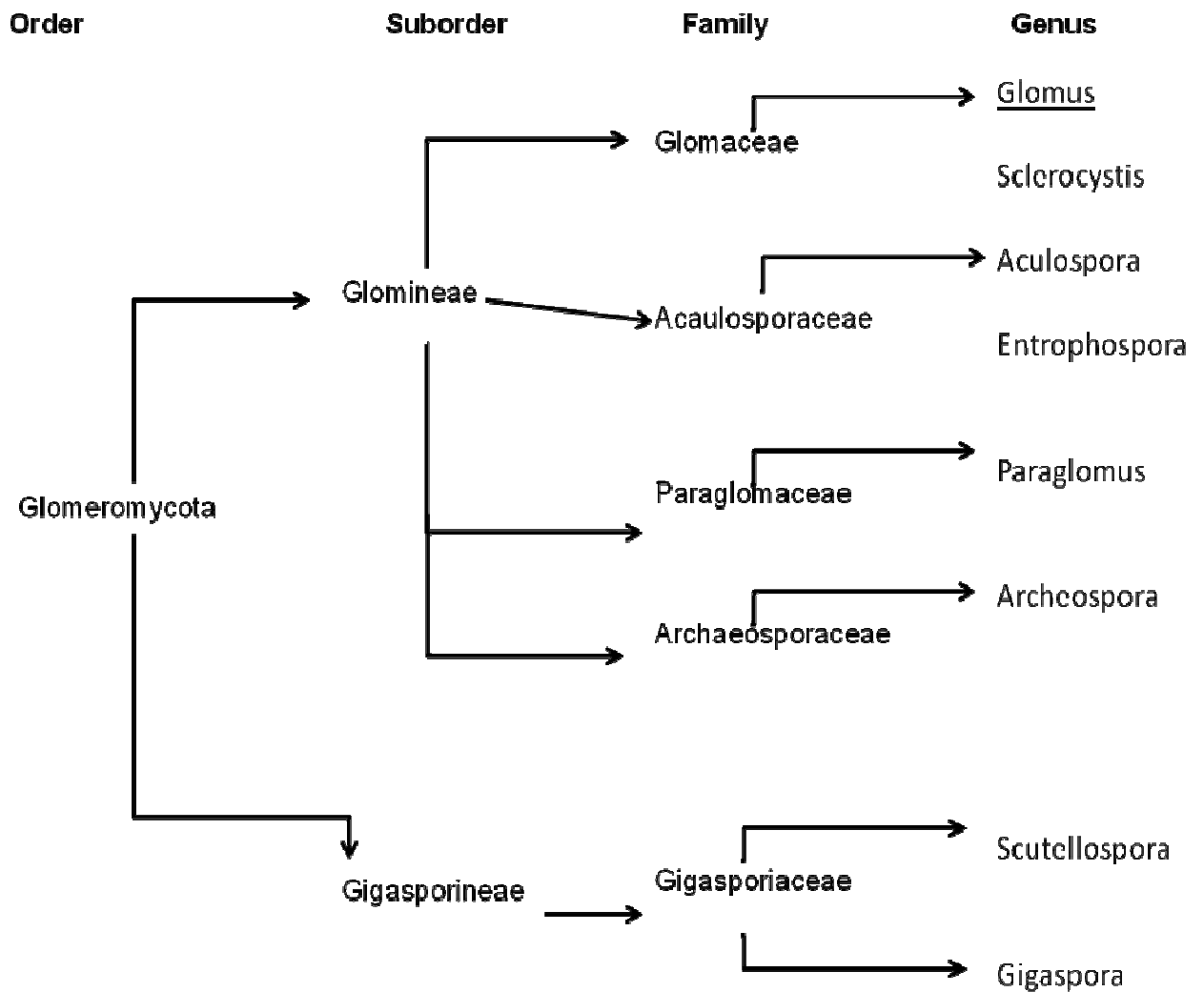
Figure 1.1: Classification of AMF, from order to genus level. Several hundred species are present for each genus. However, the genus underlined is the AMF of interest in this study. (Adapted from INVAM, 2008)

However, molecular techniques have now allowed a detailed classification to the species level by targeting the 18S ribosomal DNA region (Schuβler *et al.* 2001). Bidartondo *et al.* (2002) depicted the complete and partial 18s rDNA tree, which showed all species of AMF, only species of interest in this dissertation (underlined), *Glomus* group A- *Glomus intraradices* was depicted here (Figure 1.2)*.*
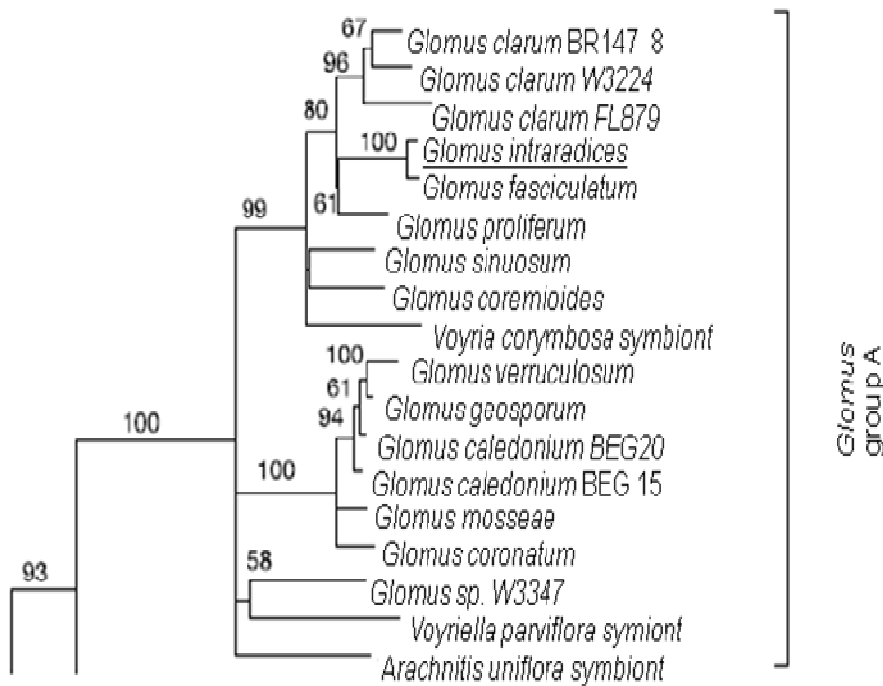
Figure 1.2: Subsection of complete 18s rDNA phylogenetic tree from *Glomus sps* of AMF known families subset myco-heterotroph symbionts, underlined was the species of study in this dissertation (adapted from Bidartondo *et al.* 2002)

AMF are dependent on the host plant for the completion of their life cycle, thus making them obligate biotrophs (Fortin *et al.* 2002). Species of AMF have a basic life cycle. However, each genus has different time periods and signalling molecules for colonization within host plants (George, 2000).
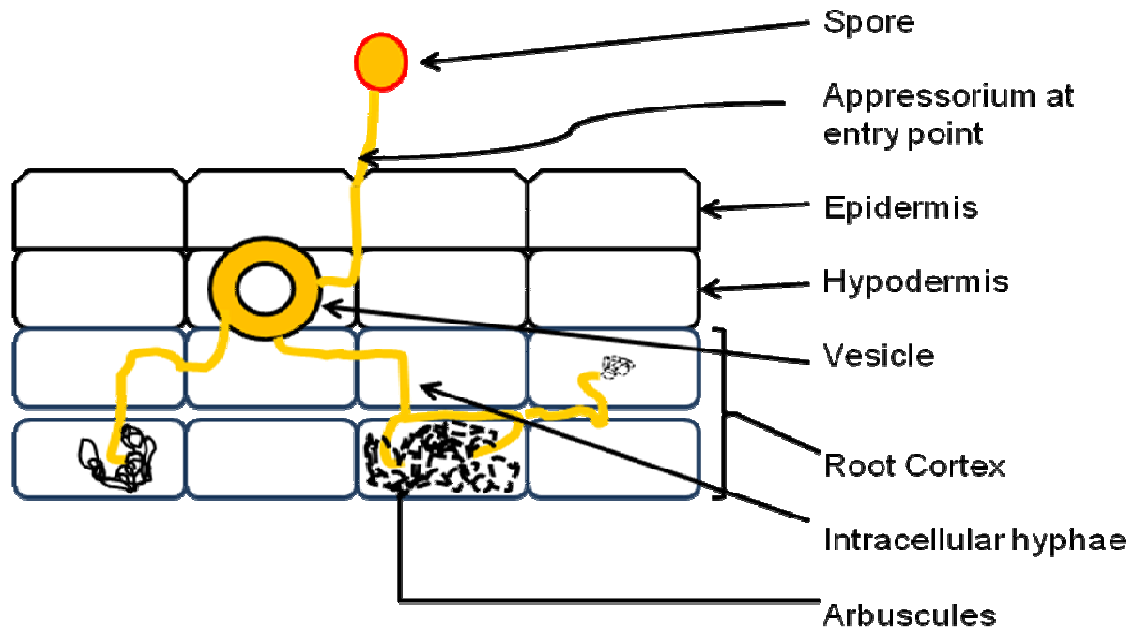
Figure 1.3 : Schematic representation of AMF infection within root cortex of host plant, which leads to a symbiotic relationship (adapted from Dhami, 2005)

Generally, AMF's are characterised by inter- and intracellular fungal growth in the root cortex, forming specific fungal structures referred to as vesicles and/or arbuscules. This characteristic growth gives the endomycorrhizae their alternate name, vesicular arbuscular mycorrhizae (Smith and Read, 1997). AMF's colonize the host root via appressorium. Once within the root cortical cells, AMF's develop highly branched arbuscules (Figure 1.3). These arbuscules act as a medium for the symbiotic relationship between the plant and fungus. Some genera are capable of forming vesicles, which are thin-walled, lipid-filled structures that usually form in the intercellular spaces. Their primary function is thought to be storage. However, vesicles can also serve as reproductive

propagules for the fungus. Reproductive spores can be formed either in the root or, more commonly, in the soil. Spores produced by fungi forming AM associations are asexual, formed by differentiation of the vegetative hyphae. For some fungi (e.g. *Glomus intraradices*), vesicles in the root undergo secondary thickening and a septum (cross wall) is laid down across the hyphal attachment, leading to spore formation. More often, spores develop in the soil from hyphal swellings (Dhami, 2005 and Sylvia, 1998). The most abundant and easily isolated AMF is the *Glomus* species. This type of genus is investigated tremendously under *in vitro* culture systems. It is widely known for intense hyphal branching and host non specificity. Thus, the genus and species of interest in this study was *Glomus intraradices* Schenck and Smith (strain 41833).

AMF's increases the uptake of nutrients from the soil, resulting in enhanced host vigour and promoting plant growth, as well as crop yield. Arbuscules provide an increased surface area for metabolic exchanges between the host and fungus. AMF develop extradical hyphae that grow into the soil, thereby increasing the potential of the root system for nutrient and water absorption, and thus soil structure is improved for better aeration and water percolation (Linderman, 2000 and Hock and Varma, 1998). This can alter membrane permeability and thus, quality and quantity of root exudation. Altered root exudation induces changes in the composition of microorganisms in the rhizophere soil (Rambelli, 1973). The net effect of these changes in plants is the ability to endure environmental stresses and tolerate or reduce the effects of plant diseases (Linderman, 1988 and Gerdemann, 1967). AMF thus protect the plant without causing symptoms contributing to induced systemic resistance (George, 2000 and Sylvia, 1990).

AMF require certain stimulants in order to colonize the plant. In this study, we assessed a strain of *Glomus intraradices* associated with sorghum, as well as the determination of root exudate properties exhibited by this crop, using *in vitro* culture systems.

Agricultural crops

In Africa, agriculture is the most important non-oil exporting economy; this income is used for the continent's population livelihood. However, farmers – particularly small-scale farmers – face many challenges, such as adverse climate change, crop nutritional requirements and pest problems. This is especially so in South Africa.

Although South Africa has a well-developed agricultural environment, small-scale farmers suffer tremendous inflation costs with regard to fertilizers, control agents and machinery. This requires them to produce high yields in order to generate a profit, as many use the funds to support their families. The major crops produced in South Africa are corn (maize), wool, sugar, peanuts and wheat (Gouse *et al.* 2005). This research focuses on *Zea mays L.* (maize) and *Sorghum moench L.* (sorghum).

Maize

Maize (*Zea mays L.)*, the second largest crop in the world after wheat, shows great genetic diversity and enables growth in different environments. Maize has many uses, such as feed, corn, alcohol, dairy and ethanol production (Sanders, 1930). Developing countries in Africa use it directly as a staple food, making it the largest production-line crop (Pingali, 2001).

Industrial farmers, unlike small-scale farmers in southern Africa, produce tremendous amounts of maize per year. Low yield production for small-scale farmers is a result of biotic and abiotic factors such as drought, low soil fertility, pests and diseases. Annually, more than 60% of maize in eastern and southern Africa suffers from devastating pest infestations, where damage due to insect pests in the field and in grain storage is detrimental to small-scale farmers. Researchers are challenged to produce maize seeds that are resistant to the above-mentioned factors (Pingali, 2001).

Currently, control methods involve chemical, biological, cultural and host plant resistance. Chemical control is the most widely used method but it is expensive and has a detrimental effect on the environment (Pingali, 2001). Biological, cultural and plant resistance methods are being studied as they are environmentally friendly. Research is now being done using microorganisms, specifically endophytes, as control agents. The best way to study maize growth and development, as well as reaction to endophytes, is via *in vitro* culture systems.

Sorghum

Sorghum is an important cereal crop throughout the world, but in Africa and India it is a staple food requirement. In Africa, it is processed into nutritious traditional foods, animal feed grain and an alternative for barley for lager beer brewing (Taylor, 2004).

Sorghum is known for its production reliability as it is drought resistant and can be grown in areas that are hot and dry (Taylor, 2004). However, this crop may be infested by a range of pests, which subsequently threaten crop yields and thus cause economic damage (Teetes, 1980). Pests may be managed via three methods: biological, chemical and cultural control. Although chemical methods are effective in preventing pest infections, they are costly and cause adverse ecological and environmental consequences, with one major disadvantage being that they also kill natural non-target organisms in the crop. Research now being conducted focuses on moving away from chemical control methods towards biological and cultural control. These methods use natural organisms such as endophytes, which act as a barrier in protecting sorghum from abiotic and biotic stresses (Taylor, 2004 and Teetes, 1980).

Aims

This study aimed at developing *in vitro* culture systems for maize and sorghum agricultural crops to investigate the interaction studies of Arbuscular Mycorrhizal fungi (AMF).

Objectives

These were as follows:

1. The development of *in vitro* culture systems for sorghum;

2. Optimization of surface sterilization of maize and introduction into *in vitro* culture systems;

3. *Glomus intraradices* strains associated with sorghum and its development were studied under *in vitro* HAM-P culture systems.

The focus was on investigating endophytic microorganisms due to their great importance for the plant host's health and survival. Not only do the endophytic strains protect the host against pests, they also cause physiological changes in their host, such as withstanding abiotic and biotic stresses. This dissertation is divided into two parts: Part A involves sorghum, focusing on the development of an *in vitro* culture system, the assessment of plant growth and exudate production in the presence of the *Glomus intraradices* strain. Part B involves maize, focusing on the development of an *in vitro* culture system, as well as the optimization of surface sterilization of maize seeds.

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PART A

Chapter Two
Development of an autotrophic *in vitro* culture system for the South African agricultural crop sorghum

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In vitro culture systems are a useful tool for investigating the development of different agricultural crop plants under sterile conditions. An *in vitro* Half Arbuscular Mycorrhizal Plant (HAM-P) culture system was developed for the sorghum crop and its development, nutritional requirements and maintenance were observed. Experiments were conducted using surface sterilized seeds. The development of HAM-P systems, initially using medium Petri plates (90 mm diameter), followed by larger Petri plates (160 mm diameter), were examined. Thereafter, nutritional requirements were investigated using the medium of Modified Strullu Romand (MSR-S-V), consisting of various chemicals that made up two concentrations: 1x and 2x *in vitro* medium. The results indicated that plants required larger systems for the effective examination of plant development. According to the results obtained for nutritional examination, it was concluded that 1x or 2x medium introduced throughout plant development decreased plant mortality and increased maintenance. However, plants that were introduced to systems initially containing 2x medium and maintained with 1x medium showed healthy growth. It was concluded that the various growth stages of sorghum require different levels of nutritional input. For instance, during its three-leaf growth stage, the plant required higher nitrogen, phosphorous and potassium (NPK) sources. However, during the five-leaf growth the plant undergoes root maturity and development, and thus the NPK sources should be low at this stage of plant development.

2.1　Introduction

Sorghum is a tropical cereal grass that is cultivated across the world in the warmer climatic areas. It is the world's fifth most important cereal grain, after wheat, maize, rice and barley. Sorghum is still largely a subsistence food crop in Africa, due to its productivity variability (Kimber, 2000). Knowledge of sorghum development and nutritional requirements is crucial for a higher crop yield production, thus *in vitro* systems are a useful tool when investigating such conditions.

2.1.1　*In vitro* culture systems

An *in vitro* system is a series of differential experiments which can be divided into three study systems. These involve "axenic systems", which refers to one organism (endophyte) growing under aseptic conditions; "monoxenic systems", referring to two organisms (endophyte and host root organ) that are grown together; and "dixenic systems", which refers to three organisms (host root organ and two types of endophytes) growing under aseptic conditions (Vierheilig and Bago, 2005).

There are many negative drawbacks when investigating plant and fungi relationships in pot culture systems. This is the reason why over the past decade *in vitro* culture systems were developed. These systems allowed us to investigate the plant and organism relationship with no interference from other organisms. However, the duplication of such systems became tedious. Voets *et al.* (2005) and Dupre de Boulois *et al.* (2006) developed two *in vitro* culture systems, namely the Half-closed Arbuscular Mycorrhizal Plant system (HAM-P) and Arbuscular Mycorrhizal Plant system (AM-P), respectively. These systems allowed for autotrophic plants and AMF to be grown in association on a synthetic medium with no sugar or vitamins. The synthetic medium contains all the macro- and micronutrients that the plant would require for development, and no excess nutrients are present as AMF need to form an association within the plant root and thus, a symbiotic relationship. This technique proved to be useful for AMF in terms of assessing the life cycle, metabolism, biochemical analysis,

symbiotic relationship, environmental factors, mass production, exudates and molecular analysis of the fungus (Vierheilig and Bago, 2005).

The system of interest and which was used in this project was HAM-P. This system was first developed to assess the spore production dynamics, intraradical root colonization, germination capacity and life cycle of AMF associated with potato plantlets (Voets *et al.* 2005). This project focused on optimizing HAM-P systems for sorghum development for the South African environment.

2.1.2 Sorghum Nutrition

In order to increase productivity of sorghum, we need to understand the growth, development and nutrient uptake of this crop. Nutritional requirements are of primary importance to crop productivity (William *et al.* 1965). Any type of deficiency/excess of macronutrients affects the growth of this crop (Table 2.1). In this study, we concentrated on the effects of macronutrients present in *in vitro* medium on plant development. Deficiencies in the macronutrients potassium, calcium and phosphorous interact with each other to negatively affect plant development (Table 2.1). Similarly, an excess of macronutrients such as magnesium ions, potassium, calcium, nitrogen, and phosphorous interact with each other to negatively affect plant development (Table 2.1).

Micronutrients play an important role in sorghum development, mainly after the third stage, bringing about differentiation and seed development (Table 2.2) (Sprague, 1961). The primary function of micronutrients is to regulate synthesis and the utilization of carbohydrates, mainly at a later stage in sorghum development (Yawalkar *et al.* 1977). Depending on the sorghum variety and hybrid type, nutritional and temperature requirements might differ and therefore, knowing the optimal requirements is imperative (Kimber, 2000).

Table 2.1: Various macronutrients' characteristic effects on sorghum crop development used under *in vitro* medium

Macronutrients	Properties			
	Function	**Excess**	**Deficiency**	**References**
Potassium	Helps in the building of protein, photosynthesis	Modifies root, which results in ion imbalance of Ca	Immobility root uptake thus lower rates of photosynthesis and water uptake, resulting in weakened grain crops and stem	• Devitt *et al.* 2006 • Abida *et al.* 2007
Calcium	An essential part of plant cell wall structure	Unable to regulate Ca^{2+} increased cation ion uptake such as K^+	Weak stem and limited root development	• Hepler, 2005 • Baker and Ray, 1965
Phosphorous	Essential part in photosynthesis, forms starches	Uptake inhibited by root modification caused by imbalance of K and Ca	Retards growth & purple discolouration of leaves	• Devitt *et al.* 2006 • Mulla and Bhatti, 1997
Nitrogen	An integral part of chlorophyll	Can modify root uptake and exudation properties. Thus, weakens roots, creating nutrient imbalance	-	• Burton and Fortson, 1966 • Wojtaszek *et al.* 1993 • Pageau *et al.* 2002
Magnesium	Part of chlorophyll	Prevented from being taken up by plant roots	-	• Abida *et al.* 2007

Table 2.2: Various micronutrients that affect sorghum development under *in vitro* systems (adapted from Sprague, 1961 and Yawalkar et al. 1977)

Micronutrients	Properties	
	Function	Deficiency
Zinc	Regulates utilisation of carbohydrates	Upper leaves turn white
Iron	Chlorophyll formation	Yellowing between vein and leaves in younger plants
Manganese	Functions with enzymes to breakdown carbohydrates	Interveinal chlorosis
Boron	Regulates nutrients and aids in production of sugars and carbohydrates	Maturation stage of plant droops
	Aids in development of seed grain	
Copper	Maturation development stage	Light colouration of entire plant
	Aids in root metabolism	Younger leaves curl, twist and turn yellow at the tip
Chlorine	Plant metabolism	Wilt and chlorosis

2.1.3 Sorghum Development

Ten different development stages occur in sorghum (stages 0-9); these stages are defined from Growth stage zero – emergence to Growth stage nine – physiological maturity (Table 2.3). The time required to reach each stage depends on both the hybrid and the environment in which it is growing.

Plant development was observed by examining leaf maturity in terms of size, shape, colour and number. These factors determine crop productivity and nutrient deficiency/excess (William *et al.* 1965). The literature indicated that many agrochemicals added after Growth stage three, since the crop is susceptible to pathogenic attacks and nutrient deficiencies (Vanderlip and Reeves, 1972). We aimed at developing and investigating an *in vitro* culture system for sorghum to test nutritional requirements under absolute controlled sterile environmental conditions.

Table 2.3: Ten growth stages of sorghum development under field conditions (adapted from Vanderlip and Reeves, 1972)

Growth name	Growth stage	Days of occurrence	Description of plant
Emergence	0	3-10	Germination of seeds leading to coleoptile visible at the soil surface.
3 leaf stage	1	10th	Collar of 3rd leaf is visible.
5 leaf stage	2	20th	Collar of 5th leaf is visible.
Point differentiation	3	35th	Plant undergoes growing point differentiation. This stage is critical as agrochemicals should be added at this point.
Final leaf	4	45th	Final leaf visible is in a whorl.
Boot	5	50th	The head is extended into flag leaf sheath. At this stage all leaves have fully expanded, the head is full size and is covered by the flag-leaf sheath. Rapid growth and nutrient uptake continue.
Half-bloom	6	60th	Half of the plants in a field are in some stage of bloom. Flowering progresses from the tip of the head downward over a period of 4 to 9 days.
Soft dough	7	70th	The grain has a dough-like consistency and grain fill is occurring rapidly.
Hard dough	8	85th	At this stage, approximately three-fourths of the grain dry weight has been attained. Nutrient uptake at this point is complete.
Physiological maturity	9	90th	This stage is determined by the dark spot seed on the opposite side of the kernel from the embryo.

2.2   Materials and Methods

All methods for the experiments were carried out according to Voets *et al.* (2005) with modifications indicated.

2.2.1   Medium preparation

Two concentrations of Modified Strullu Romand (MSR) medium (Refer to appendix 1 for stock solution make up and suppliers details), lacking vitamins and sugars, were used in the following experiments (Table 2.4). The following stock solutions were prepared for construction of MSR-S-V: Macro-elements (KNO_3, KCL and $MgSO_4.7H_2O$), Calcium Nitrate, NaFeEDTA, Micro-elements ($MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, $CUSO_4.5H_2O$ and $(NH_4)_6Mo_7O_{24}.2H_2O$). Millipore water was added to dissolve the constructs (Table 2.4) and brought to a volume of 1 litre, while the pH was adjusted to 5.5 using Microprocessor pH 211 meter (Hanna instruments). The solution was solidified with 3g/l Phytagel. Medium was then Autoclaved (Speedy vertical type) for 15 minutes at 121°C.

Table 2.4: Preparation of 1 litre of Modified Strullu Romand lacking sugars and vitamins (MSR-S-V) medium concentration, thereafter steam sterilized at 121°C for 15min

Stock solutions	Concentration of MSR-S-V medium (ml/l)	
	1x	2x
Macro-elements*	10	20
Calcium Nitrate*	10	20
NaFeEDTA*	5	10
Micro-elements*	1	2

*Key: * Refer to appendix 1 for stock solution preparation and/or ordering information.*

2.2.2 Plant material

An unknown variety of untreated South African sorghum seeds was surface sterilized with Sodium hypochlorite (3.5%) in a 50ml sterile falcon tube (Merck chemical and lab supplies) for 15 minutes, then rinsed four times in sterile deionized water for ten minutes. Using sterilized stainless steel forceps, surface sterilized seeds were then plated into medium Petri plates (Merck chemical and lab supplies) (90mm diameter, five seeds/plate) containing 1 x concentrated MSR-S-V media. Petri plates were then sealed with Paraflim (Merck chemical and lab supplies), followed by incubation for three days in dark incubator (Stay cold incubator set at 27˚C), to allow for germination, with all seed germination points were placed in one direction into the plate. Thereafter, germinated seeds were placed in a light phytotron (22˚C day/ 18˚C night) for photosynthetic tissues to form in the plant.

2.2.3 Development of an autotrophic culture system

The system used for this experiment was the HAM-P culture system and set up was done by constructing a hole on the edge of the lid and base of Petri plate (either 90mm (Merck chemical and lab supplies) and/or 150mm diameter (Capital lab supplies) containing either 1x/2x concentrated MSR-S-V medium. Seven-day-old germinated Sorghum seeds were transferred to each Petri plate. Under a laminar flow hood (horizontal), close to a Bunsen burner, sterilized stainless steel forceps were used to aseptically transfer one germinated seed into 150mm diameter Petri plate, with roots placed inside the plate on the medium and the shoot protruding outwards through the hole of each Petri plate. The Petri plates were sealed with Paraflim (Merck chemical suppliers) and the area with the hole was further covered with sterilized silicon grease (Hardware outlets) to avoid any contamination. Thereafter, the plates were covered with an opaque plastic bag (to allow a dark environment for root development) and incubated in a phytotron with well regulated moisture and phototrophic settings*. Every two weeks (depending on plants nutrient requirements) *in vitro* HAM-P

culture systems were maintained, by adding MSR-S-V into each Petri plate. This was done to provide renewed nutrients to the plant for optimum development.

*Phytotron setting:
- ○ 22˚C/18˚C day/night
- ○ 16 hours photoperiod
- ○ For moisture, 4x 2-litre containers were filled with water and placed around the plants. These containers were filled with clean water every 5 days.

Table 2.5: Experimental design of HAM-P culture systems, to determine the optimum growth of Sorghum plants, in terms of MSR-S-V nutrient requirements and size of systems (n=10)

Petri plate diameter	MSR-S-V concentration used in sorghum HAM-P systems	
	Newly set up HAM-P system	Maintenance
90mm (Merck chemical and lab supplies)	1x	1x
150mm (Capital lab supplies)	1x	1x
150mm (Capital lab supplies)	2x	2x
	2x	1x

Four different HAM-P culture systems were designed for this experiment, in order to determine optimum growth of the sorghum plant (Table 2.5). All parts of the experiment were carried out aseptically under the laminar flow hood (Horizontal).

The table above describes the Petri plate diameter size and concentration of the MSR-S-V medium used in each experiment.

It was important to note that at different stages, maintenance occurred when roots were no longer submerged or in contact with growing medium. Hence, 20-30ml of respective concentration medium was added to *in vitro* systems containing sorghum.

2.3   Results

Sorghum seed germination occurred between three to five days after sterilization (Section 2.3.1). System development sizes were compared between medium (diameter 90mm) and large Petri plate systems (Section 2.3.2). Nutritional requirements of sorghum were investigated in large systems (Section 2.3.3). All table results are average values of each system (refer to appropriate Appendices for each system results). All shoot lengths were measured from stem to end of highest leaf. Systems were observed weekly for plant development. All four experiments were repeated various times (n=10).

Seed sterilization and germination

On the third day, all germinated seeds showing root growth were placed in an upright position in the dark incubator, and left for a further two days. This promoted sorghum geotropism and allowed for root development in one direction (Figure 2.1a). On the fifth day, the germinated seeds were placed in phytotron with light, to strengthen the seedlings and allow for photosynthetic tissues to form in plant (Figure 2.1b). After a week of germination, the emergence stage (GS-0) of sorghum was observed, whereby coleoptile was easily noted (Figures 2.1b and 2.2). Following this observation, HAM-P systems were developed (Figure 2.2) and sorghum growth was thereafter observed.

Plant development in HAM-P systems differing in size

Results indicated significant differences in plant development between the 90mm (medium) (Table 2.6 and Figure 2.3) and 160mm diameter (large) (Table 2.7 and Figure 2.4) Petri plates.

Table 2.6: Average plant development of HAM-P culture systems in medium Petri plates with 1x concentration, observed weekly and incubated in a phytotron

| Weeks | Average plant development | | | Contamination | Figure |
	Root growth	Shoot (cm)	Maintenance		
1	++	4.35	0	0	2.3a
2	++	13	1	0	2.3b
3	+++	21.25	1	1	2.3c
4	++++	26.11	1	1	-

Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots; ++++, root system starting to discolour; 1, yes and 0, no. The table above illustrates average values of Appendix 2.

Table 2.6 and Figure 2.3 indicated plant development in medium HAM-P systems. It could be clearly observed that sorghum had a healthy development until the third week, where the plant roots system covered the entire plate and discolouration occurred (Figure 2.3). Weekly maintenance was required, resulting in contamination.

The results indicated three growth stages: GS-0, three-leaf (GS-1) and death during five-leaf stage (GS-2). Growth stages were observed in accordance to root and leaf development.

Week 1 was GS-0, which moved towards GS-1, during which root development occurred rapidly for metabolites production. This rapid root development carried on for the next two weeks, which resulted in weekly maintenance to replace nutrients utilized by the rapidly developing plant. Developing roots are sensitive to environmental changes that surround it. Thus, by maintenance occurring

weekly, roots were easily subjected to opening and closing of Petri plates, which results in them being easily contaminated.

GS-1 persisted for three weeks until death of plant. This was due to roots adapting to the environment and in search of new space and nutrients. Due to the root space deprivation, the plant underwent stress, resulting in the drying of leaves. The main reason for death of plant was due to the abundance of roots, which resulted in insufficient nutrient uptake to the rest of the plant for further development, leading to plant death. Space deprivation had many drawbacks, such as nutrient uptake, root abundance and stress, contamination and death of plant. This system was tedious and the depiction of plant development in terms of leaves and roots was unclear. Thus, smaller systems proved to be inefficient for sorghum studies in upcoming chapters.

The above results were compared to larger system plates. It was proved that larger plates were easier for sorghum leaf and root studies (Table 2.7 and Figure 2.4).

The results indicated slow development of the plant from week 1, during which

Table 2.7: Average plant development of HAM-P culture systems in large Petri plates, with 1x concentration medium, observed weekly and incubated in a phytotron

| Weeks | Average plant development | | | Contamination | Figure |
	Root growth	Shoot (cm)	Maintenance		
1	+	4.1	0	0	-
2	++	7.8	0	0	-
3	+++	10.2	1	0	2.4a
4	+++	13.6	0	0	2.4b
5	++++	13.5	1	0	2.4c

Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots; discolouring of roots observed; ++++, root system starting to discolour and dry; 1, yes and 0, no. The table above shows average values of Appendix 3.

the leaf length increased gradually until the last week, with constant increases in leaf length being observed. Apart from the constant increase in leaf length; the discolouration of the leaves indicated a nutrient deficiency (Figure 2.4). Conversely, large systems proved to be beneficial for sorghum studies as less maintenance of the systems was required, while space deprivation was not a critical issue and lastly, there was no contamination.

The results represented in Table 2.7 and Figure 2.4 were unusual as GS-1 developed quicker in the first two weeks of plant development. However, when the plants were introduced into HAM-P systems, the GS-0 and GS-1 matured within seven days (Table 2.7). It was observed that as plants matured towards GS-2, their roots and leaves discoloured (Figure 2.5).

The results indicated that the emergence stage requires higher nutrients, as the plant leaves turned purple at the beginning of week 2 and matured with purple discolouration, eventually leading to plant death (Figure 2.4). Plant death resulted when further nutrients could not be located within the medium (Figure 2.4c).

One major factor that affected sorghum plant development was nutrient concentration at the different stages. Large systems proved to be beneficial for observing nutrient requirements.

Plant development in large HAM-P systems with varying nutrient concentrations

Large systems proved to be beneficial for studying the development of sorghum. One major factor taken into account was the nutrient concentration supply to crops at different growth stages. This section investigated sorghum nutrient factors. Sorghum was developed into systems containing 2x medium throughout its growth (Table 2.8 and Figure 2.5). These results were compared to sorghum development in systems initially containing 2x medium, thereafter maintained with 1x medium (Table 2.9 and Figure 2.6). Growth stages were determined according to healthy leaf number and root development. GS-0, a coleoptile, was clearly noted (Figure 2.2). The next stage, known as GS-1, involved the coleoptile drying and newer leaves forming. GS-2 indicated the maturation of leaves and the formation of newer leaves (Figures 2.5 and 2.6). The leaf numbers in the tables below include healthy and drying leaves.

As observed in Table 2.8, a greater nutrient concentration was not necessarily beneficial for plant development at the different growth stages. As plants acclimatized to these conditions, they required greater concentrations of macronutrients and micronutrients. As these nutrients were limited, the plants suffered a nutrient imbalance and would cease to develop (Figure 2.5). The results in Table 2.8 indicated the fast development of plants from week 1, when the plant leaf length number increased. Maintenance occurred in the third week of development. However, it was noted that leaf numbers remained constant (Figure 2.5).

Table 2.8: Average plant development of HAM-P culture systems in large Petriplates, with 2x concentration medium, observed weekly and incubated in a phytotron

| Weeks | Average plant development | | | | Contamination | Growth stages |
	Root growth	Leaf numbers	Shoot (cm)	Maintenance		
1	++	6	6.8	0	0	GS-1
2	+++	6	12.6	0	0	GS-1
3	++++	7	14.7	1	0	GS-2
4	++++	5	18.4	0	0	GS-2
5	++++	5	21.4	1	0	GS-2

Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots and discolouring of roots observed; ++++, root system starting to discolour and increase at a constant rate; 1, yes and 0, no. The table above shows average values of Appendix 4.

According to Table 2.8 and Figure 2.5, GS-1 persisted for two weeks and thus root development increased in this time, while the leaves discoloured due to a nutrient imbalance. The plants required more nutrients in the third week. As the nutrients were insufficient, leaf length increased slowly and root development remained constant in GS-2. As nutrients were not provided, the plants ceased to develop, resulting in plant death (Figure 2.5).

Table 2.9 indicated that the systems were beneficial for the studying of sorghum development. Maintenance occurred once every third week. Root development increased at a constant rate.

Table 2.9: Average plant development of HAM-P culture systems in large Petriplates, with 2x concentration medium and maintained with 1x concentration medium; plants were observed weekly and incubated in a phytotron

| Week | Average plant development | | | Maintenance | Growth stages |
| | Root | Shoot | | | |
		Length (cm)	Number		
0	+	5	2	0	GS-O
1	++	8.55	5	1	GS-1
2	++++	12.5	7	0	GS-2
3	++++	15.05	7	0	GS-2
4	++++	17.35	7	0	GS-2
5	++++	19.5	5	1	GS-2
6	++++	22.25	5	0	GS-2
7	++++	23.65	7	0	GS-2
8	++++	26.05	9	1	GS-3

Key: +, growth with new lateral roots; +++, growth with numerous lateral roots and discolouring of roots observed; ++++, root system starting to discolour and increase at a constant rate; 1, yes and 0, no. The table above shows average values of Appendix 5.

The results indicated that GS-2 persisted for one week (Table 2.9), which was quicker than the two weeks persistence (Table 2.8). This system showed crucial keys of sorghum developmental stages. As observed in the average plant development of HAM-P culture systems in large Petri plates, with 2x concentration medium and maintained with 1x concentration medium (Table 2.9), plants required higher nutrient concentrations for germination and GS-0. Thereafter, plants required lower nutrient concentrations for GS-1 and GS-2, as these two stages involved root development and leaf maturity. These two stages produce root exudates mainly for plant protection. The results illustrated that GS-2 persisted for 39 days, which did not match the literature provided. Leaf length increased at a constant rate and was healthy during these two stages (Figure 2.6). However, the plant root system increased gradually, as did the leaves (lengths and numbers) (Figures 2.6 a-d and Table 2.9). Plant leaves remained healthy, with no deficiencies noted during these weeks of development. However, during the seventh to eighth week of plant development, nutrient deficiencies were noted (Figures 2.6e-f). This signalled that plants required increased nutrient concentrations from this point onwards for the next stage of plant development, Growth point differentiation (GS-3).

2.4   Discussion and Conclusion

This chapter dealt with the development of an *in vitro* culture system for sorghum. Additionally, we studied the nutrient concentration supply for sorghum developmental stages. This chapter investigated four stages (GS-0, GS-1, GS-2 and GS-3). Our results were in accordance with that of Vanderlip and Reeves, (1972) in that each stage required different concentrations of nutrients for optimum growth and yield. Additionally, sorghum growth was stunted due to insufficient space, thus the root system underwent stress, which resulted in sorghum plant death.

Nutrients played an important factor in plant development (Tables 2.1 and 2.2). All macronutrients interact together to provide optimal plant development (Devitt *et al.* 2006). Potassium was important for plant functioning, in terms of enzyme activity and increased uptake of ions, such as calcium ions. A deficiency in potassium leads to weak stem development (Devitt *et al.* 2006). Generally, Potassium allows for the uptake of calcium ions required for the absorption of Phosphorus. Phosphorus was an important factor in plant development in terms of energy transfer and photosynthesis interaction (Hepler, 2005). A deficiency in Phosphorus resulted in plant leaves exhibiting purple discolouration. Phosphorus was absorbed mainly by roots and then transported to the rest of the plant; a lack thereof resulted in the extension of root systems in search of accessible Phosphorus (Devitt *et al.* 2006). This extension of roots resulted in decreased space within the *in vitro* system, thus plants underwent abiotic stress, leading to an insufficient nutrient supply to other parts of the plant. Our results indicated that larger systems were beneficial for studying this cereal crop. Once plants had sufficient space for root development, the different stage requirements for the healthy growth of the plant could be investigated.

The first stage of sorghum development was GS-0. According to Vanderlip and Reeves, (1972) GS-0 usually occurred between three to ten days. However, in this study it occurred on the seventh day, when coleoptile was easily noted. According to Vanderlip and Reeves, (1972) this stage requires a high nutrient supply, as leaf development and maturity occurs here. GS-0 determines the time taken for the plant to increase to maturity. If fewer and/or excess nutrients were supplied, the subsequent stages produced discoloured leaves (Sprague, 1961). Discolouration of leaves indicated imbalance of nutrients present in the growing medium, with leaf colour indicating the type of deficiency/excess. This was indicated in our results. More media for maintenance of plant growth was only added once the medium level in the Petri plate receded below the root level, i.e. roots no longer submerged in the media.

GS-0 proceeded towards GS-1 for sorghum development. Compared to twenty days of development for GS-0 and GS-1 combined (Vanderlip and Reeves, 1972), our results indicated quicker development within ten days of the combined stages. Upon GS-0 maturity, GS-1 used up the excess nutrients for leaf maturity. Vanderlip and Reeves, (1972) concluded that GS-1 occurred around the tenth day and matured for 10 days towards GS-2. Yet our results showed quicker maturity of GS-1 towards GS-2, within eight days. GS-1 mainly involved adaptability and the production of metabolite, which allowed for rapid root development in GS-2. Our results indicated that if excess nutrients are supplied at GS-2, a nutrient imbalance occurred, resulting in root exudates being modified. Thus the plant required different nutrient compounds in order to meet the demands of plant development (Pardales and Kono, 1990).

The next stage of sorghum development was GS-2, in which excess root development occurred. This complicated order of lateral branching gives rise to many lateral roots all differing in age, size and metabolic activity (Pardales and Kono, 1990), so that the plant can produce the metabolites required to protect it whilst it matures towards GS-3 (Vanderlip and Reeves, 1972). GS-2 does not require any excess nutrients, which may be harmful to further development. This was clearly observed when plants were subjected to double concentrated medium throughout their development, as indicated in our results. As indicated previously, all macronutrients interact together, to provide optimal plant development (Devitt *et al.* 2006). Increased Potassium in the plant leads to increased calcium ion uptake. This in turn increases the uptake of nitrates and phosphorus, which can be beneficial, but not during the young stage in sorghum development (Vanderlip and Reeves, 1972).

The increased uptake of nitrates at GS-2 modifies the roots (Pageau *et al.* 2002). According to Muchow's, (1998) findings, sorghum's nitrogen use efficiency (NUE) is highly variable under different climatic, growth stage and soil and management conditions. Excess nitrogen uptake resulted in lower NUE, by allowing excess nitrogen compounds to be stored as the non-toxic nitrogen-rich compound, asparagines, a stimulant of the parasite *Striga hermonthica*. These

excess amino acids produced modified root uptake behaviour, and thus nutrients required for plant development would be inaccessible to the plant (Pageau *et al.* 2002). By decreasing the uptake of macronutrients such as Potassium and Phosphorus, plants can undergo various deficiencies, including the purpling of leaves and weakened stems. Due to root modification there is a decrease in the absorption of Potassium and Phosphorus, resulting in an increased concentration of these two macronutrients in the rhizosphere. There is then an inhibition of magnesium ion uptake, resulting in the plant suffering from chlorotic stress (Abida *et al.* 2007). Our results indicated that when the plant moves onto this stage, leaves produced deficiencies that could act as a signalling method for excess nutrient concentration supplies, which was in accordance with Vanderlip and Reeves, (1972). Plant leaves discolouration observed in Figure 2.4 were possibly due to a deficiency of Potassium, Calcium and Phosphorus macronutrient supplies. GS-2 persisted for four weeks until plant death (Table 2.7), probably due to the plant attempting at this stage to recover from nutrient stress by extending the roots in search for nutrients.

Growth point differentiation is a crucial stage for farmers, as the plant requires more nutrients from this stage onwards. Farmers would have to invest in fertilizers to sustain plants for increased productivity. If nutrients are deficient at this point of differentiation, the crop would have low productivity. The main nutrient sources at this stage are potassium and Phosphorus. Increasing these two sources consecutively would increase interaction with other macronutrients, as well as with micronutrients (Subba *et al.* 1993). This fact was clearly observed in our results with the double-single concentration system, where leaves showed a purple discolouration indicative of phosphorous deficiency. Weak stem development was also observed, indicating a potassium deficiency (Devitt *et al.* 2006).

Nutrients play an important role in plant development. Excess nutrients at the incorrect stage of development can result in plant root modification, subsequently affecting other nutrients adversely and depriving the plant. It was clearly observed that sorghum development understanding is crucial for crop productivity. GS-0-1 required a high amount of nutrients, so that the plant can develop and mature to the next stage. However, at GS-2, the plant required minimal nutrients, as this stage mainly involved root development and leaf maturation. Once the leaves had matured and the plant for their protection secreted root exudates, GS-3 was then accomplished. At this stage, many farmers use Agrochemicals for fertilization as growth is at a point of differentiation and the plant is one third close to maturity of grains. After stage 3, nutrient uptake becomes crucial and farmers spend a tremendous amount of money fertilizing their crops for increased productivity. It is also important to note that temperature and climate play a vital role in development. Nutritional knowledge is only beneficial when sorghum is produced under optimum temperature and climatic conditions. According to the determined *in vitro* results for South African sorghum variety, the perfect temperature was 22°C, with a 16 hour photoperiod.

2.5   Figures
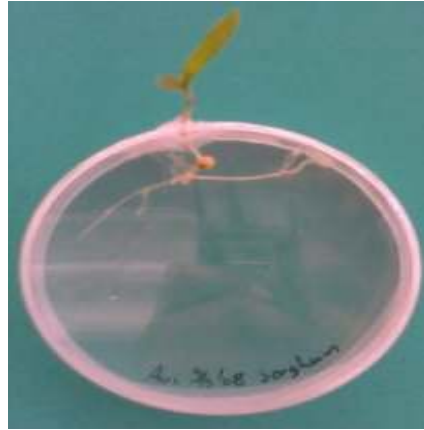
Figure 2.1:   Newly set up HAM-P culture system of a 5-day-old germinated sorghum seed, 90mm Petri plate, coleoptile easily noted.
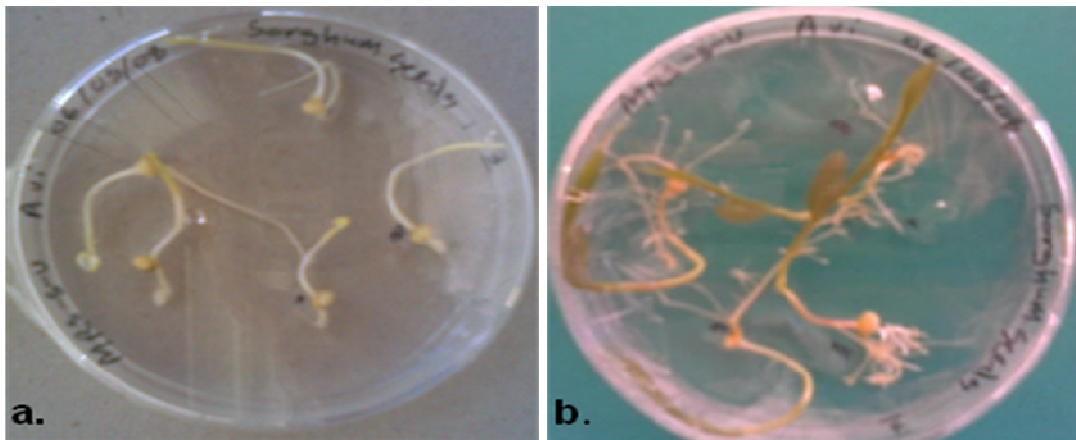
Figure 2.2: Surface sterilized germinated sorghum seeds in MSR-S-V plates: a. 3-day-old germinated seeds, where plates were incubated in one position and b. 5-day-old germinated sorghum seeds showing coleoptile, ready to be introduced into HAM-P systems.
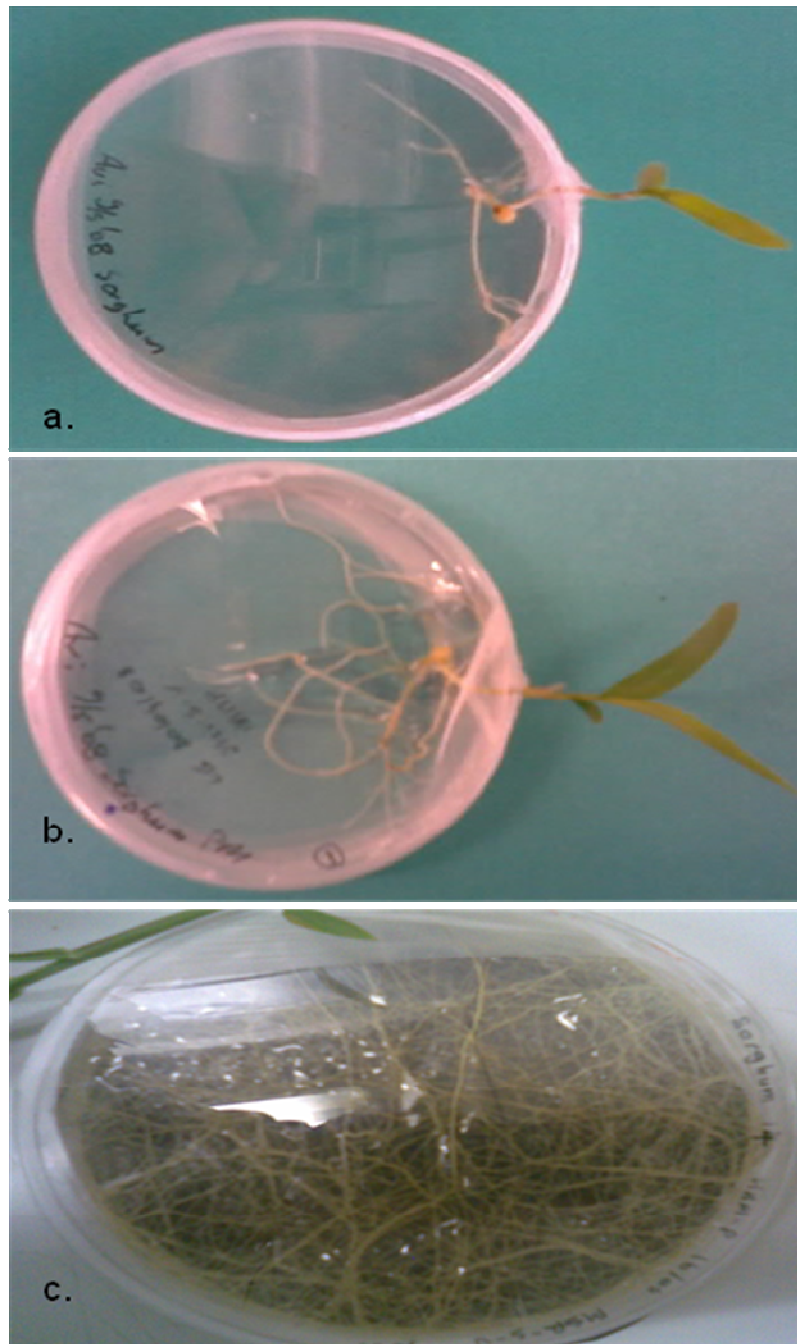
Figure 2.3: Weekly observation of sorghum development in HAM-P culture system using smaller systems (90mm diameter): a. one-week-old system, b. two-week-old system, and c. three-week-old system. The rapid root development was clearly noted.
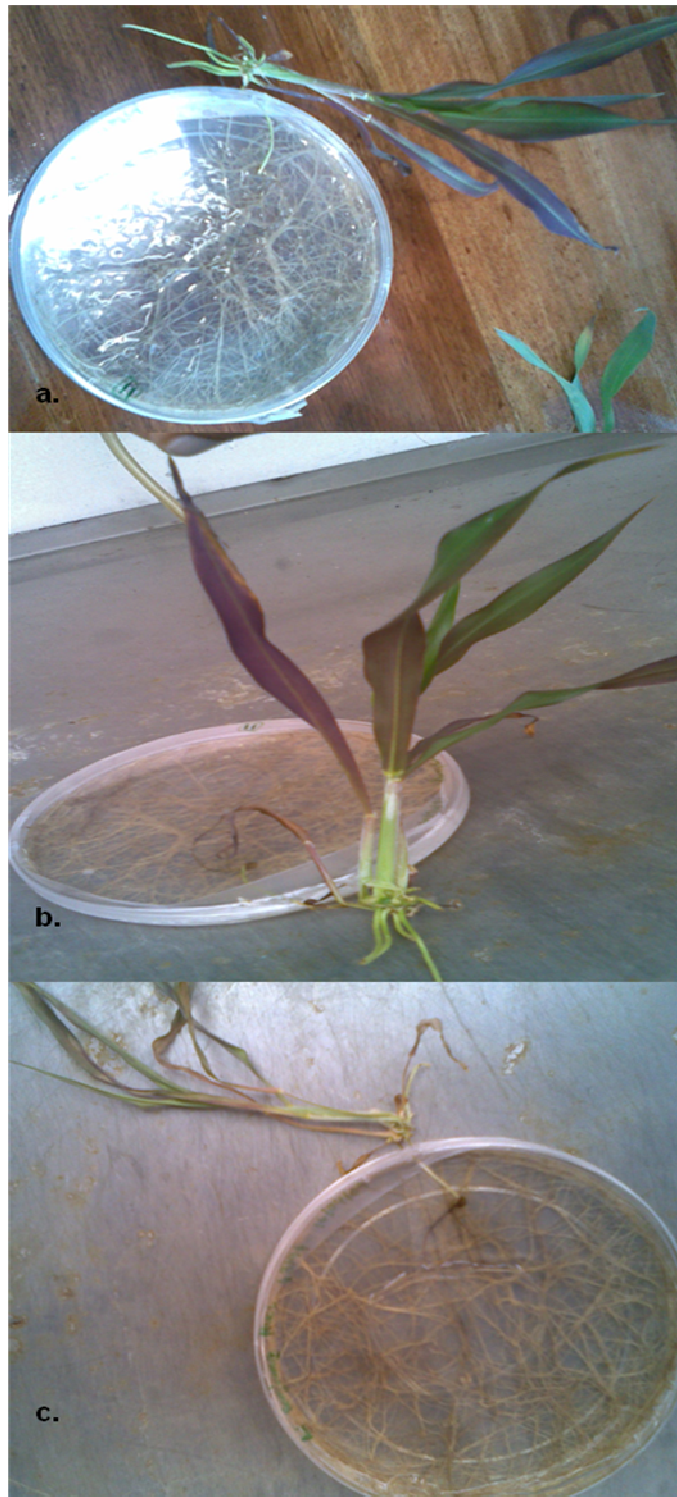
Figure 2.4: Weekly observation of sorghum development in HAM-P large Petri plate culture system (1x concentration medium). All figures were at GS-2: a. three-week-old system, b. four-week-old system, and c. five-week-old system. Images above depict colour change due to nutrient deficiency.
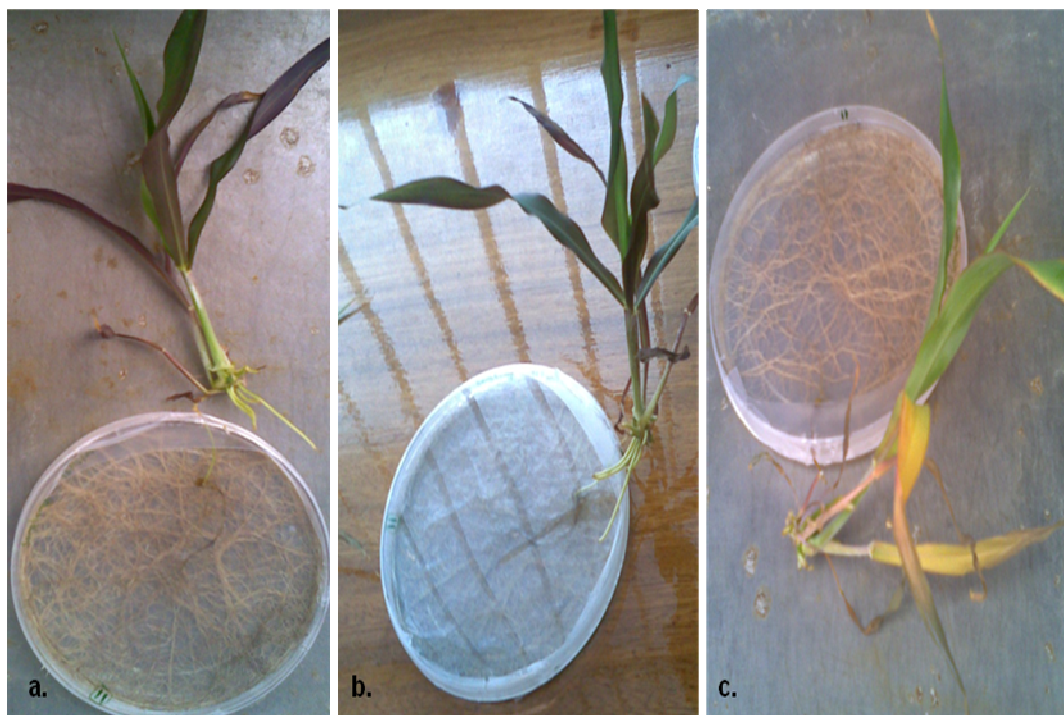
Figure 2. 5: Weekly observation of sorghum development in HAM-P large Petri plate culture system (2x concentration medium): a. two-week-old system, b. four-week-old system, and c. five-week-old system. Images above depict colour change due to nutrient deficiency.
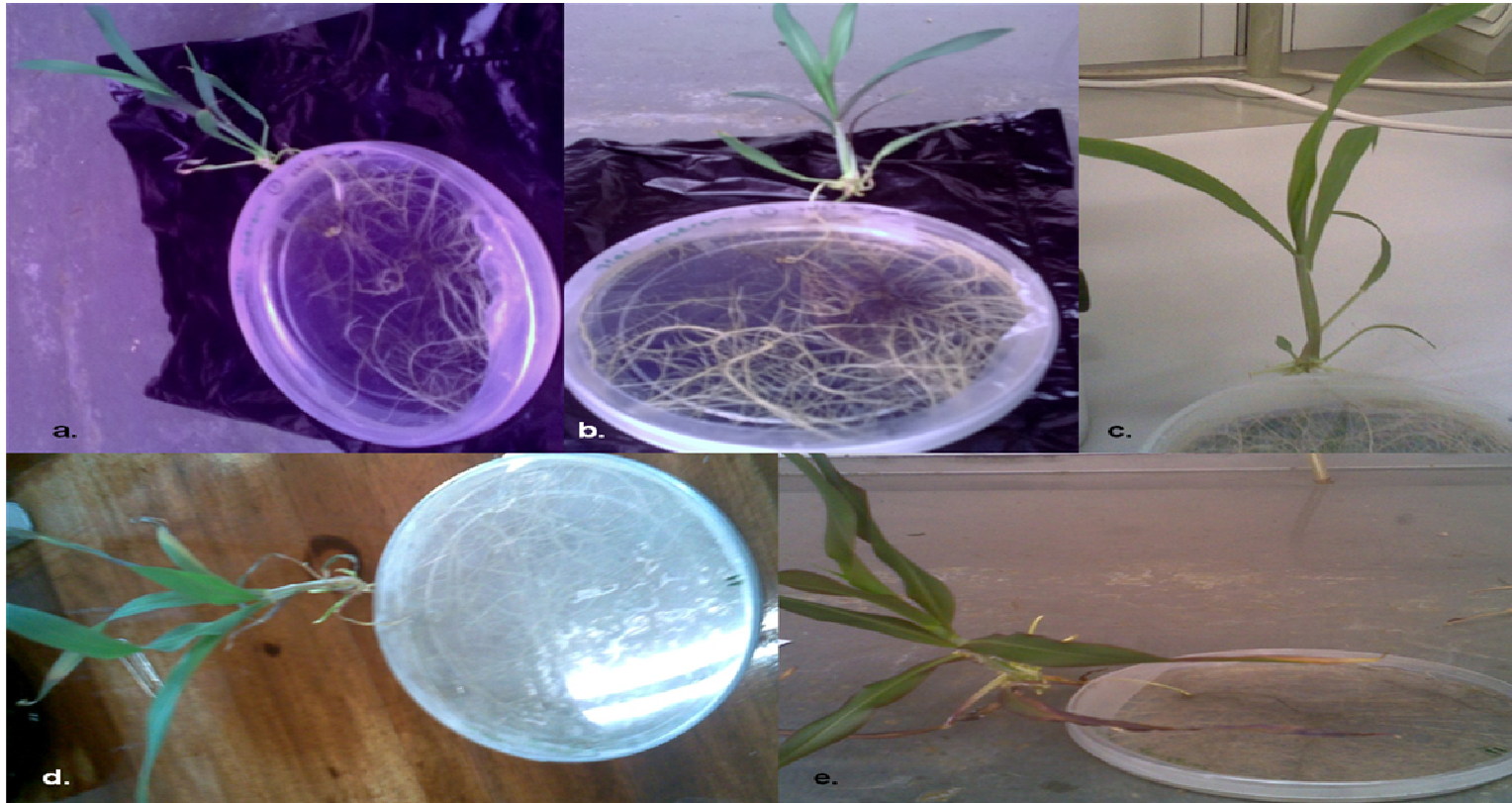
Figure 2.6: Weekly observation of sorghum development in HAM-P large Petriplate culture system (2x-1x concentration medium): a. second week, b. third week, c. fifth week, d. sixth week and e. seventh week. The number of leaves and length increased gradually, while root development remained constant after the third week of plant growth.

2.6   References

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Chapter Three
Assessment of root exudates produced by sorghum associated with arbuscular mycorrhizal fungus *Glomus intraradices* cultured under *in vitro* systems

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Secondary metabolites secreted by plant compounds are important signals for symbiotic plant-microorganism interaction. *In vitro* systems are useful tools for investigating agricultural crop plant-endophytic interactions under sterile conditions. Secondary metabolites produced by the association of arbuscular mycorrhizal fungi (AMF) species *Glomus intraradices* with sorghum under *in vitro* culture system conditions were investigated. Exudates produced by the plant roots associated with or without AMF were analysed using high performance liquid chromatography (HPLC), with the retention time and mass spectrometric detectors. The results indicated in the third week that root exudates acted as chemoattractants, stimulating germination and hyphal formation of AMF. After the sixth week of plant development, AMF hyphal development was restricted, which could be due to phytotoxic compound production. AMF development was observed by Trypan blue staining. It was concluded that when the plant underwent stress it produced exudates, which destroyed symbiotic organisms around the sorghum rhizophere.

3.1 Introduction

Cultivated sorghum is not only the fifth most important crop in the world, but the second most important crop in Africa, as a subsistence food crop. It is well known for its production reliability against biotic and abiotic stresses. This ingenuity is due to the root exudates it produces as phytochemicals (Ludwig-Muller, 2000; Kimber, 2000 and Taylor, 2004).

Root exudates are valuable secondary metabolites that are exuded by the plant. These low molecular weight compounds surround the rhizophere, as either defence or attraction chemical compounds, commonly known as phytochemicals (Bais *et al.* 2006). All plants have the capability of producing phytochemicals and to understand their interaction is of utmost importance. Over the past decade, researchers have focused on cereal crop capability to produce these phytochemicals and studied their interaction in the rhizophere. One main cereal crop under such scrutiny was sorghum (Muratova *et al.* 2009).

Certain flavonoid, phenolic acid and strigolactone classes are well documented phytochemicals that are exuded by sorghum roots. These phytochemicals can act either as a positive or negative effect on the plant and/or surrounding rhizophere organisms.

Strigolactones are a group of sesquiterpene lactones (Figure 3.1) that are excreted by many plant species as root exudates. Sorghum mainly exudes sorgolactones and strigol derivatives (Akiyama and Hayashi, 2006). These exudates function as a seed germination stimulant for the root-parasitic weeds *Striga* and *Orobanche* species (Bouwmeester et al. 2003). Alternatively, the same phytochemicals have been detected as stimulants for beneficial fungal symbionts, Arbuscular Mycorrhizal fungal (AMF) species, and germination and colonization within plant (Giovannetti *et al.* 1994).

Under abiotic stress, sorghum root exudates produce phenol groups such as, flavonoids and phenolic acids, at different concentrations. At low concentration, these phytochemicals act as a chemoattractant for symbionts, Arbuscular mycorrhizal fungi spore germination and colonization within host plants

(Vierheilig *et al.* 1998). Subsequently, under severe abiotic stress, plants recognise any type of organism in the rhizosphere as a pathogen and produce the same phytochemicals at high concentrations, which act as phytotoxic compounds resulting in the destruction of these organisms (Hirsch *et al.* 2003). One prime example of phytotoxic effects on AMF species was investigated by Hahn *et al.* 1983. It was noted that at high concentrations, flavonoid exudates have a negative effect on AMF development and thus colonization into host plant was inhibited.

Researchers pay much attention to AMF-host interaction, as this forms a mutualistic association with a variety of crops. Upon fungal colonisation, these obligate biotrophs have the capability to provide immobile soil nutrients and water to their host, as well as increase pathogen resistance. The pathogen resistance in sorghum plants is well documented, with colonized AMF change root exudate properties, thus making it undesirable to pathogenic organisms such as *Striga* species, resulting in an increased crop yield of sorghum (Rillig, 2004 and Akiyama & Hayashi, 2006). In order for the symbiotic relationship to occur, appropriate root exudates are required as stimulants for AMF species. To study the exudates produced by plants for AMF colonization, *in vitro* systems are useful. AMF studies are based on *Glomus* species isolates as they are easily isolated and over 100 species are known. Thus, this genus life cycle is well documented under *in vitro* culture systems (Dalpé *et al.* 2005). This chapter focuses on *Glomus intraradices* species under *in vitro* conditions.

In vitro culture systems are important tools in understanding the behaviour of plant and/or organism, without interference from the changing environment. An *in vitro* system is a series of differential experiments which can be divided into three study systems. These involve "axenic systems", which refers to one organism (endophyte) growing under aseptic conditions; "monoxenic systems", referring to two organisms (endophyte and host root organ) that are grown together; and "dixenic systems", referring to three organisms (host root organ and two types of endophytes) growing under aseptic conditions (Vierheilig and Bago, 2005).

Voets *et al.* (2005) developed an *in vitro* culture system, namely the Half-closed Arbuscular mycorrhizal plant system (HAM-P). This system allowed for autotrophic plants and AMF to be grown in association on a synthetic medium with no sugar or vitamins. The synthetic medium contains all macro- and micronutrients that the plant would require for development. No excess nutrients are present as AMF need to form an association within the plant root and thus form a symbiotic relationship. This technique proved to be beneficial for AMF in terms of assessing the life cycle, metabolism, biochemical analysis, symbiotic relationship, environmental factors, mass production, exudates and molecular analysis of the fungus (Vierheilig and Bago, 2005). Many analytical methods are used to detect exudates produced by the plant and/or organism under *in vitro* systems.

An analytical method that is as beneficial in the identification of chemical compounds is High Pressure Liquid Chromatography (HPLC). The basic procedure of HPLC is when extracted compounds are separated on a column (Stationary phase) by pumping compounds using a solvent (mobile phase) through the column. However, depending on the affinity of each compound between the stationary and mobile phases, these compounds will pass through the column at different times (retention time), as well as be detected at different wavelengths. Each compound has a unique wavelength and retention time at which they are detected (Synder *et al.* 2000), making the identification of compounds easier to observe using HPLC.

HPLC has two system operations – isocratic flow and gradient flow. The system used in this study was gradient flow, whereby the mobile phase composition does not remain constant throughout the process. This system allows for the mobile phase to change during the separation process. An example is when the gradient starts at 90% methanol and ends at 10% methanol after 30 minutes. The component found in the mobile phase acts as a carrier of the extracted compound to the column, which can either be water, methanol, acetonitrile or ispropanol. The use of any one of these components depends of the type of extracted compounds being detected (Synder and Dolan, 2006).

There are many types of HPLC that follow basic principle methods. HPLC uses different modes to analyse samples. These types include partition, normal phase, displacement, reverse phase, size exclusion, ion exchange and bioaffinity chromatography (Synder and Dolan, 2006). All types of chromatography can be combined with many detectors, to be beneficial in identifying compounds. These detectors are Absorbance (UV with Filters), IR Absorbance, Fluorescence, Refractive-Index, Evaporative Light Scattering Detector (ELSD), Electrochemical, Mass-Spectrometric and Photo-Diode Array (Skoog *et al.* 1998).

The detector of interest in this experiment was liquid chromatography mass-spectrometric (LC/MS), which follows the same procedure as normal HPLC, with mass analytic capabilities. Compounds are identified according to their molecular weight as well as their absorbencies and retention times, which are unique for each compound (Synder *et al.* 2000). This analysis has been combined with the Electrospray ionization (ES) technique for efficient use in identification of possible Strigolactones and Flavonoids that might present in each sample. In the ES method, highly charged droplets are sprayed onto possible compounds that pass through the column, and as a result the compound can become negatively/positively charged, thus leading to identification. ES has two modes of identification – negative and positive. The positive mode releases hydrogen or sodium adduct ions to the molecule, which join to the investigated compound, and an intensity peak can be detected. However, in the negative mode, molecules release hydrogen ions mainly and are thus detected. Compounds can be detected either in single or both mode/s at similar retention times (Kauppila *et al.* 2004).

The aim of this chapter was to assess the root exudate properties of sorghum with AMF, *Glomus intraradices* species association, under *in vitro* culture systems. In addition, we investigated the effects of possible exudates produced on AMF development and colonization using the LC/MS technique.

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3.2   Materials and methods

All methods for the experiments were carried out according to Voets *et al.* 2005, with modifications indicated.

3.2.1   Medium preparation

Two concentrations of Modified Strullu Romand (MSR) medium (Refer to appendix 1 for stock solution make up), lacking vitamins and sugars, were used in the following study (Table 3.1). The following stock solutions were prepared for construction of MSR-S-V: Macro-elements (KNO_3, KCL and $MgSO_4.7H_2O$), Calcium Nitrate, NaFeEDTA and Micro-elements ($MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, $CUSO_4.5H_2O$ and $(NH_4)_6Mo_7O_{24}.2H_2O$). Millipore water was added to dissolve the constructs (Table 3.1) and brought to a volume of 1 litre. The pH (Microprocessor pH 211 metre- Hanna instruments) was adjusted to 5.5. The solution was solidified with 3g/l Phytagel (Sigma). The medium was then Autoclaved (speedy horizontal) for 15 minutes at 121°C. A double concentrated medium was used for the initial step of the HAM-P set up. A single concentrated medium was used for seed germination and system maintenance.

Table 3.1: Preparation of 1 litre of Modified Strullu Romand lacking sugars and vitamins (MSR-S-V) medium concentration, thereafter steam sterilized at 121°C for 15min

Stock solutions	Concentration of MSR-S-V medium (ml/l)	
	Single	Double
Macro-elements*	10	20
Calcium Nitrate*	10	20
NaFeEDTA*	5	10
Micro-elements*	1	2
pH	5.5	
Phytagel (Sigma Aldrich)	3g/l	
Millipore water	Dissolve stock solution until 1 litre was reached	

*Key: * Refer to appendix 1 for stock solution preparation and/or ordering information.*

3.2.2 Plant material

An unknown variety of untreated South African sorghum seeds was surface sterilized with Sodium hypochlorite (3.5%) in sterilized 50ml falcon tubes for fifteen minutes, then rinsed four times in sterile deionized water for ten minutes. Surface sterilized seeds were then plated in medium Petri plates (Merck chemical and lab supplies) (90mm diameter, five seeds/plate) containing 1x concentrated MSR-S-V media. Petri plates were then sealed with Paraflim (Merck chemical and lab supplies), followed by incubation for three days in dark incubator (Stay cold incubator set at 27°C), to allow for germination, with all seed germination points were placed in one direction into the plate. Thereafter, germinated seeds were placed in a light phytotron (22°C day/ 18°C night) for photosynthetic tissues to form in the plant.

3.2.3 Arbuscular mycorrhizal fungi cultures

Glomus intraradices Schenck and Smith MUCL 41833 were supplied in 90mm diameter Petri plates colonized with Medicago truncatula CV Jemalong A17 *in vitro* plants, from GINCO and MUCL laboratories (Alternate strain for sale at GINCO lab is 43194: webites www.mbla.ucl.ac.be/ginco-bel), Belgium, The Petri plates containing above mentioned Medicago and MUCL 41833 strain, were incubated and maintained for a period of six months in an inverted position in a phytotron (settings*). This resulted in the mass production of spores, which were used in the present experiment.

3.2.4 Development of an autotrophic culture system

The system used for this experiment was the HAM-P culture system and set up was done by constructing a hole on the edge of the lid and base of Petri plate (150mm diameter (Capital lab supplies) containing either 1x/2x concentrated MSR-S-V medium. Seven-day-old germinated Sorghum seeds were transferred to each Petri plate. Under a laminar flow hood (horizontal), close to a Bunsen burner, sterilized stainless steel forceps were used to aseptically transfer one germinated seed into 150mm diameter Petri plate, with roots placed inside the plate on the medium and the shoot protruding outwards through the hole of each Petri plate. The Petri plates were sealed with Paraflim (Merck chemical suppliers) and the area with the hole was further covered with sterilized silicon grease (Hardware outlets) to avoid any contamination. Thereafter, the plates were covered with an opaque plastic bag (to allow a dark environment for root development) and incubated in a phytotron with well regulated moisture and phototrophic settings*.

*Phytotron setting:
- 22˚C/18˚C day/night
- 16 hours photoperiod

- o For moisture, 4x 2-litre containers were filled with water and placed around the plants. These containers were filled with clean water every 5 days.

3.2.5 Association of AMF to autotrophic systems

Two weeks later, using sterilized stainless steel scalpel (number 4) with sterilized stainless steel blades (number 24), twenty to thirty *Glomus intraradices* spores with medium were cut out from cultured plates (described above) and associated to two-week-old HAM-P sorghum roots. Thereafter, the Petri plates were sealed with Paraflim (Merck chemical suppliers) and the area with the hole was further covered with silicon grease (sterilized at 121°C for 15 minutes) to avoid any contamination. Thereafter, the plates were covered with an opaque plastic bag (to allow a dark environment for roots to develop) and incubated in a phytotron with well regulated humidity and phototrophic settings.

Fortnightly, *in vitro* HAM-P culture systems were maintained by adding single concentrated MSR-S-V into each Petri plate. This was done in order to provide renewed nutrients to the plant for optimal development. All parts of the experiment were carried out aseptically under the laminar flow hood. Systems were observed in the third, fifth, sixth and eighth weeks of development. Control systems were not associated with *Glomus intraradices* species.

3.2.6 System observation

Prescribed week systems were observed for root change, abundance and AMF spore germination, and hyphal elongation using microscopy techniques. AMF colonization within the root was investigated using non-vital stain procedure.

Microscopy techniques:

a. Dissecting microscope

Root samples associated with/without spores were observed for root exudation properties, colour change and the development of AMF. Each HAM-P system was placed under dissecting microscope (Zeiss Stemi DVH Dissecting microscope) and magnification was adjusted to view specific features. Images were captured with Canon PowerShot A640 10 Mega Pixel camera attached to the dissecting microscope.

b. Environmental scanning electron microscope (ESEM)

This technique allows for the outer surface of samples that could not be clearly viewed under dissecting and light microscope; to be viewed at a higher resolution. Using sterilized stainless steel scalpel (number 4) with sterilized stainless steel blades (number 24), Root samples associated with/without spores were removed aseptically from HAM-P systems and mounted onto carbon-taped stainless steel stubs. These stubs containing samples were viewed using Phillips XL30 Environmental Scanning Electron Microscope (ESEM), under low vacuum and spore formation towards roots was observed.

Presence of AMF colonization within roots using Trypan blue stain:

Trypan blue staining for AMF colonization was carried out according to Phillips and Hayman (1970), with modifications by Dr Ingrid M van Aarle at MUCL lab, Belgium, and myself. 1 gram of root samples containing spores were aseptically cut (using sterilized stainless steel scalpel (number 4) with sterilized stainless steel blades (number 24)) into pieces and placed in a sterile 50ml falcon tubes. The samples were cleared at 60°C for 15hours with 20ml 10% KOH (Merck chemicals). Thereafter, the samples were rinsed with distilled water, followed by a minute acidification with 1% HCL (Merck chemicals and lab supplies). Acidified roots were then stained with 0.1% Trypan blue (1:2:2 of Lactic

acid:glycerol:water and 1gram/litre Trypan blue) for 2 hours and 30 minutes. Thereafter, the roots were destained with 50% glycerol overnight and viewed under light microscope (Olympus AX 70 Provis light microscope with soft imaging system analysis 3.0 software) for observation of colonized AMF within roots. *NB: due to Trypan blue toxicity and carcinogenic effects for humans, great care was taken when this type of stain was used. Gloves and fume-hood were at all times.*

3.2.7 Analysis of possible root exudates

Exudates were extracted according to Hertog *et al.* (1992), with modifications. In the second, third, fifth, sixth and eighth weeks, root samples were aseptically cut (using sterilized stainless steel scalpel (number 4) with sterilized stainless steel blades (number 24)), out of HAM-P systems and placed into a test-tube containing 20ml 100% methanol (Merck chemicals and lab supplies). These samples were then subjected to overnight heating in a water bath set at 60°C, in order to extract free exudates that the roots had excreted. Thereafter, the samples were vortexed for 30 seconds and filtered through Whatman number 1 filter paper. Methanol was used to rinse the root samples until colour was no longer released. Filtrates were dispensed into glass scintillation vials (Anatech) and concentrated overnight in a speed vacuum at high speed (Speed Vac SC200 with refrigerated condensation trap RT 400) (with drying rate and freezing condensation on). Dried concentrated samples were then re-suspended in methanol and centrifuged (Genfuge 24 D Progen table top Centrifudge) at 10000rpm for 30 minutes. Supernatants were filtered through 0.45µm nylon filter (Anatech) into HPLC vials, which were sealed with septa and caps (Anatech).

An analysis was carried out by using gradient Ultra pure liquid chromatography with Waters LCT Premier mass spectrometry as a detector. Samples were placed into an auto-sampler, where extracted exudates were injected and passed through Water BEH C18 column (1.7µm particle size and 2.1x100mm). This was connected to a Hewlett-Packard photodiode array detector (absorbance detected between 210-400nm). Samples were passed through the

column and PDA using Carriers' methanol and water. These carriers had an elution gradient over a period of 30 minutes (Table 3.2). Mass spectrometry had two sources of detectors – Electron spray ion (ES) positive and negative mode. Each mode had specificity when identifying the molecular masses and compounds of each sample, which occurred at a varied wavelength and retention time. Reference points were taken from various published papers (Sato *et al,* 2003 and Prasain *et al*, 2004); which used similar gradient flow methods.

Table 3.2: HPLC/MS Gradient flow set up from carrier solvents methanol to water over a period of 30 minutes

Initial time	Water %	Methanol %
0	90	10
10	90	10
12.5	70	30
15	0	100
28	0	100
28.5	90	10
30	90	10
Flow rate of sample and carrier		0.35 ml/min

3.3 Results

3.3.1 Autotrophic culture systems

Systems were observed weekly for AMF and plant development. Low molecular weight compounds were observed in the second week of HAM-P development (Figure 3.2). The third week of plant development after association of *Glomus intraradices* resulted in positive effects on root exudates to *Glomus intraradices* hyphal development. In the sixth to the eighth weeks, plants were under severe stress, thus resulting in photochemical production in a higher concentration, which recognized *Glomus intraradices* as pathogen, resulting in spore and hyphal disintegration. In control plants, root exudate production was only observed at higher concentrations towards the eighth week of plant development. A summary of all molecular weights detected at different modes and retention times over the eight weeks with and without *Glomus intraradices* was tabulated in Table 3.3.

Table 3.3: LC/MS Summary of all molecular weights detected at different ES modes and retention times over the eight weeks with and without *Glomus intraradices*

Week	ES modes	Molecular weight of various exudates detected at different retention times (m/z)					
		Retention times (minutes)					
		0 - 9.59	**10 – 12.59**	**13 – 14.59**	**15 – 19.59**	**20 – 24.59**	**25 – 30**
2 (non-AMF)	**Negative**	329.05, 346.03	-	713.39	399.14, 399.20	399.20, 399.20	399.20, 399.20
	Positive	365.12, 447.16, 332.14	-	701.52	239.16, 804.54, 326.38	413.27, 413.27	413.27, 413.27
3 (AMF)	**Negative**	-	-	304.04	367.13, 399.16	399.16, 399.16, 399.16	399.16
	Positive	-	-	331.20, 245.07	333.16, 803.45. 413.25	-	413.25, 413.25, 413.25
5 (AMF)	**Negative**	209.96	431.12	551.16	339.20, 279.24, 256.25, 236.28, 399.26, 399.26	399.36, 399.26	399.36, 399.26
	Positive	365.13	-	465.30, 553.35,	301.15, 803.57	413.29, 413.29,	413.29, 413.29,

				701.53		413.29	413.29
6 (AMF)	**Negative**	209.92, 537.12	-	431.04, 609.09, 264.99	255.18, 842.52, 682.52	399.19	399.19, 399.19
	Positive	191.05	399.14, 399.15	543.16, 701.48	804.52, 639.47	413.26, 413.26	413.26, 413.26
8 (AMF)	**Negative**	191.03, 490.09, 449.05, 320.96	-	577.05, 327.17	339.14, 399.19	399.19, 399.19	399.19, 399.19
	Positive	381.06, 267.14, 334.09, 332.13	561.19	543.19, 615.13	301.13, 803.47	413.26	-
8 (non-AMF)	**Negative**	341.06, 237.04, 267.05	563.06, 563.06	327.07, 713.36	325.11, 281.20	399.19, 399.19	399.19, 399.19
	Positive	365.10, 261.13, 305.15, 349.18, 383.20, 437.23	481.26	475.32	539.54, 803.45	413.26	413.26, 413.26, 413.26

Above results indicated that upon association of AMF to plant; the plant produced more compounds, in order to act as a stimulant. However, when the plant underwent stress, they produced compounds in a high intensity on LC/MS

and concentration in Petri plate, which in turn was negative to AMF formation and colonization. The phytotoxic compounds were produced to protect the plant, consequently certain phytotoxins produced by the plant at higher concentration, negatively affect the plant and AMF development.

HPLC with Mass spectrometric detectors (LC/MS) used for the following experiments have been combined with the Electrospray ionization (ES) technique for efficient use in the identification of possible Strigolactones and Flavonoids that might present in each sample. All possibly identified compounds were compared to respective journal articles as reference points.

3.3.1.1   Non-AMF plant systems (Control)

Second week HAM-P system

The second week of development was the most crucial stage (Chapter 2), where beneficial root exudes are produced for plant development and protection. As a result, *Glomus intraradices* was associated at this stage, as plant roots would produce certain chemoattractants for AMF colonisation. At the start of this sorghum growth stage, LC/MS analysis of root exudates was investigated (Figure 3.2).

High intensity compounds were detected between the retension times 15-22 minutes (Figure 3.2). One compound that occured numerously and was detected at high intensities in the ES negative mode had a molecular weight of 399m/z, while the ES positive mode had indicated these compounds at a molecular weight of 413m/z (Figure 3.2 and Figure 3.3). Since the retention times and peaks of both the compounds were similar, it could most probably be the same compound, presented and identified as isomers (Figure 3.2 and Figure 3.3). In the ES positive mode, other compounds with the molecular weights of 239m/z and 804m/z (Figure 3.2) were also detected. These compounds could not be conclusively identified.

Eight week old HAM-P system

When compared to the two-week-old system (Figure 3.2), the results indicated additional compounds of different masses being produced. One major organic compound that plants produce are flavonoids. These could most probably be Quercitin derivatives such as Quercetin-3-O-trisaccharides and Quercetin-3-O-glucosides. Comparing Figure 3.2a to Figure 3.4a, it was clearly noted that after eight weeks of plant development, more exudates were produced. New exudates were detected at retention times between 7-12 minutes at low intensities. Similarly, comparing Figure 3.2b to Figure 3.4b, new exudates were detected at retention times between 1-13 minutes at low intensities. It was noted that as the plant matures it produces new compounds to aid in the growth and maturity stage, as well as producing defense metabolites to protect and regain plant health. This usually occured when the plant underwent stress. The main stress could be due to space deprivation of root formation (Figure 3.14). As a result, the plant root darkened (Figure 3.14). This is synomous with flavonoids as they are produced when the plant undergoes stress.

3.3.1.2 AMF associated to sorghum under HAM-P *in vitro* culture systems

Third week HAM-P system with AMF

When comparing the non AMF plant (Figure 3.5a) with the AMF plant (Figure 3.5b), the control plant leaves looked healthy, with the older coleoptile leaf drying out. With the *Glomus intraradices* associated plant systems, the leaves were healthy and the coleoptile leaf did not dry out. The roots of the non AMF plant were clear (Figure 3.5c), while the roots of the AMF plant had begun to change colour around the *Glomus intraradices* associated zone (Figure 3.5d). The results indicated that around the discolouring zones of roots, hyphal elongation occured (Figure 3.5d). Around the discolouration, part of the root germinated spores then formed runner hyphae (Figures 3.5 e-f), as well as

germ tubes which moved towards the roots of sorghum (Figure 3.5g). The trypan blue stain showed no colonization of AMF to Sorghum root (Figure 3.5h).

Roots that were discoloured and had AMF activity were observed for exudates that were formed to stimulate AMF germination. Upon LC/MS results, it was noted in the vicinity of AMF association that roots exuded a possible chemoattractant for AMF hyphal extension (Figure 3.6). This compound was found in higher intensities under negative ES mode. Further investigation indicated the resemblance of Sorgolactones (Figure 3.6), in terms of its ionization mode and retention time detection. The results also indicated that roots away from AMF presence did not excrete these molecular weight compounds (Figure 3.7). Two-week-old control systems produced major compounds with high intensities between retention times of 17-29 minutes (Figure 3.2). These were similar to three-week-old samples (Figure 3.7). However, low intensity compounds that occured in the control system (Figure 3.2) were not noted in the three-week-old system with the presence of *Glomus intraradices* (Figure 3.7). These unidentified compounds could have been used up by the plant to produce a chemoattractant for AMF spore germination and hyphal extension.

Upon LC/MS analysis, new molecular weights were detected in the positive mode, which was located around AMF zone. On the basis of the literature, these compounds were most probably Sorgolactones (Figure 3.6).

Fifth week HAM-P system development

The results indicated root discolouration throughout the plate containing AMF-plant, with darker discolouration around *Glomus intraradices* vicinity (Figure 3.8d). It was further noted that fungal runner hyphae elongated and spread around the plate, probably in search for roots (Figure 3.8e). Upon HPLC analysis, it was noted that the root exudate compounds seemed to spread throughout the Petri plate, as similar results were obtained from associated *Glomus intraradices* away and around the root (Figures 3.9 and 3.10).

Control plants indicated the drying and death of older leaves and abundant root formation, with a cream colouring (Figure 3.8a). When compared to the AMF associated plant system, the leaves remained healthy, while leaf length increased from the previous week and maturity of leaves occurred with no nutrient deficiency (Figure 3.8b). The roots of the AMF system showed browning (Figures 3.8b and d) and hyphal elongation was noted. Although runner hyphae extended towards the roots (Figure 3.8e), no colonization was observed (Figure 3.8f).

LC/MS analysis detected that similar molecular weight exudates were produced close and away from the vicinity of AMF. However, the compound with high intensity that was present in both samples was similar to the compound detected in the third week sample exudate under ES positive mode (Figures 3.9 and 3.10). Compared to the week two and three results, more compounds were detected (Table 3.3). Week five seemed to produce more compounds at different retention times, while the week three sample had fewer compounds (Figure 3.7 and Figure 3.9). The systems showed that as AMF-plant systems aged they produced more exudates, which could have acted as a stimulant for AMF (Figure 3.9). A compound that resembled the patterns produced in LC/MS (Figure 3.8 and Figure 3.9) was strigolactones. Reference points in the literature were detected according to specific retention times and mass, specifically in the ES positive mode (Figures 3.9b and 3.10).

Sixth week of HAM-P system development

It was indicated that the control plants suffered nutrient deficiency as leaf discolouration was observed (Figure 3.11a). The roots covered the entire plate with decolourisation (Figure 3.11c). The decolourization was not as drastic as in the systems associated with *Glomus intraradices*. AMF-plant leaves exhibited signs of chlorotic stress and thus the older leaves dried out (Figure 3.11b). Subsequently, around AMF-root associated zones, the root colour blackened and darker root exudates were observed (Figure 3.11d), leading to a negative

development of AMF in that runner hyphal production weakened and broke off from the root (3.11e-f). Trypan blue staining indicated no AMF colonization (3.11g).

Dark exudates produced by AMF-plant root systems were detected using LC/MS. The results indicated three peak wavelengths between 209-210nm at a retention time of 14-16 minutes (Figure 3.12a), with values indicating a methanol presence. According to the ES negative mode, fewer masses were noted when compared to week five LC/MS results (Figure 3.9b and Figure 3.12b). The relative masses of 399.28m/z (detected at different retention times) occurred at higher intensities than that in Figure 3.9b. New masses were detected between the retention times of 14-16 minutes (Figure 3.12b). Comparing Figure 3.9c and Figure 3.12c, fewer masses of 413.29m/z occurred at higher intensities in the sixth week. According to HPLC, for efficient identification of unknown compound of interest, their masses should be produced at high intensities under the different modes.

New masses were detected between the retention times of 10-17 minutes (Figure 3.12c). Upon further analysis of the retention times of 14-16 minutes on absorbance and ES negative and positive mode, the results indicated a specific mass in ES negative mode and wavelength (Figure 3.13). The most probable compound was flavonoid, as this exhibited the properties of absorbance patterns (Figure 3.13b) synonymous with typically occurring flavonoids. According to HPLC/MS results, associated *Glomus intraradices* species away and around sorghum roots excreted similar compounds.

Eighth week of HAM-P system development

Figure 3.14a indicated that the control plant was suffering from a nutrient deficiency and was thus drying; the roots were abundant with brown darkening (Figure 3.14c). All systems with *Glomus intraradices* showed the older leaves were drying and the stems were weakened (Figure 3.14b). Roots covered the entire plate and a blackening appearance was observed throughout the plate

(Figures 3.14d and e). At this point, all traces of *Glomus intraradices* hyphal and spore structures disintegrated into the medium (Figure 3.14f). Upon Trypan blue staining, no root colonization was observed (Figure 3.14g). Exudates were dispersed throughout the medium, as the roots were abundant (Figure 3.15), with more molecular weight compounds being detected at an early retention time (Figure 3.15). Control plant LC/MS analysis produced similar results to the week two control. In week two, more low intensity molecular weight compounds were detected at an early stage (Figure 3.4). Upon further investigation, our results indicated absorbance peaks when compared to an eight-week-old control system (Figure 3.4 and Figure 3.15). A variety of compounds was detected in Figures 3.15b and c, which occurred at high intensities. The compounds that were produced in AMF-plant system may possibly be phenolic and flavonoids, due to their absorbance peaks.

A combination of all exudates from week two to week eight with/without AMF indicated that upon association of AMF to plant, the plant produced more compounds in order to act as a stimulant. However, when the plant underwent stress, it produced compounds in a high intensity on LC/MS and concentration in Petri plate, which in turn was negative to AMF formation and colonization. The phytotoxic compounds were produced to protect the plant and consequently, certain phytotoxins produced by the plant at a higher concentration negatively affected the plant and AMF development.

3.4 Discussion and conclusion

Root development and exudate formation occurs at the 3-leaf stage (GS-1). According to Vanderlip and Reeves, (1972) Emergence (GS-0) and GS-1 take approximately 20 days to produce. Yet under *in vitro* systems, it was accomplished within ten days of development. GS-1 mainly involved adaptability and production of the metabolite stage, which allows for rapid root development in the 5-leaf stage (GS-2). The roots can be used to excrete exudates and metabolites that protect the plant while it matures towards the growth point differentiation stage (Pardales and Kono, 1990). This was

observed in our results, which showed that two-week-old control systems produced high intensity compounds, although these compounds could not be indentified conclusively. literature states that in order for sorghum to accomplish essential root development, plant acclimatising and maturity, they need to produce neccessary sugars, amino acids and organic compounds (Neumann and Römheld, 2001).

Root exudates that act as a chemoattractant at the symbiotic stage are usually produced at GS-2 of sorghum development. Hence, *Glomus intraradices* species were associated to sorghum roots under *in vitro* HAM-P culture system in the second week of plant development. Usually, *Glomus intraradices* has five stages in order to complete its life cycle. In this study, only the first three stages of the life cycle were observed, viz. spore germination, pre-symbiotic mycelium and host root connecting stage (Dalpé *et al.* 2005).

Although the germination stage was usually not affected by the chemoattractants produced by the plant host, the germination rate can be stimulated if plants produce chemoattractants (Dalpé *et al.* 2005). Completion of this stage took from a few days up to six months and involved the inner spore wall being forced through the lumen of the subtending hyphae and directly through the outer spore wall (Gilmore, 1968). Our results indicated the initiation of germination, as spores formed subtending hyphae in the third week of HAM-P development.

Glomus intraradices spore germinated as a result of certain stimulants present within the *in vitro* systems that were produced as exudates by roots. The literature states that metabolic activation was a prerequisite for spore germination since it triggers signals from the host plants (Tsai *et al*. 1991). Sorghum has been reported to secrete strigolactone, to stimulate the cellular functioning of AMF. Strigolactones are a group of four sesquiterpene lactones. A group within the family that can increase the spore germination rate of *Glomus intraradices* was sorgolactones (Figure 3.16) (Akiyama and Hayashi, 2006). According to Sato *et al.* (2003), sorgolactones' parent molecule has a mass of 317m/z [M] and was detected at a retention time of 14-17 minutes.

Under HPLC/MS detection, two main sorgolactone daughter ions are detected in the ES positive mode: 242m/z [M+Na-97] and 301m/z [M+H-17]. The results of Sato *et al.* (2003) were used as a reference point for the identification of possible unknown compounds in this study. These authors concluded that the electron spray under the positive mode of HPLC/MS indicated that Sorgolactone structure are cleaved at the vinyl ether bonds between the C and D rings (Figure 3.16); resulting in D-ring moiety via a collision with Argon molecules. The D-ring loss results in sodium adduct ions attaching to the new daughter ion, thus forming a mass of 242m/z in ES positive mode. The other daughter ion detected in the positive mode of HPLC/MS has a mass of 301m/z, which results in the Sorgolactone structure losing a Hydroxyl group and obtaining Hydrogen adduct from electron spray. The results of Sato *et al.* (2005) were similar to our results obtained in HPLC, where masses of 242m/z and 301m/z were observed at high intensities in the ES positive mode. These compounds could have resulted in *Glomus intraradices* spore germination. To account for the instability and attraction function of Strigolactone groups produced in sorghum roots, they can change root enzyme functioning and produce certain flavonoids (Akiyama and Hayashi, 2006).

Sorghum produces various flavonoids, which vary in functioning. However, when produced at low concentrations, these can act as a chemoattractant for symbiotic organisms. The well-documented example of Quercetin (Figure 3.17) was produced by sorghum at low concentrations to increase spore germination and promote the AMF life cycle to the next stage, Pre-symbiotic (Dalpé *et al.* 2005; Gioninazzi-Pearson *et al.,* 1989; Tsai and Phillips, 1991).

In the pre-symbiotic stage, the Quercetin produced allowed for an increase in AMF hyphal formation, as well as increased root and leaf development, and maturity (Gioninazzi-Pearson *et al.* 1989). Our results indicated that GS-2 was reached faster with AMF, as it promotes flavonoid production and thus has a positive effect on plant growth. Quercetin typically produces a darkening of the appearance of exudates and roots over time.

Quercetin is a plant-derived flavonoid or more specifically, a flavonol. It has been shown to have phytotoxic or chemoattractant properties at different concentrations in soil (Häkkinen, 1999). Quercetin has a parent mass of 303m/z. It is detected under HPLC/MS ES negative mode at daughter masses of 301m/z [M-2H$^+$] and 302m/z [M-H$^+$], with an absorbance of 330-350nm and a retention time of 14-15 minutes (Prasain *et al.* 2004). These were observed clearly in our results. According to Prasain *et al.* (2004) HPLC/MS ES negative mode identified quercetin as two daughter masses, which are identified when electron spray allows Hydrogen ions to be removed from the parent compound. The most stable and higher occurring percentage daughter compound is 301m/z.

At the pre-symbiotic mycelium stage, the spore metabolizes compounds that it obtains from host root exudates, which leads to runner hyphae formation. Runner hyphae explore the environment by successive branching into filaments (Declerck *et al.* 2000). If runner hyphae do not obtain root contact or host signal detection within a few days, this results in the termination of germ tube growth (Bécard and Piché, 1989). Our results indicated that runner hyphae were formed within the fifth week of HAM-P development. Hyphal roots did not make contact with roots within the fifth to sixth week of HAM-P development, which resulted in hyphal breakage. Certain cases indicate hyphal structure breakage, resulting from host exudate secretion. Sorghum roots exude phytochemicals that act as phytotoxic compounds when undergoing abiotic stress (in our results, this was root space deprivation). These phytotoxic compounds allow for sorghum to move into plant 'survival mode'. Consequently, they recognize any foreign organisms (including symbiotic organisms such as AMF by recognizing their chitin wall) as pathogens, resulting in the death of any organisms surrounding the plant (Fries *et al.* 1997). In our results, the death of *Glomus intraradices* by the weakening of hyphal growth was clearly observed. These phytotoxic compounds are usually flavonoids in a higher concentration, which initially acted as a chemoattractant for *Glomus intraradices*. In the sixth and eighth week of HAM-P development, the results indicated a dark brown exudate

formation throughout the plate and upon HPLC/MS analysis, it was identified as Quercetin.

The host root connection stage occurs when AMF hyphae reach the root. At this point, communication between the plant and AMF is crucial. This communication is due to the signals produced by host root exudates, which have to be produced in the correct concentration (Giovannetti *et al*. 1994). Sorghum undergoes abiotic stress. It produces exudates in a higher concentration and these exudates can change the properties of root uptake, which negatively affects plant and fungal development (Pageau *et al.* 2002). Our results indicated that when roots produced high concentrations of flavonoids, these compounds adversely affected AMF development and plant development.

3.5 Figures:

Strigol   sorgolactone   orobanchyl-acetate

Orobanchol   5-deoxystrigol
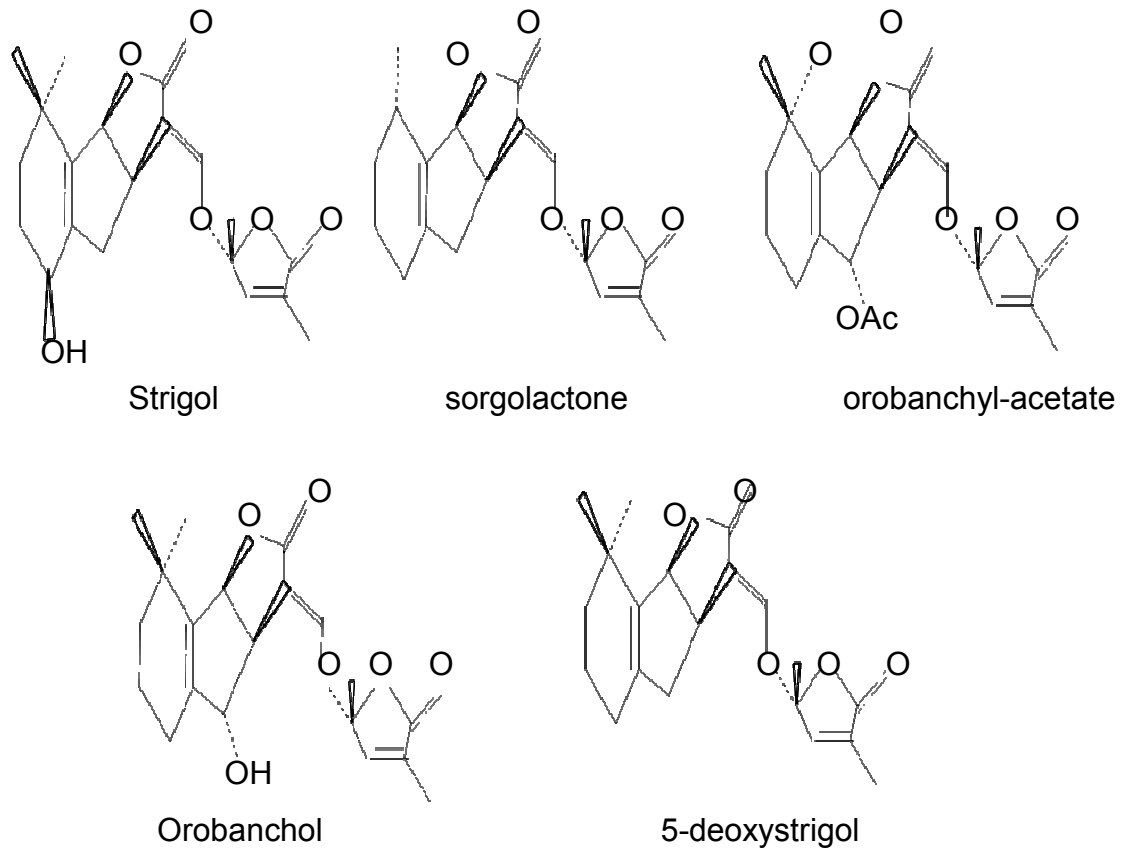
Figure 3.1: Chemical structures of groups of strigolactones commonly exuded by plant species (Adapted from Sato *et al*. 2003).
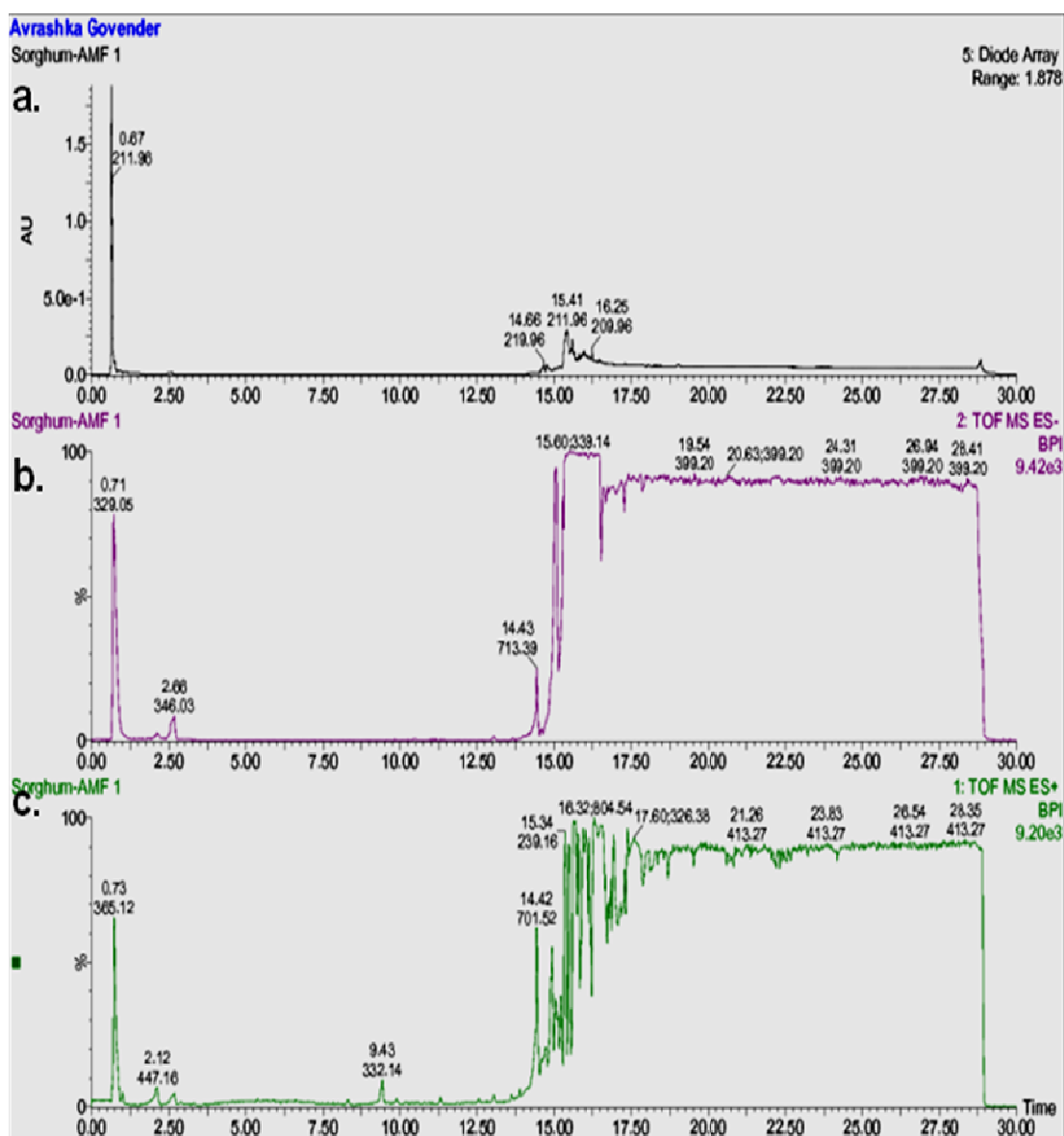
Figure 3.2: LC/MS graphical representation of methanol-based root exudate extract from two-week-old sorghum plant showing low molecular weight compounds occurring at high intensity: a. Chromatogram detected, b. ES negative mode and c. ES positive mode.
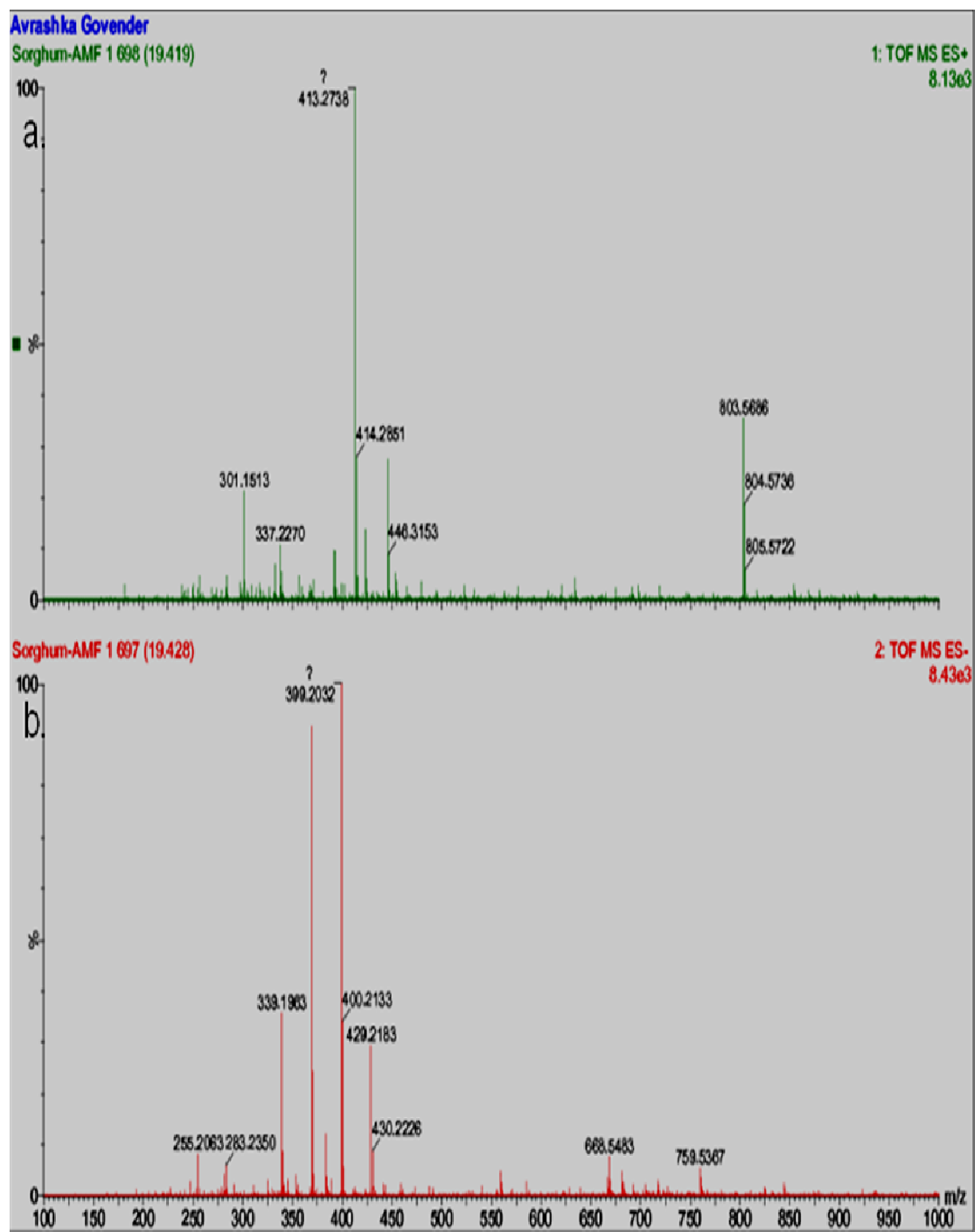
Figure 3.3: Two-week-old mass spectrum of an unknown compound detected in abundance at: a. ES negative mode: 399m/z and b. ES positive mode: 413m/z. Question mark usually represents oversaturation and concentration of that compound within the extract.
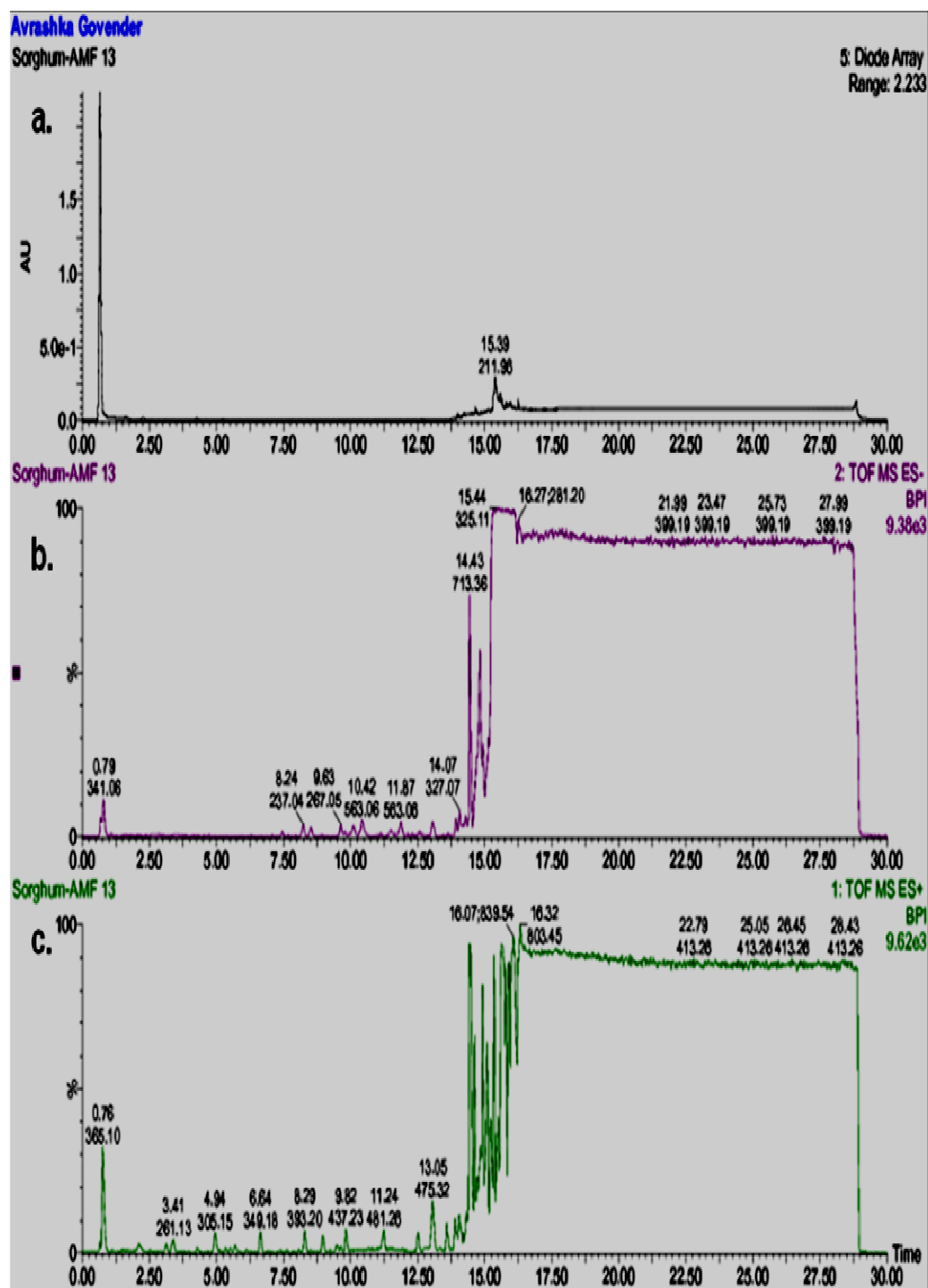
Figure 3.4: LC/MS graphical representation of methanol-based root exudate extract from eight-week-old sorghum control plant, showing more compounds being detected at low intensities: a. chromatogram detected, b. ES negative mode, and c. ES positive mode.
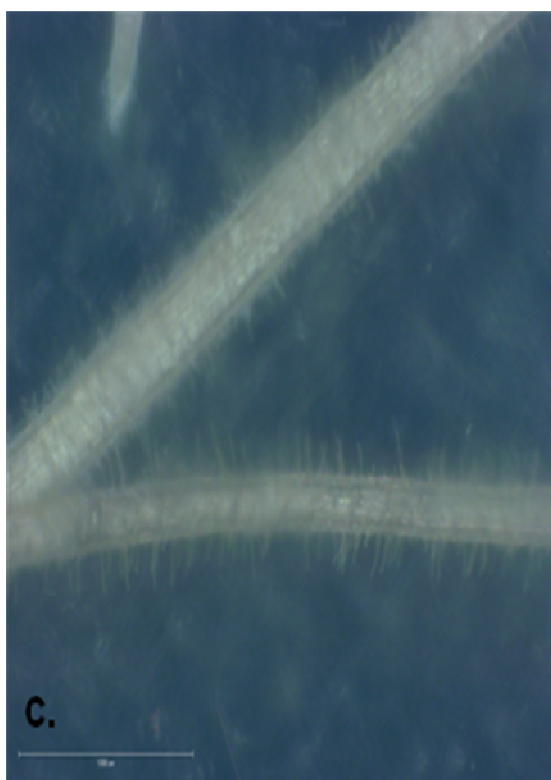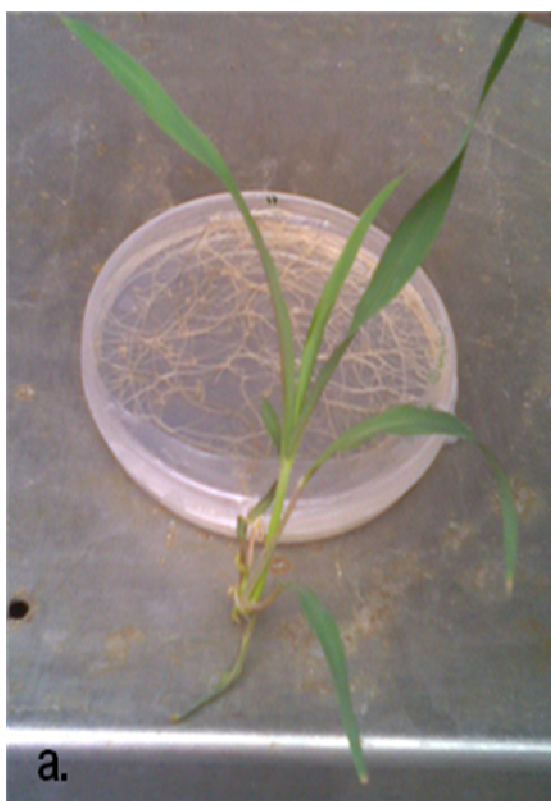
Image continued on next page.
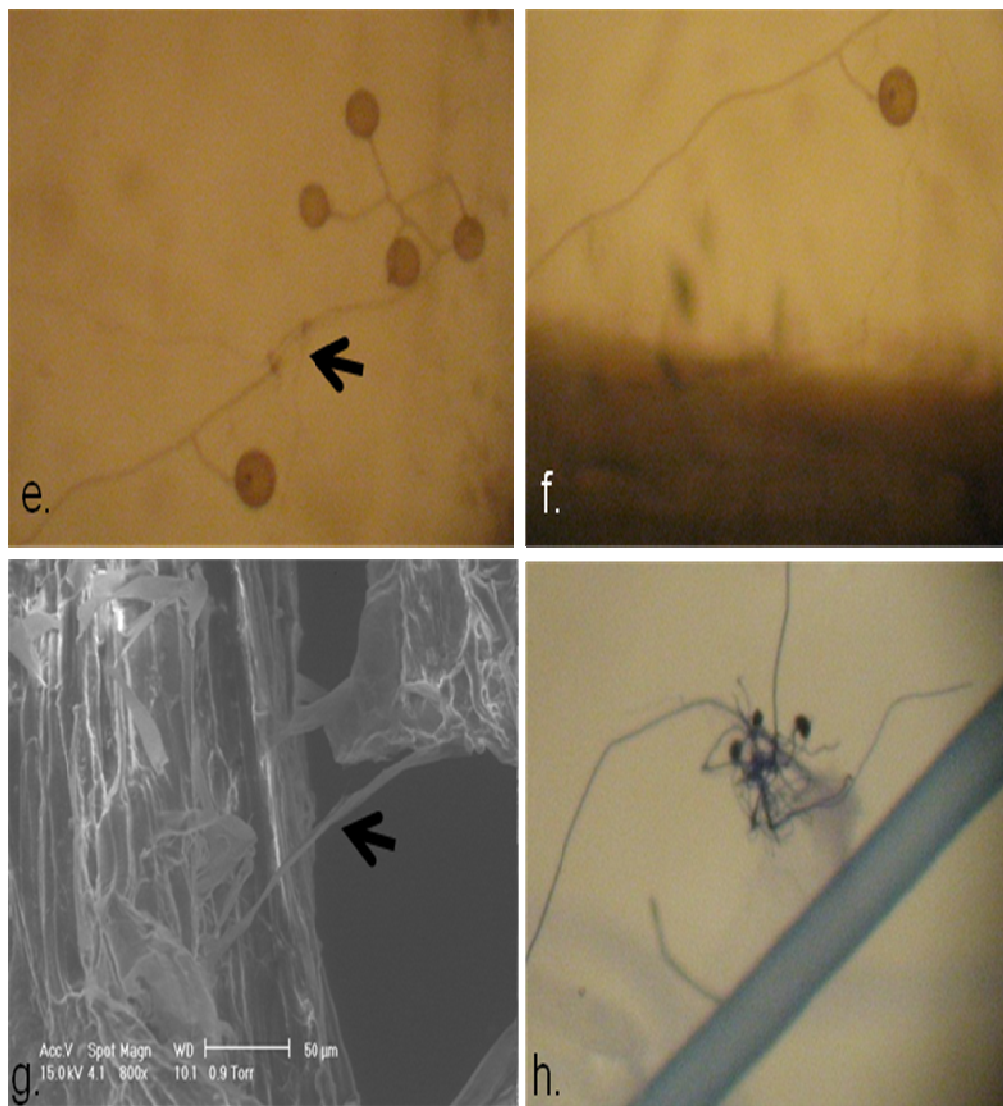
Figure 3.5: Three-week-old HAM-P system development: a. Control plant with no root discolouration; b. *Glomus intraradices* associated plant system, root discolouration observed; c. Dissecting microscope image of control root, clearly indicating little/no exudate production; d. Dissecting microscope image of root with associated *Glomus intraradices*, root change observed, as well as hyphal elongation (arrow head); e and f. Dissecting microscope image of *Glomus intraradices* germination and hyphal development (arrow head); g. ESEM image of hyphal extension towards root (arrow head) and d. Trypan blue staining of root, showing no colonization within root.
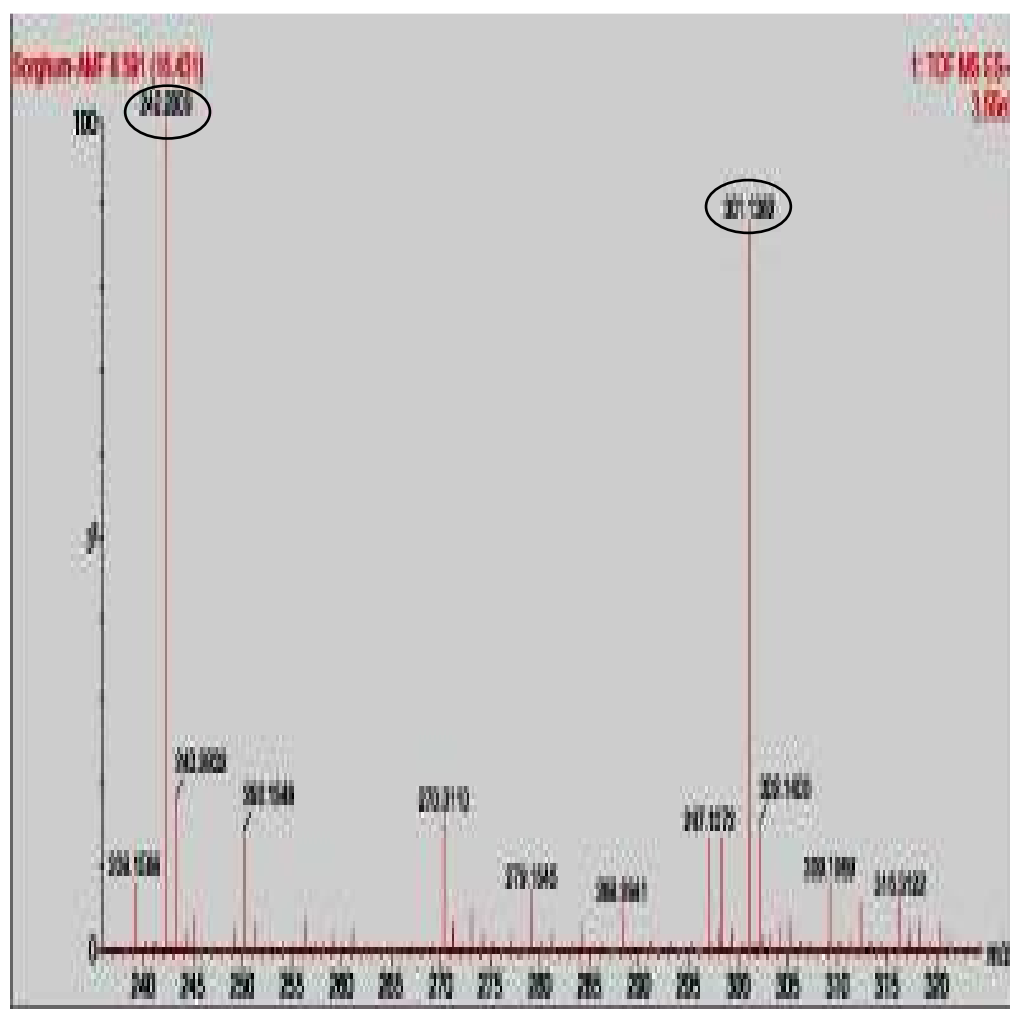
Figure 3.6: LC/MS graphical representation of possible exudate, showing two daughter Sorgolactone ions (MW circled) produced by roots within the vicinity of *Glomus intraradices*, after three weeks of HAM-P development in the ES positive mode.
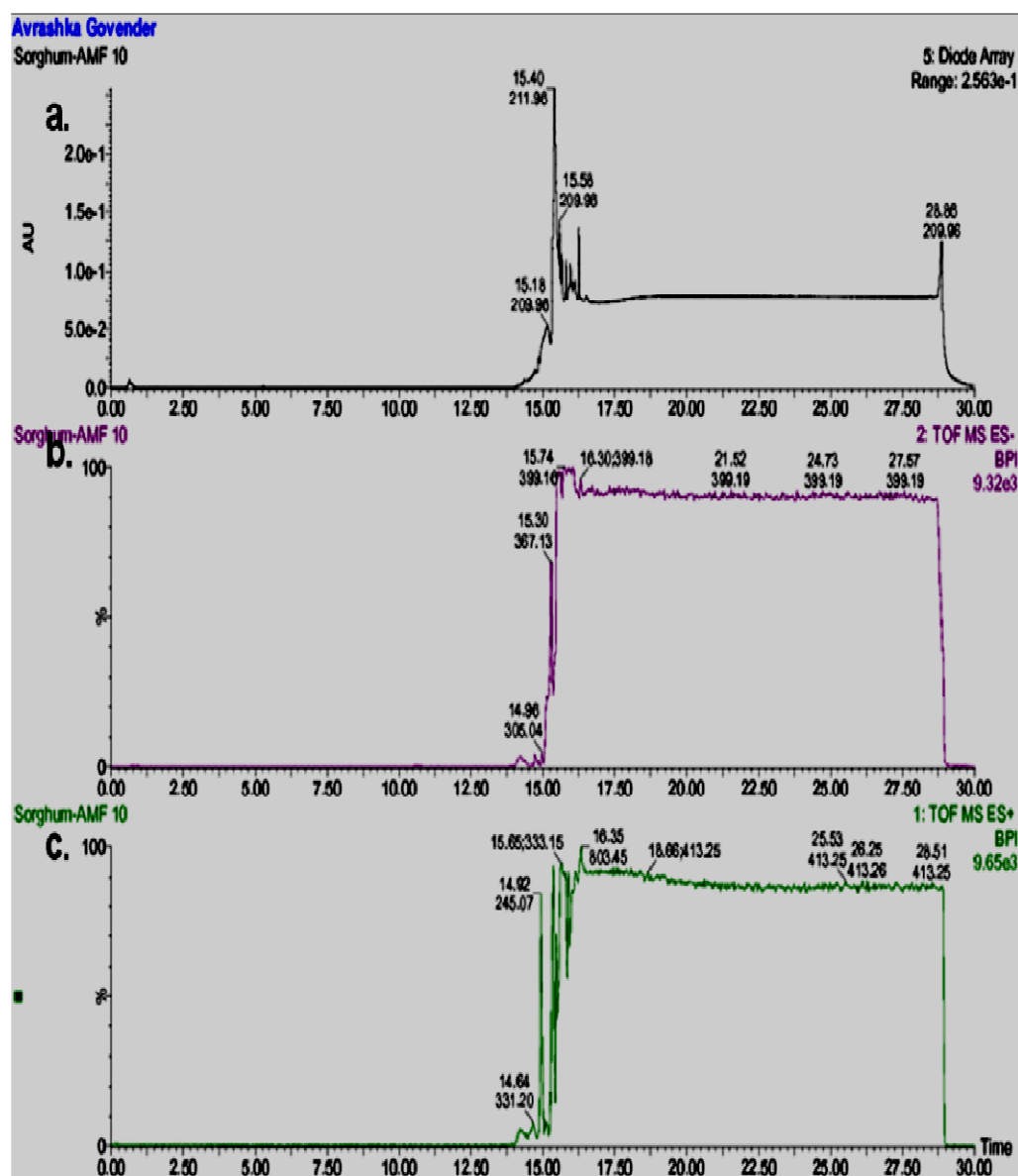
Figure 3.7: LC/MS graphical representation of methanol-based root exudate extract from three-week-old sorghum plant. Root sample analysis was taken from plates that were further away from associated *Glomus intraradices*: a. Chromatogram detected, b. ES negative mode, and c. ES positive mode.
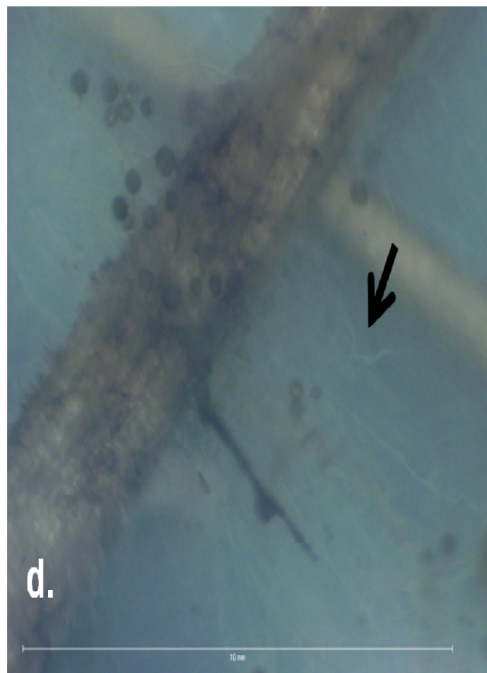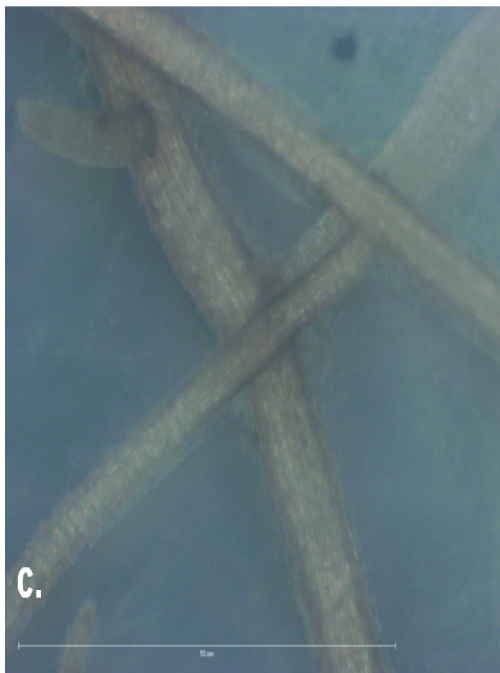
Image continued on next page.
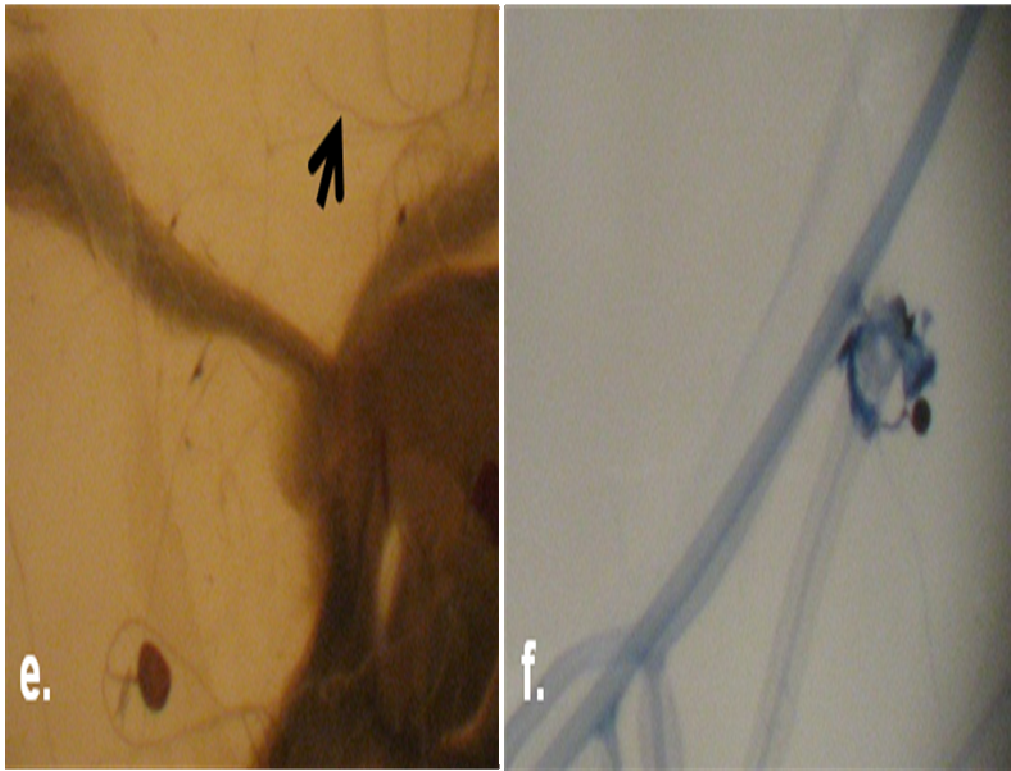
Figure 3.8: Five-week-old sample showing plant development: a. Control plant; b. *Glomus intraradices* associated plant; c. Root of control plant; d. *Glomus intraradices* associated plant, with hyphal development (arrow head); e. Microscopy images of root development with associated *Glomus intraradices* showing germination and hyphal development (arrow head), and f. Microscopy image of Trypan blue staining of root with *Glomus intraradices*.
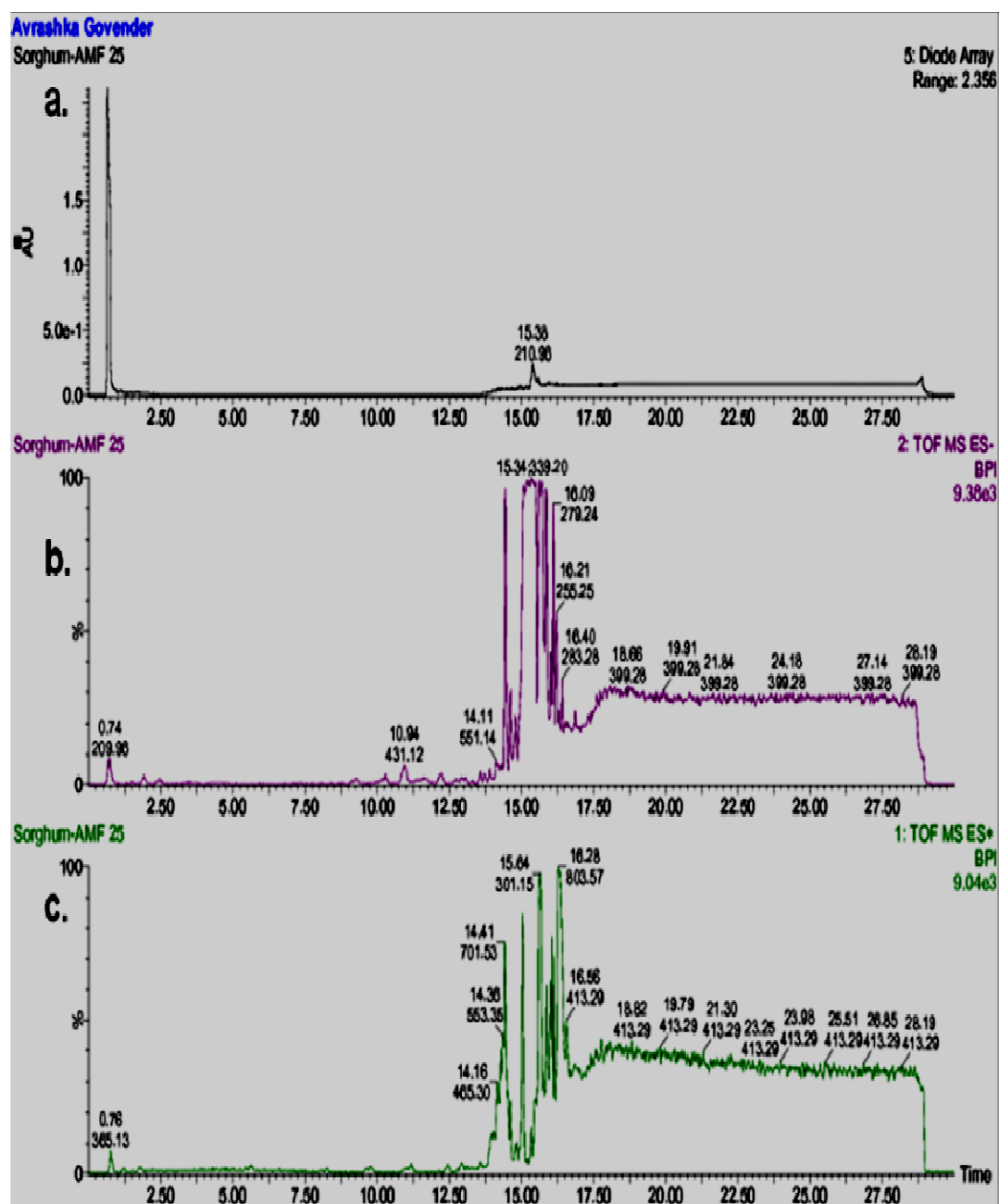
Figure 3.9: LC/MS graphical representation of methanol-based root exudate extract from five-week-old sorghum plant. Root sample analysis was taken from plates that were further away from associated *Glomus intraradices*: a. Chromatogram detected, b. ES negative mode, and c. ES positive mode.
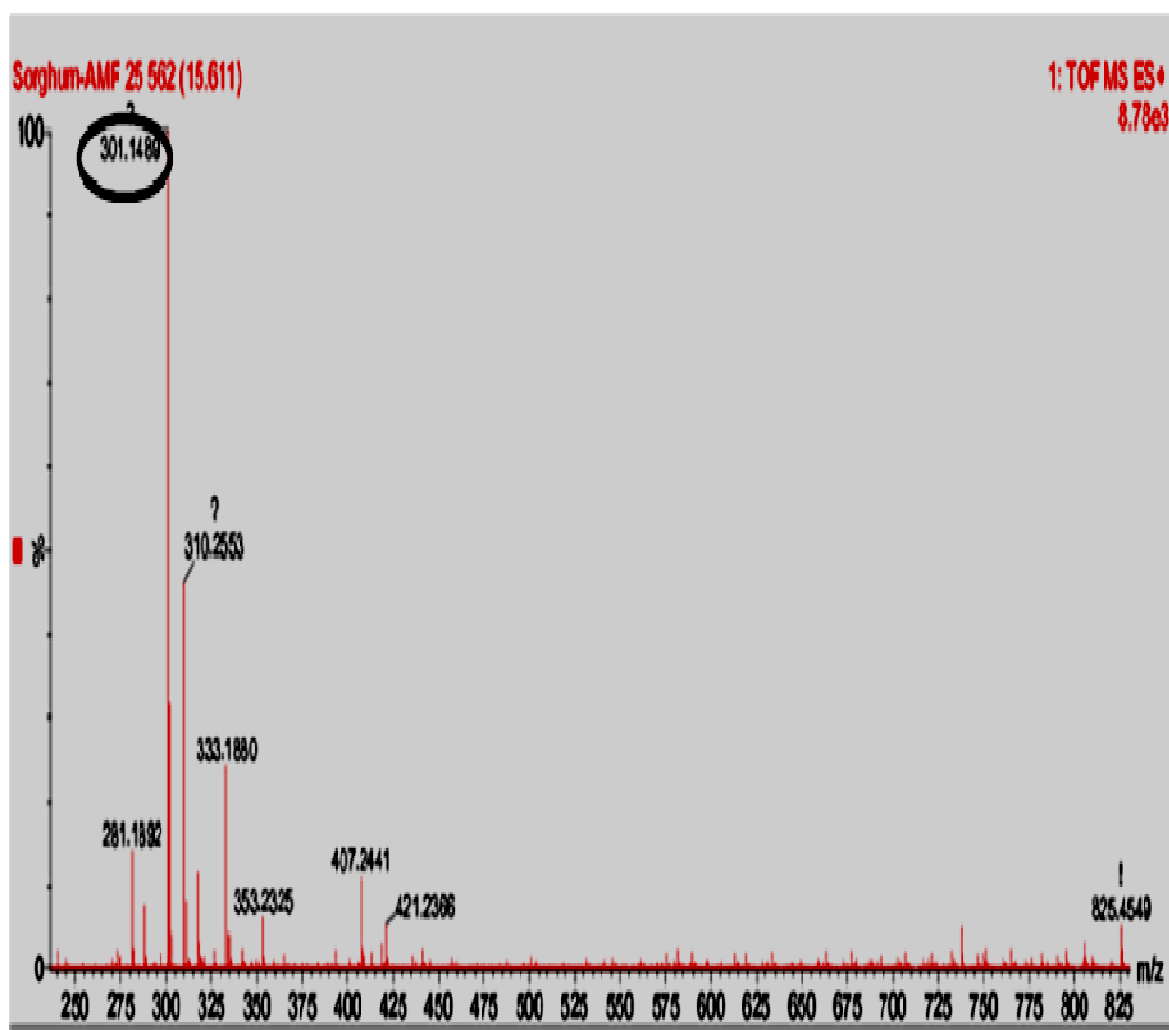
Figure 3.10: Mass spectrometry graphical representation of possible exudate, daughter ions of Sorgolactones (MW circled) produced by roots in the vicinity of *Glomus intraradices* in the ES positive mode after five weeks of HAM-P development.

Image continued on next page.
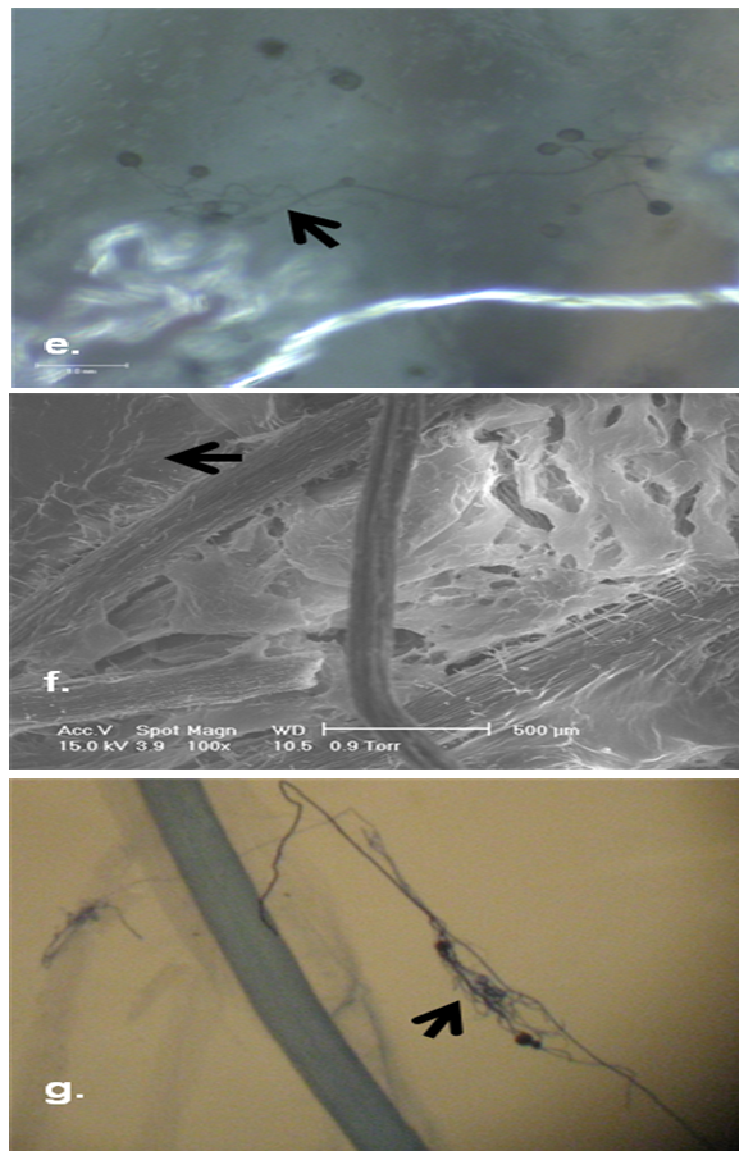
Figure 3.11: Six-week-old sample showing plant development: a. Control plant; b. *Glomus intraradices* associated plant; c. Root of control plant, showing slight discolouration; d. *Glomus intraradices* associated plant, with root blackening in the vicinity of AMF; e. Dissecting microscopy image of *Glomus intraradices* around root showing weakened hyphal development (arrow), f. ESEM image of *Glomus intraradices* breaking off from surface of root (arrow) and g. Microscopy image of Trypan blue staining of root showing no *Glomus intraradices* colonization and hyphal structure breaking (arrow).
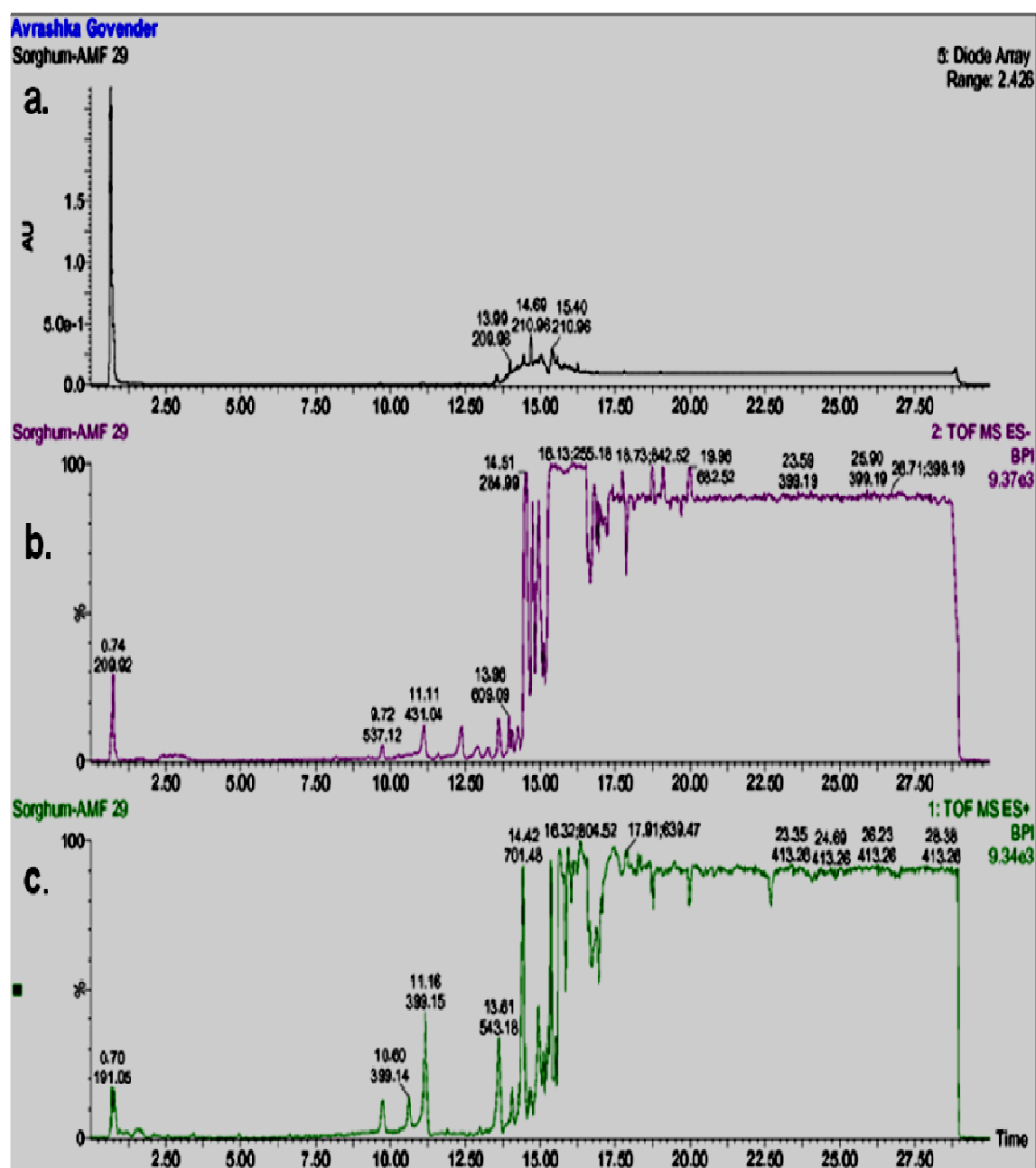
Figure 3.12: LC/MS graphical representation of methanol-based root exudate extract from six-week-old sorghum plant. Root sample analysis was taken from plates that were further away from associated *Glomus intraradices*: a. Chromatogram detected, b. ES negative mode and c. ES positive mode.
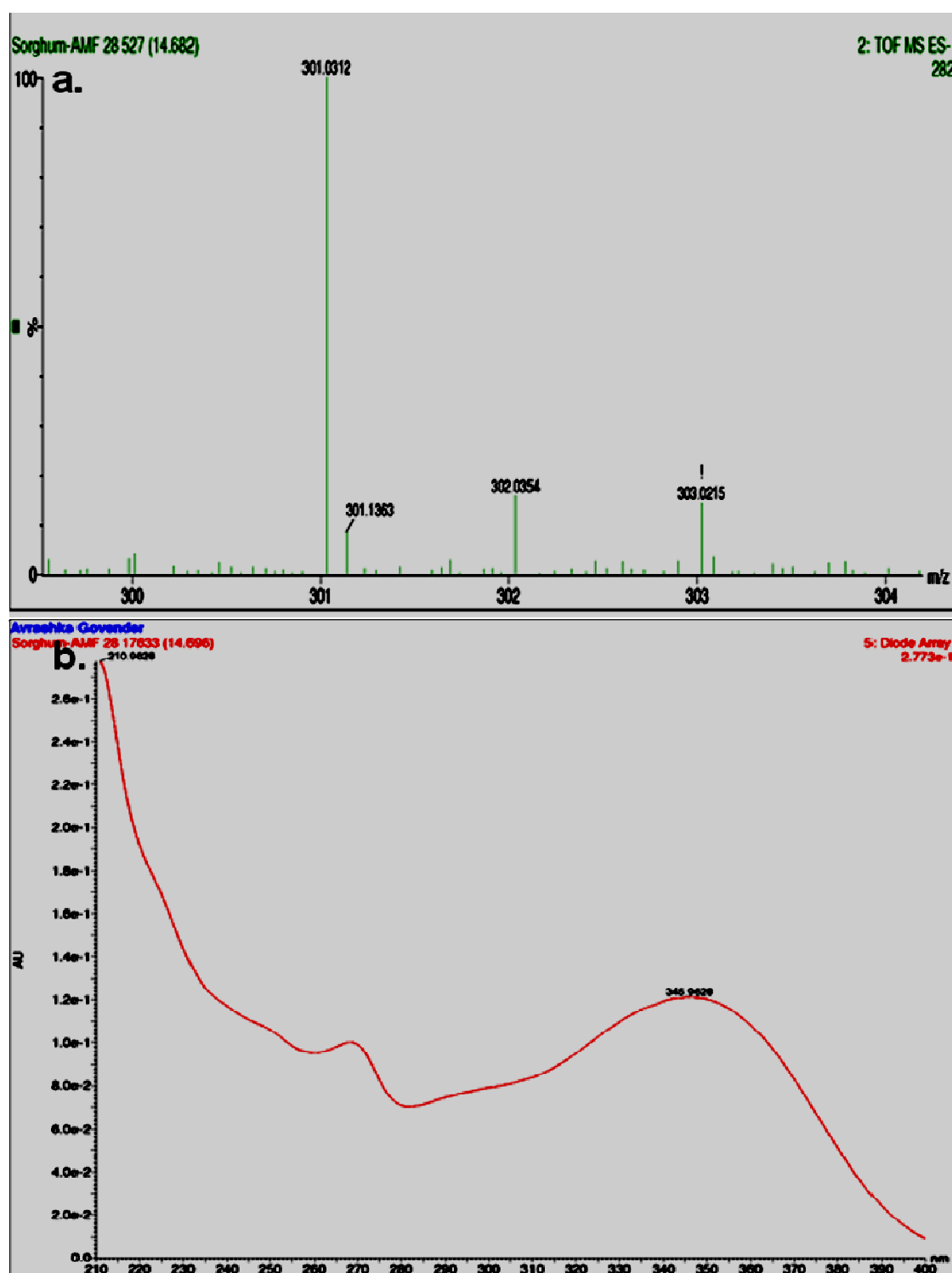
Figure 3.13: HPLC/MS graphical representation of suspected flavonoid, Quercetin ions, in the sixth week of sorghum plant-AMF development: a. Detected molecular weight in the negative mode, and b. Detected absorbance under diode array detector, showing two peak wavelength possibilities for Quercetin detection.
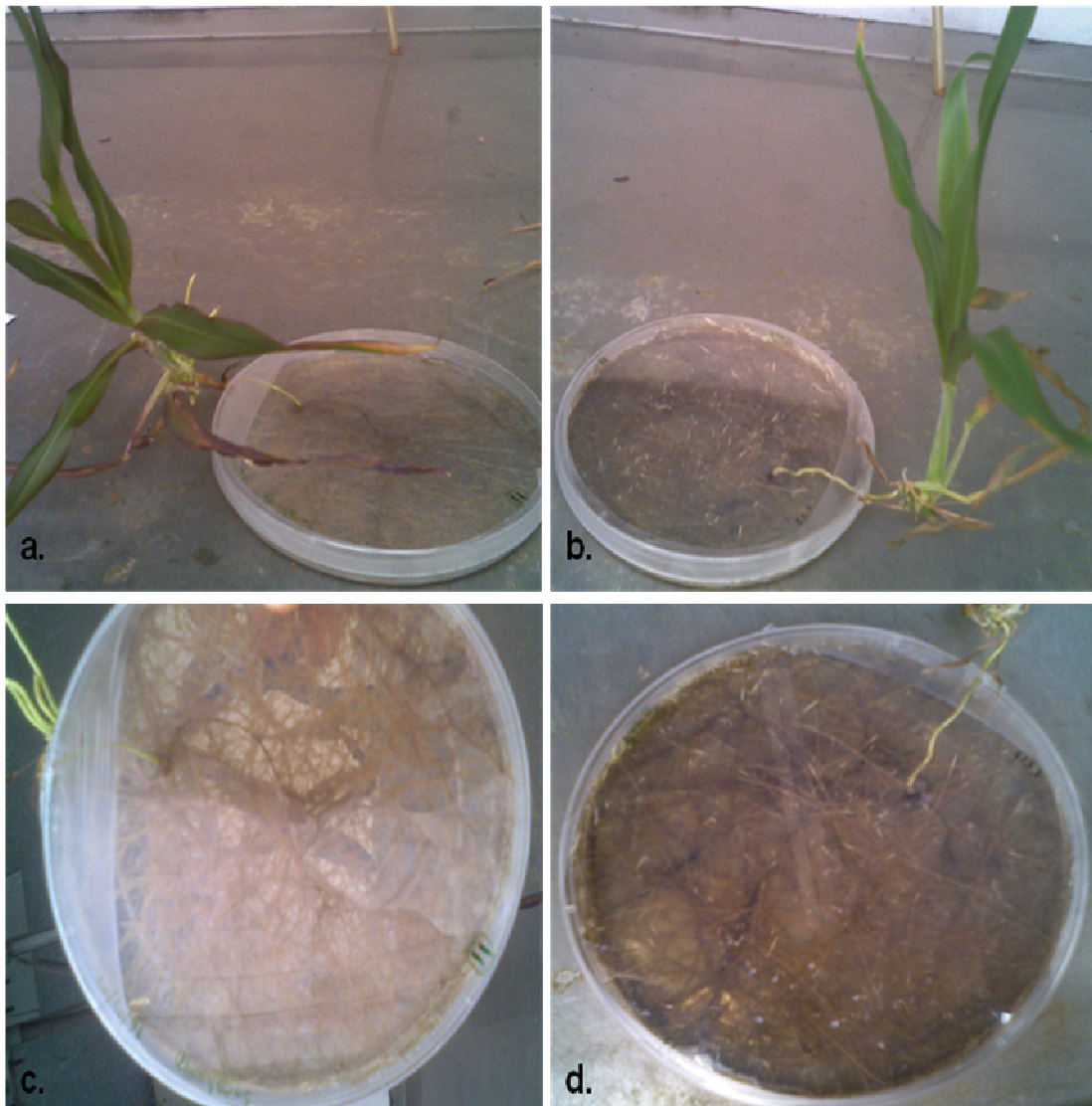
Images continued on next page.
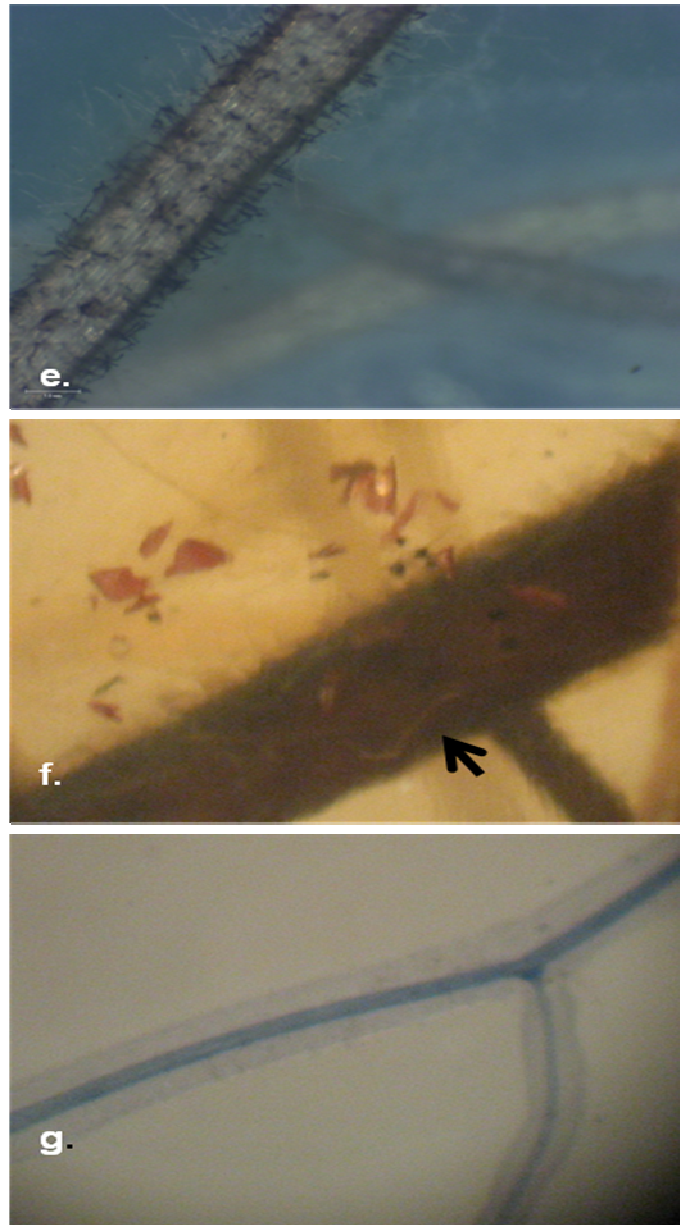
Figure 3.14: Eighth-week-old sample showing plant development: a. Control plant; b. *Glomus intraradices* associated plant; c. Root of control plant; d. *Glomus intraradices* associated plant, with root blackening in the vicinity of AMF and throughout the plate; e. Dissecting microscopy image of root showing exudate accumulation around the root, *Glomus intraradices* hyphal structures disintegrated; f. Dissecting microscope image of *Glomus intraradices* spores bursting (arrow) and hyphal structures disintegrated, and g. Microscopy image of Trypan blue staining of root showing no *Glomus intraradices* colonization.
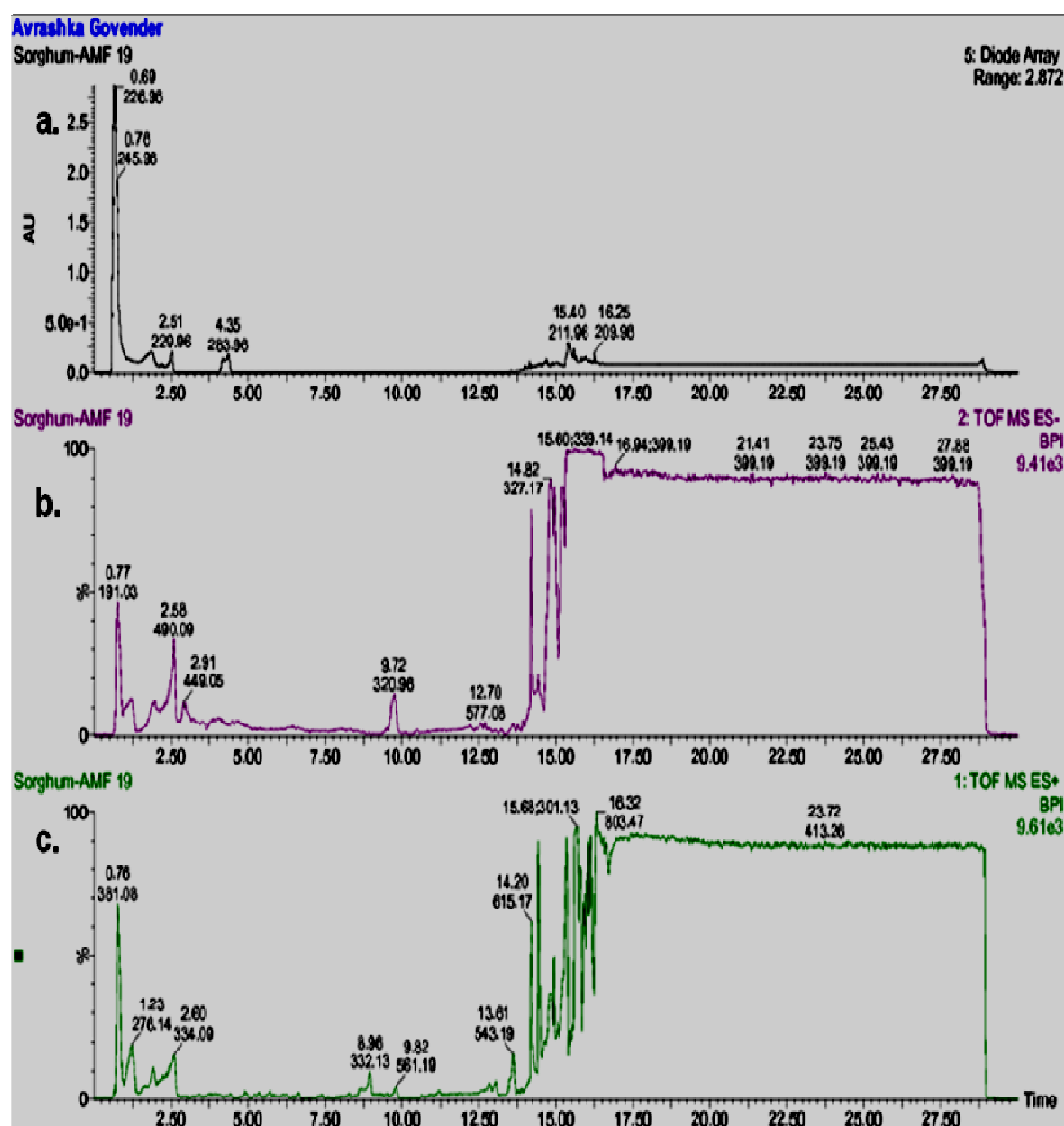
Figure 3.15: LC/MS graphical representation of methanol-based root exudate extract from eight-week-old sorghum plant associated *Glomus intraradices*, showing various compounds detected at different intensities and retention times: a. chromatogram detected, b. ES mode, and c. ES positive mode.
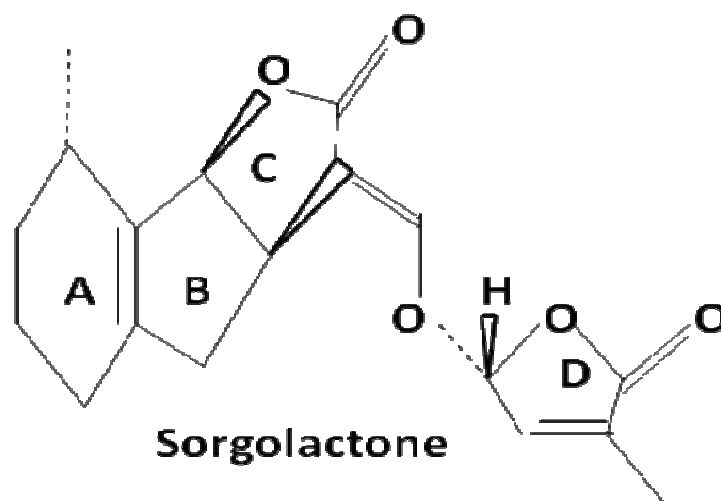
Figure 3.17: Typical structure of sorgolactones produced by sorghum, with four ring structures (A-D) (adapted from Sato *et al.* 2003).
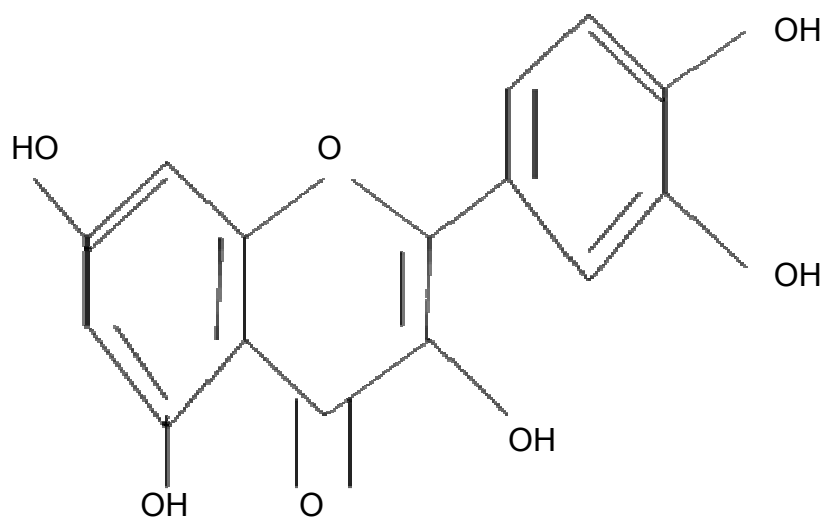
Figure 3.16: Typical chemical structure of flavonoid, Quercetin (Prasain *et al.* 2004).

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PART B

Chapter Four
Optimization of seed surface sterilization method under autotrophic *in vitro* culture system for South African agricultural crop:
Maize

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***In vitro* systems were developed for investigating the development of various agricultural crops under sterile conditions. The optimization of sterilization method provided new avenues for maize studies, such as nutritional development under *in vitro* culture systems. The purpose of this study was to optimize the seed surface sterilization method for the maize crop under the *in vitro* HAM-P culture system and observe its development, maintenance and contamination (after surface sterilization). Several experiments were conducted using three types of surface sterilization methods. The first was sterilizing maize seeds as a whole. It was observed that culturable fungal endophytes emerged and all systems did not survive more than three weeks. The second sterilization involved removal of the tip cap, followed by germination onto a non-supplemented medium. The results indicated that after the fifth week the systems suffered nutrient deficiencies. The third sterilization method required the removal of the tip cap, followed by germination onto a medium supplemented with various antibiotics. The initial results showed no contamination, although stunted growth and slow root development were observed. Plants adapted to HAM-P systems at a rapid rate, but nutrient deficiencies were observed after the sixth week. The importance of the removal of the tip cap from the maize kernel to ensure sterile plantlets was concluded. This will be beneficial when studying maize effects and growth patterns.**

4.1   Introduction

Maize is one of the most important grain crop in South Africa. It is produced throughout the country in diverse environments. Successful maize yields depend on the correct application of production inputs that will sustain the environment as well as maize productivity, viz. adaptable cultivars, fertilisation and pest control (Plessis, 2003).

Maize is known to have two main growth stages: vegetative and reproductive. The vegetative stage is subdivided into germination, maturity and the growth of leaves, tassels and ear structures, which under adequate nutrient availability and warm conditions (22˚C-30˚C) can last up to seven weeks until the next stage takes over. The reproductive stage is mainly the production of flowering structures and the maturation of cobs. This stage is variable depending on hybrid used and climatic conditions present (Hanway, 1966; Chase and Nanda, 1967). To fully understand what occurs within each stage, *in vitro* tissue culturing systems were developed.

In vitro tissue culture techniques require not only an aseptic work environment but also contaminant-free starting materials. One major factor in introducing maize into *in vitro* culture systems is the sterilization of kernels or embryos. Many researchers have successfully developed *in vitro* plantlets of maize using callus (Somers and Hibberd, 1994), somatic cells (Armstrong, 1994), meristem tissue culture (Irish, 1994) and whole kernels (Martinez, 2008). However, these methods are tedious and can take up to three days to produce a completely sterile plantlet showing coleoptile emergence. The reason for longer sterilization periods is due to endophytes residing within maize seeds. These endophytes can be either beneficial (Wagner or Lewis, 2000) or harmful (Glenn *et al.* 2001). However, to fully understand maize development, 'clean'/sterile (culturable endophyte-free) systems need to be developed.

In vitro culture systems are used to investigate the plant and microorganism relationship with no interference from other organisms. However, the duplication of such systems became tedious. Voets *et al,* (2005) and Dupre de Boulois *et al.* (2006) developed *in vitro* culture systems, namely Half-closed Arbuscular mycorrhizal plant system (HAM-P), which was optimized for this study. This system was first developed to assess the spore production dynamics, intraradical root colonization, germination capacity and life cycle of AMF associated with potato plantlets (Voets *et al.* 2005).

These systems allowed for autotrophic plants and AMF to be grown in association on a synthetic medium with no sugar or vitamins. The synthetic medium contains all macro- and micronutrients that the plant would require for development and no excess nutrients are present as AMF need to form an association within the plant root and thus form a symbiotic relationship. This technique proved to be useful for AMF in terms of assessing the life cycle, metabolism, biochemical analysis, symbiotic relationship, environmental factors, mass production, exudates and molecular analysis of the fungus (Voets *et al.* 2005).

The aim of this study was to develop a fast sterilization protocol, which will produce culturable endophyte-free sterile plantlets.

4.2 Materials and Methods

All *in vitro* culture systems utilized in the following experiment were followed according to Voets *et al.* (2005), with modifications.

4.2.1 Medium preparation

The three types of Modified Strullu Romand medium, lacking vitamins and sugars (MSR-S-V), used were as follows: single and double concentrations (Table 4.1), as well as a single concentration medium supplemented with various antibiotics (Table 4.2). All antibiotics were filtered in an autoclaved medium. The following stock solutions were prepared for the construction of MSR-S-V: Macro-elements (KNO_3, KCL and $MgSO_4.7H_2O$), Calcium Nitrate, NaFeEDTA and Micro-elements

($MnSO_4.4H_2O$, $ZnSO_4.7H_2O$, H_3BO_3, $CUSO_4.5H_2O$ and $(NH_4)_6Mo_7O_{24}.2H_2O$). Millipore water was added to dissolve the constructs (Table 4.1) and brought to a volume of 1 litre, while the pH was adjusted to 5.5 using Microprocessor pH 211 meter (Hanna instruments). The solution was solidified with 3g/l Phytagel. Medium was then Autoclaved (Speedy vertical type) for 15 minutes at 121°C.

Table 4.1: Preparation of 1 litre of Modified Strullu Romand lacking sugars and vitamins (MSR-S-V) medium concentration, thereafter steam sterilized at 121°C for 15min

Stock solutions	Concentration of MSR-S-V medium (ml/l)	
	Single	Double
Macro-elements*	10	20
Calcium Nitrate*	10	20
NaFeEDTA*	5	10
Micro-elements*	1	2
pH	5.5	
Phytagel (Sigma Aldrich)	3g/l	
Millipore water	Dissolve stock solution until 1 litre was reached	

Table 4.2: Supplementation of antibiotics into steam sterilized single concentrated MSR-S-V medium

Antibiotics	Quantity used in medium/litre	Supplier
Pimaricin	20ml	Sigma Aldrich
Flutriafol	2ml	Sigma Aldrich
Chloramphenicol	50mg	Sigma Aldrich

All antibiotics were aseptically filter sterilized (using PALL Filters: Sterile Acrodisc PF Syringe filter, 0.8/0.2µm membrane with Low protein binding and Non-pyrogenic PN 4187; attached to disposable syringe 3ml/cc (Jung/Won Medical Industry Co Ltd)) into sterilized 1x MSR-S-V media (steam sterilized at 121˚C for 15 minutes) in a horizontal laminar flow.

4.2.2 Plant material

An untreated variety of maize seeds was tested (kindly supplied by the Plant Pathology Department at the University of KwaZulu-Natal). Three methods of sterilization were conducted, as follows:

a. Sterilization 1: Whole seed (tip cap not removed) (Figure 4.1a)

Approximately 5 maize seeds were placed in falcon tubes (Merck chemical and lab supplies) then rinsed with absolute ethanol (Merck chemical and lab supplies) for 10-20 seconds (depending on the condition of the seeds), then surface sterilized with a mixture of 3.5% sodium hypochlorite and tween 20 (Merck chemicals and lab supplies) (4 drops per 100ml of 3.5% sodium hypochlorite) for 10 minutes, and thereafter rinsed five times in sterile deionized water for a period of 15 minutes each. The surface sterilized seeds were then plated in Petri plates (Merck chemical and lab supplies, 90mm diameter, 2-3 seeds/plate) containing single concentrated MSR-S-V medium, with all seeds' germination points (Tip cap) placed in one direction. . Petri plates were then sealed with Paraflim (Merck chemical and lab supplies), followed by incubation for five days in dark incubator (Stay cold incubator set at 27˚C), to allow for germination, with all seed germination points were placed in one direction into the plate. On the fifth day, plates containing germinated seeds were incubated (upright - roots facing downward and shoots upward for growth) in a light phytotron (22˚C day/ 18˚C night) for photosynthetic tissues to form in the plant. On the sixth day, the germinated seeds were introduced to HAM-P systems (section 4.2.3).

b. Sterilization 2: Seed with tip cap removed – germinated onto single concentrated MSR-S-V

The tip cap (approximately 0.5cm) of the maize seeds were removed using a scalpel (Surgical sterile blades, number 24 was attached to Stainless steel scalpel, number 4) (Figure 4.1b), followed by surface sterilization as above (4.2.2a). The surface sterilized seeds were then plated in Petri plates (Merck chemical and lab supplies, 90mm diameter, 2-3 seeds/plate) containing single concentrated MSR-S-V medium, with all seeds' germination points (Tip cap) placed in one direction. . Petri plates were then sealed with Paraflim (Merck chemical and lab supplies), followed by incubation for seven days in dark incubator (Stay cold incubator set at 27˚C), to allow for germination, with all seed germination points were placed in one direction into the plate. On the eighth day, plates containing germinated seeds were incubated (upright - roots facing downward and shoots upward for growth) in a light phytotron (22˚C day/ 18˚C night) for photosynthetic tissues to form in the plant. On the ninth day, the germinated seeds were introduced to HAM-P systems (section 4.2.3).

c. Sterilisation 3: Seed with tip cap removed – germinated onto single concentrated MSR-S-V supplemented with antibiotics

The same procedure was followed as in sterilization method (4.2.2b). However, The surface sterilized seeds were plated in Petri plates (Merck chemical and lab supplies, 90mm diameter, 2-3 seeds/plate) containing single concentrated MSR-S-V medium, supplemented with antibiotics (Table 4.2), with all seeds' germination points (Tip cap) placed in one direction. . Petri plates were then sealed with Paraflim (Merck chemical and lab supplies), followed by incubation for nine days in dark incubator (Stay cold incubator set at 27˚C), to allow for germination, with all seed germination points were placed in one direction into the plate. On the ninth day, plates containing germinated seeds were incubated (upright - roots facing downward and shoots upward for growth) in a light phytotron (22˚C day/ 18˚C night) for photosynthetic tissues to form in the plant. On the eleventh day, the germinated seeds were introduced to HAM-P systems (section 4.2.3).

4.2.3 Development of an autotrophic culture system

The system used for this experiment was the HAM-P culture system and set up was done by constructing a hole on the edge of the lid and base of Petri plate (150mm diameter (Capital lab supplies)) containing 2x concentrated MSR-S-V medium. Under a laminar flow hood (horizontal), close to a Bunsen burner, sterilized stainless steel forceps were used to aseptically transfer one germinated seed into 150mm diameter Petri plate, with roots placed inside the plate on the medium and the shoot protruding outwards through the hole of each Petri plate. The Petri plates were sealed with Paraflim (Merck chemical suppliers) and the area with the hole was further covered with silicon grease (sterilized at 121°C for 15 minutes) (Hardware outlets) to avoid any contamination. Thereafter, the plates were covered with an opaque plastic bag (to allow a dark environment for root development) and incubated in a phytotron with well regulated moisture and phototrophic settings*. Through observation, every two weeks *in vitro* HAM-P culture systems were maintained, by adding 20-30ml 1x MSR-S-V into each Petri plate. This was done to provide renewed nutrients to the plant for optimum development.

*Phytotron setting:
- o 22°C/18°C day/night
- o 16 hours photoperiod
- o For moisture, 4x 2-litre containers were filled with water and placed around the plants. These containers were filled with clean water every 5 days.

It was important to note that at different stages, maintenance occurred when roots were no longer submerged or in contact with growing medium.

4.3 Results

4.3.1 Plant material

Maize seed surface sterilization was the first factor to consider when developing *in vitro* culture systems. The first method involved surface sterilization of the kernel, with the tip cap intact. The results indicated fungal endophytes resided within the seed and surfaced eight days after seed germination or within a week in HAM-P systems. This led to the development of two alternative sterilization methods, which involved the removal of the tip cap from the kernel, followed by the normal sterilization procedure. The second method consisted of seed germination onto normal MSR-S-V medium. The third method involved germination of the kernel onto MSR-S-V medium that contained various antibiotics. Although the plant growth was stunted during the first three weeks of development, this system proved to be healthy for the plant and normal growth followed in the third week of HAM-P development.

The indicated results are the average values of each system (see Appendices for actual stats, n=10). All shoot lengths were measured from the stem to the end of the highest leaf.

a. Sterilization 1: HAM-P culture system development – tip cap not removed

Table 4.3 indicated that in some systems, the cultural fungal endophyte emerged in the second week of development (Figure 4.2c). By the third week, the plant system was fully contaminated with fungal growth (Figure 4.2d). Rapid germination was observed. However, some trials exhibited endophytic growth (Figure 4.2a).

Table 4.3: Average maize development in HAM-P culture systems, with tip cap intact, observed weekly and incubated in phytotron

Weeks	Plant development			Endophyte	Figure
	Root	Shoot (cm)	Maintenance		
1	++	6.3	0	0	5.2b
2	+++	10.5	1	0	5.2c
3	++++	9.05	1	1	5.2d

Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots; ++++, root system discolouring; 1, yes; and –, no.

The first week of development indicated rapid plant growth, when introduced into HAM-P systems. Thick primary roots gave rise to numerous lateral roots. This showed that the adaptability of the maize plant to *in vitro* systems occurred rapidly. Although leaf length increased, the colour of leaves indicated nutritional deficiencies. No maintenance was required at this point (Table 4.3 and Figure 4.2b).

The results indicated that the plants developed normally until the second week when endophytes emerged, causing stunted growth and the withering of leaves (Table 4.3 and Figure 4.2c). Plants exhibited chlorosis, the curling and drying out of leaves, as well as weakened stems (Figure 4.2b-d). The root system developed rapidly and was formed mainly around the peripheral region of the Petri plate (Figure 4.2c). Due to this rapid development of the roots and endophyte emergence, systems were maintained in the second week.

The third week development indicated the endophyte out-competed the plant for nutrients, thus resulting in plant death. Roots and leaves dried and withered, while the culturable endophyte continued to develop (Table 4.3 and Figure 4.2d). Some systems still showed no emergence of endophytes, until the plant was renewed with nutrients in the third week. A new protocol was designed, which aimed at eliminating culturable endophytes.

b. Sterilization 2: Maize system with tip cap removed – germinated onto single concentrated MSR-S-V

Table 4.4: Average maize development in HAM-P culture systems, with tip cap removed and developed onto non-supplemented MSR-S-V medium, observed weekly and incubated in phytotron

Weeks	Plant development			Figure
	Root	Shoot (cm)	Maintenance	
1	++	9.7	0	5.3a
2	++	17.6	0	5.3b
3	+++	22.05	1	5.3c
4	++++	25.4	1	5.3d
5	+++++	30.06	1	5.3e
6	+++++	35.29	0	5.3f
7	+++++	37.67	1	5.3g
8	+++++	39	1	5.3h

Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots; ++++, good thick root system; +++++, roots are thick and covering entire plate; 1, yes; and 0, no.

It was observed that once the plant system leaves reached above 36cm, they required weekly maintenance. The root system developed rapidly and thus the plant suffered nutrient deficiencies after the fifth week in the system (Figure 4.3a-h and Table 4.4). The protocol above involved removing the tip cap to allow the ethanol and sodium hypochlorite with tween 20 to enter the kernel and remove possible culturable endophytic fungi. Not all systems were totally stripped of culturable fungal endophytes. However, certain systems experienced mycotoxins produced by the endophytes present within the plant between the fourth to sixth weeks of plant development (Figure 4.4).

The results indicated primary root development with numerous lateral roots in the first two weeks (Table 4.4 and Figure 4.3a-b). In the third week, roots formed abundantly around peripheral regions of the Petri plate (Figure 4.3c). In the fourth

week, roots developed abundantly throughout the Petri plate and remained constant between the fourth to eighth week of plant development (Figure 4.3d-h).

Leaf length seemed to be constant throughout plant development. However, as the weeks passed, the leaves thinned and eventually by the sixth week the leaves suffered from nutritional deficiency (Figure 4.3a-d). However, our purpose in this chapter was to optimize the sterilization protocol. Further studies for maize growth within systems provide future work.

c. Sterilization 3: Maize system with tip cap removed – germinated onto single concentrated MSR-S-V supplemented with antibiotics

Table 4.5: Average maize development in HAM-P culture systems, with tip cap removed and developed onto non-supplemented MSR-S-V medium, observed weekly and incubated in phytotron

Weeks	Plant development			Contamination	Figure
	Root	Shoot (cm)	Maintenance		
1	0	3.9	0	0	5.4a
2	+	5.8	0	0	5.4b
3	++	13.8	1	0	5.4c
4	+++	21.1	0	0	5.4d
5	+++	30.05	0	0	5.4e
6	++++	37	1	0	5.4f

Key: +, Primary growth beginning; ++, growth with new lateral roots; +++, growth with few lateral roots; ++++, good thick root system; 1, yes; and 0, no.

In the first two weeks, the plant showed slow development. However, once the plant was being maintained in the third week, it developed much faster and no nutrient deficiency was observed. Leaves developed on average at 7cm per week (Figure 4.4a-f). In this protocol, germination medium was supplemented with three fungal

antibiotics in order to remove the suspected endophytes that resided within the maize (Figure 4.2 and 4.4).

Due to supplementation of the antibiotics, root development was stunted. No roots were observed in the first week of plant development within HAM-P systems (Figure 4.4a and Table 4.5).

The results indicated that root development affected leaf development. In the first week, no root development was noted (Figure 4.4a), while in the second week, the primary root started to form at a slow rate (Figure 4.4b). In the third week, the primary root formed with few lateral roots and the leaves lengthened more quickly (Figure 4.4c). As a result of maintenance occurring in the third week, the plants seemed to adapt to their environment in the fourth week, with numerous lateral roots observed, as well as longer length leaves (Figure 4.4d). In the sixth week, the plants showed a nutritional deficiency. However, it was noted that culturable endophytes were not present during the plant development.

4.4   Discussion and Conclusion

Fungal endophytic species are present in a wide range of plant species and can reside within the vascular system or intracellular spaces of plants. Endophytes are defined as microorganisms that are detected after the surface sterilization of a plant part and are assumed to originate from the seed and/or the surrounding environment. Some of these fungal endophytes are pathogenic to the plant, such as *F. verticillioides* in maize kernels (Rheeder *et al.* 1990; Sumner, 1968 and White, 1999). In order to study the development of maize plants from kernel to maturity, it was important for the optimization of sterilization protocols for kernels. Certain methods may eradicate endophytic organisms, but have a negative impact on plant development.

Normally, maize requires increased supplementation of macronutrients during the vegetative growth phase (Devitt *et al.* 2006 and Abida *et al.* 2007). This was clearly indicated in our results, as plants showed a nutritional deficiency during the fourth week of development. In terms of root development, Hanway, (1966) indicated that field trials showed the emergence of thick lateral roots after the sixth week of

germination. However, *in vitro* systems indicated lateral root emergence after the third week of plant development.

The first sterilization showed endophyte emergence occurring at different times, depending on favourable growth conditions. According to Hanway, (1966), under favourable temperatures, the emergence of the coleoptile occurs between the seventh to the tenth day of plant development. This in turn would give rise to culturable endophytes that resided within the kernel. According to Plessis, (2003) endophytes residing within maize kernels utilize the nitrogen and potassium that are required by the plant, thereby adversely affecting plant growth. This could be due to the endophyte utilizing the nutrient supply present in the medium after the emergence of the coleoptile. This allowed for easier access to the nutrients in the medium, thus depriving the plant and resulting in its death.

As the type of culturable endophytes present within the maize kernels was unknown, these were seen as contaminants of *in vitro* culture systems. Thus, three types of antibiotics were used. These were Flutriafol, which is effective against maize pathogens that cause major crop losses (Ward *et al.* 1997); Chloramphenicol and Pimaricin, which are effective antibiotics against *Fusarium* and *Aspergillus* species, as well as mould-forming fungi (Day *et al.* 2008). *Fusarium* and *Aspergillus* species are more common as endophytes in maize kernels (Glenn *et al.* 2001).

Fungistatic antibiotic are known to control fungal growth without killing them, this concept is well known for two antibiotics in this study, namely Pimaricin and Flutriafol. While, fungitoxic antibiotics are known to eradicate fungi; in this study chloramphenicol was investigated.

Pimaricin is produced during fermentation by Streptomyces *natalensis,* these antibiotics is known to bind and form complexes with sterols of certain fungal membranes; in turn they prevent the organism from growing (Franklin and Snow, 1998). According to Ward *et al.* (1997), fungistatic Flutriafol, is an agricultural triazole fungicide tested previously on maize against fungal diseases, provided effective control of diseases and a longer period of protection for the crop. It also gave 1x higher crop yield when compared with untreated maize. The positive effects of maize growth were observed, in terms of leaf development.

Our results indicated noticeable magnesium and zinc deficiencies, as well as decreased root development. This retardation was due to the chloramphenicol, which not only had an effect on the endophyte, but plant growth as well. According to Yang and Scandalios (1977), chloramphenicol has an inhibitory affect on specific mitochondrial proteins in *Zea mays*, which are used for certain organelle production or the maturity of certain structures. However, according to Day *et al.* (2008), chloramphenicol had a positive effect on eradicating Fusarium and Aspergillus species at low concentrations, due to its Quinone derivative chloroform, this antibiotic has a fungitoxic effect against *Aspergillus* and *Fusarium* species. When the plant was maintained after the third week, normal growth was then observed. All antibiotics worked together to eradicate endophytes within plants and clean plants were observed after six weeks. This was possible since, both fungistatic antibiotics control the possible endophytes while the fungistatic eradicates the unknown endophyte; the quick eradication was brought about by the fungistatic antibiotics, as they leave the endophytic fungi more prone to destruction.

These results clearly indicated that all three antibiotics worked in conjunction, which resulted in a positive effect of inhibiting possible culturable endophytes. Subsequently, this slowed down the growth of the plant in the first three weeks of its development. It was also important to note that the antibiotics could not work at a stand-alone, due to their mode of action. Antibiotic sterilization also had a positive effect on plant growth. However, future work could focus on maize plant development.

4.5 Figures:
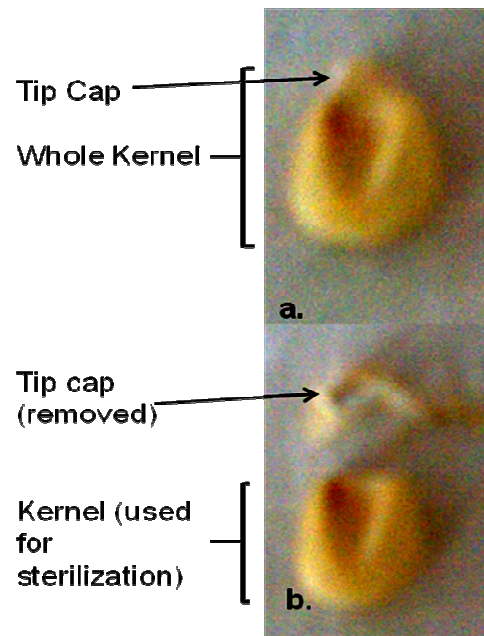
Figure 4.1: Diagrammatic representation of maize seeds before surface sterilization: a. Tip cap intact and b. Tip cap cut off from the maize seed (photographed by Avrashka Govender, 2009).

Sterilization protocol 1:
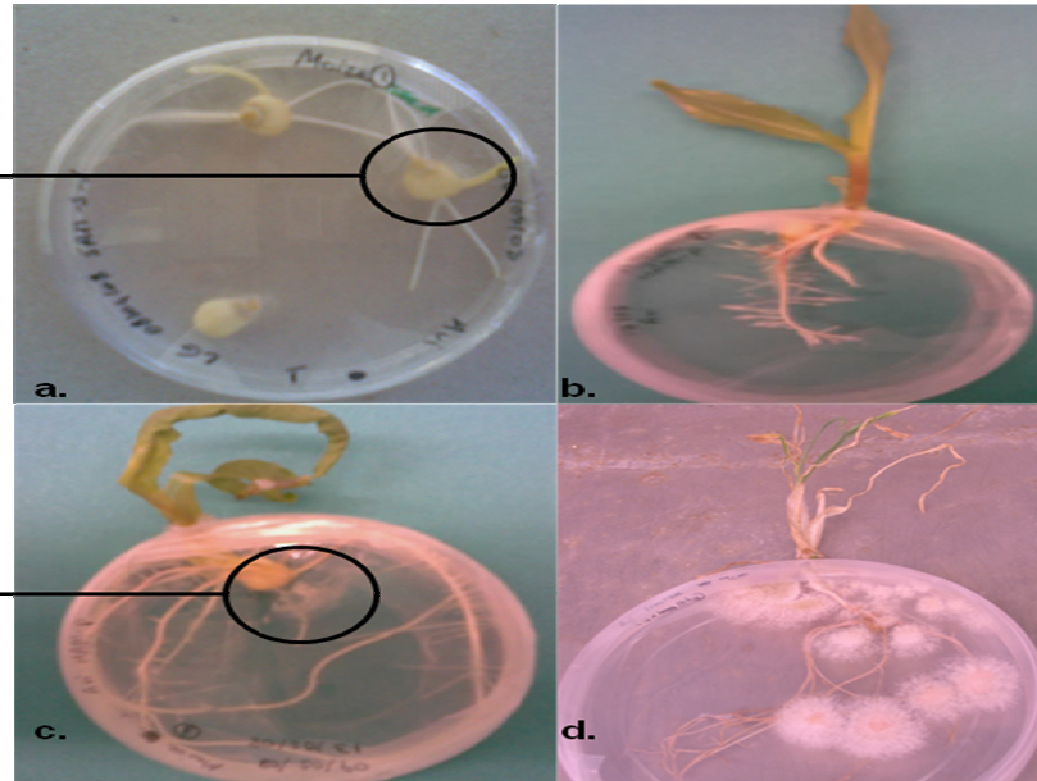
Figure 4.2: Maize development in HAM-P system, observed over a period of four weeks: a. five-day-old germinated seeds with one seed showing endophytes (circled), b. One-week-old HAM-P system, c. Two-week-old HAM-P system showing endophyte emergence (circled) and d. Three-week-old system showing whole system contaminated with fungal endophyte, which resulted in maize death.

Sterilization protocol 2:
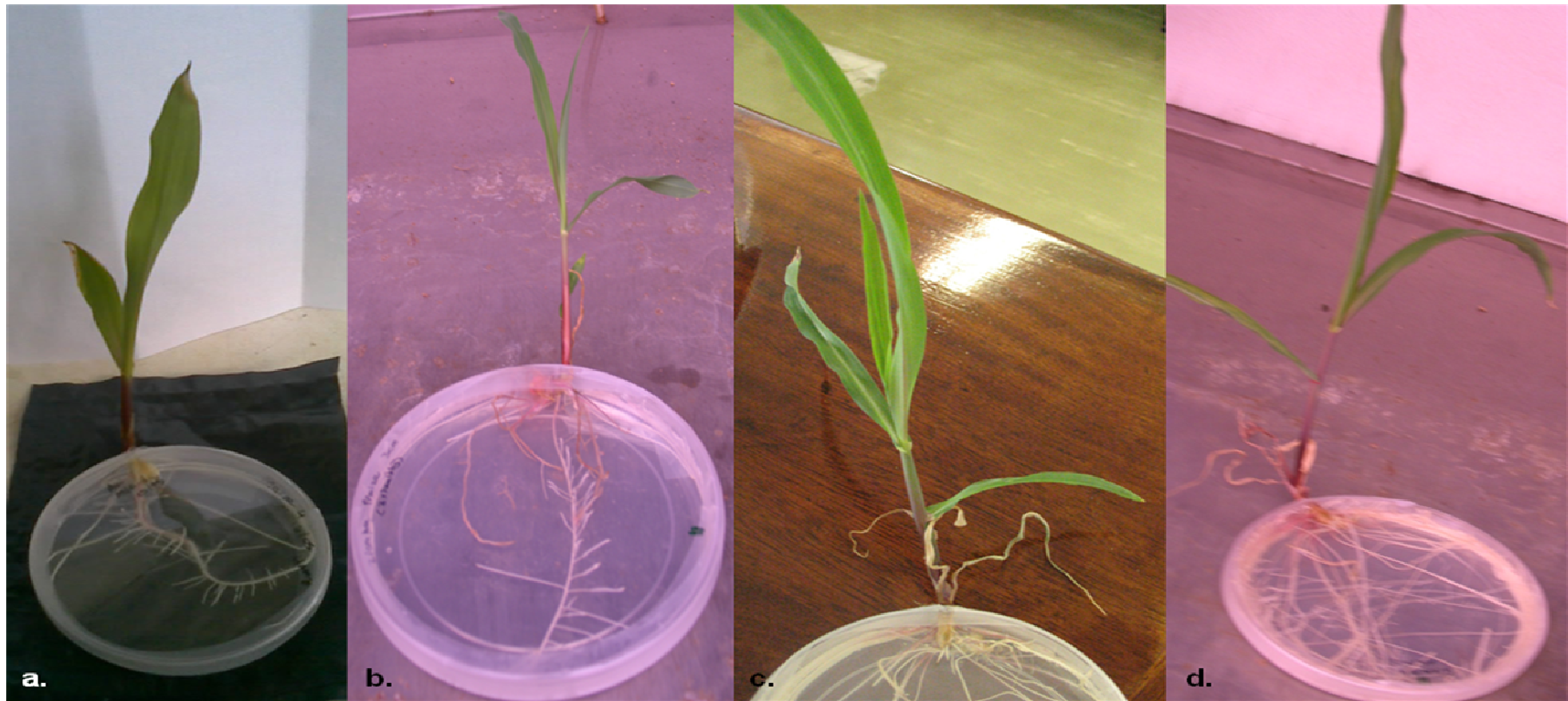
Figure 4.3: Maize HAM-P system development, over a period of eight weeks: a. One-week-old system, b. Two-week-old system, c. Three-week-old system, d. Four-week-old system, e. Five-week-old system, f. Six-week-old system, g. Seven-week-old system and h. Eight-week-old system.
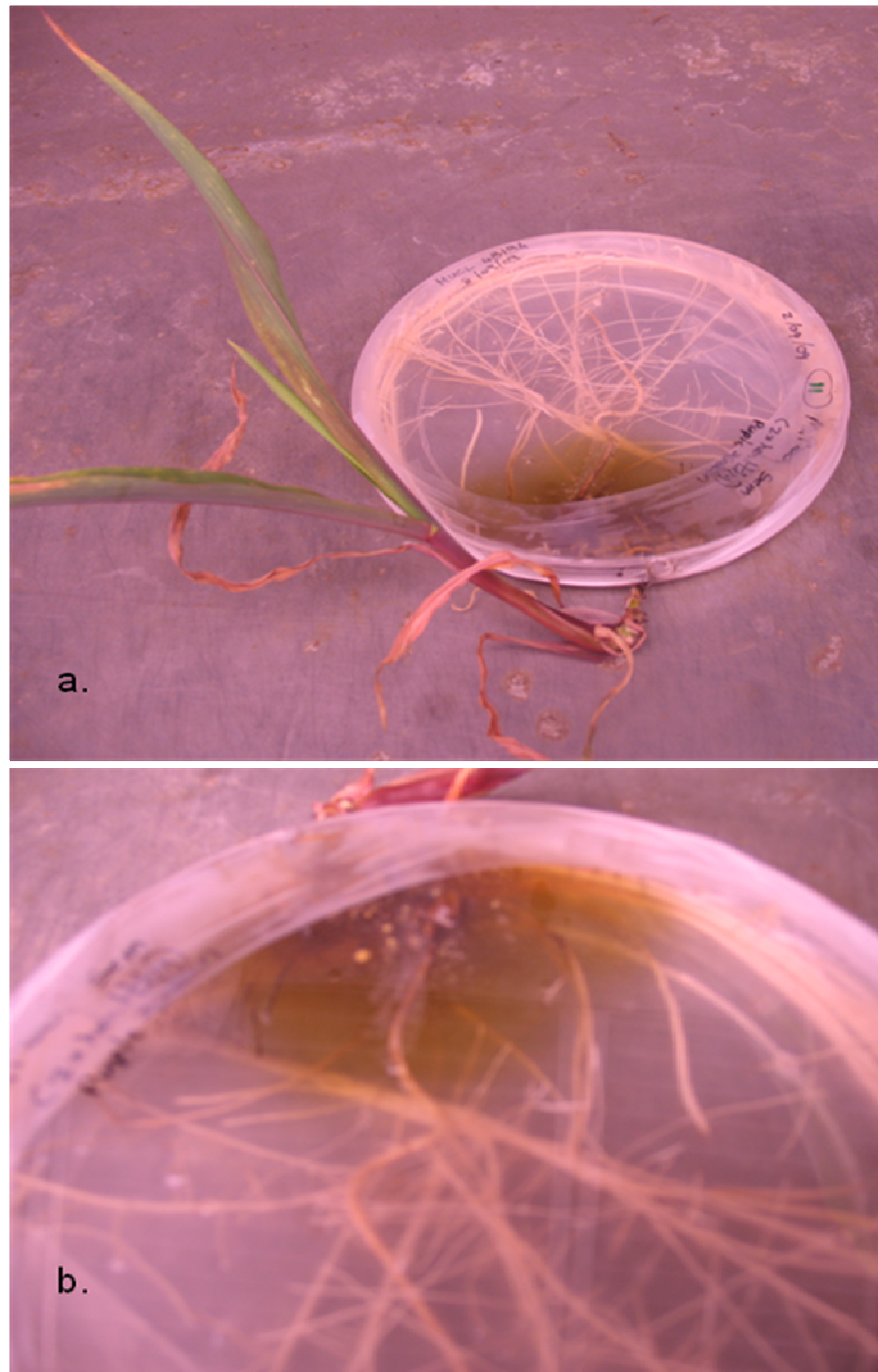
Figure 4.4: Eight-week-old maize HAM-P system showing fungal endophyte emergence: a. Whole system result, and b. Rotting/darkening of seed and mycotoxin, produced within the Petri plate.

Sterilization protocol 3:
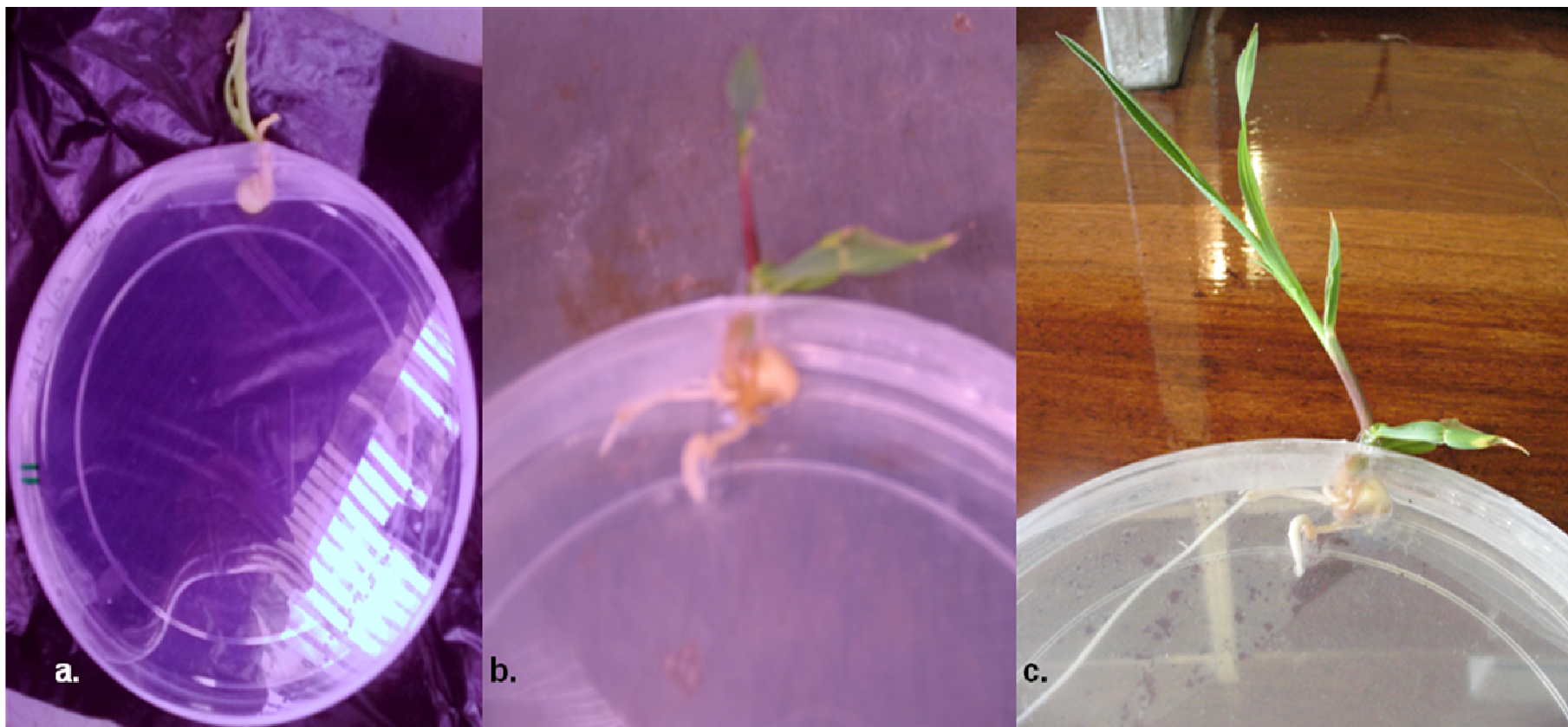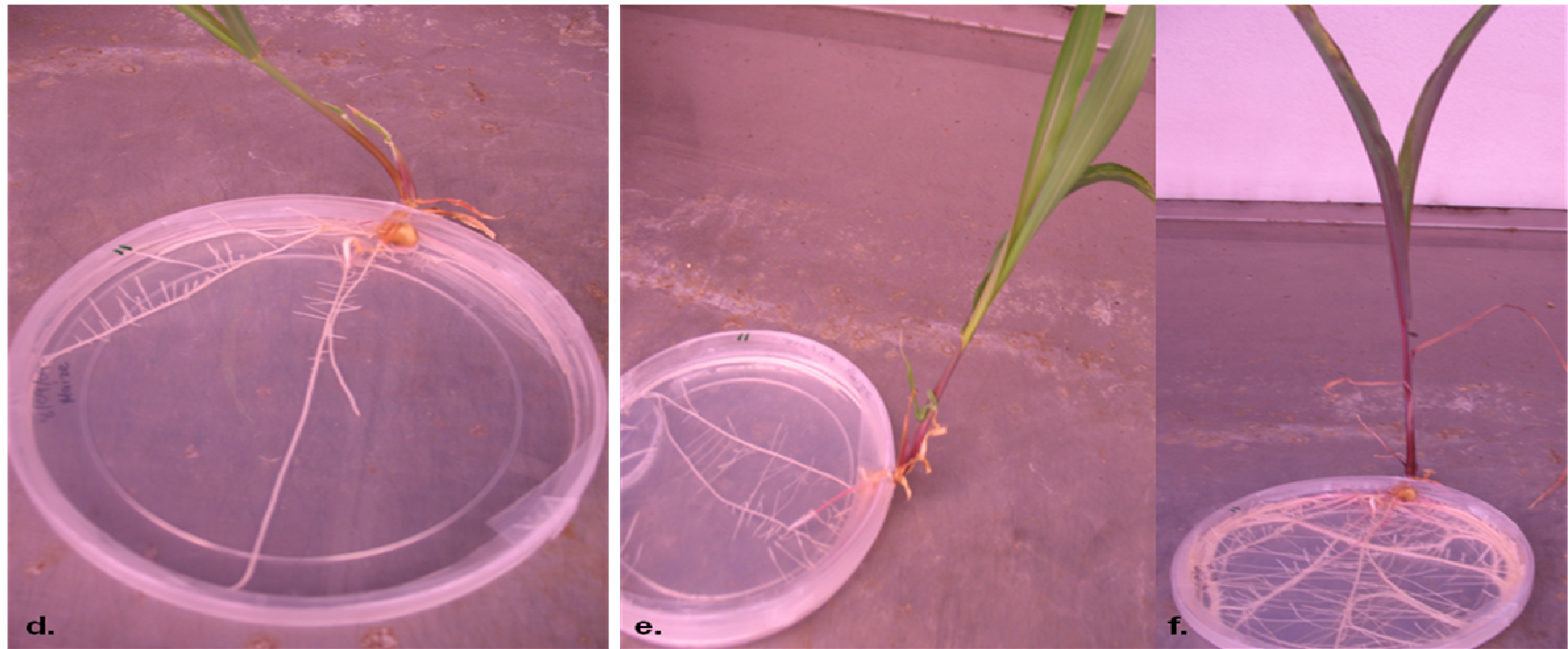
Figure 4.5: Maize HAM-P system development, over a period of eight weeks: a. One-week-old system, with no root, b. Two-week-old system, primary root observed at 3cm, c. Three-week-old system, primary root system with few secondary roots, d. Four-week-old system, with few secondary roots, e. Five-week-old system, few secondary roots and f. Six-week-old system, where secondary roots fill the entire plate; however, nutrient deficiency was observed at this point.

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Chapter Five
General discussion and future work

Cereal and grain crops are the most important dietary and livestock feed in South Africa. These crops are also crucial as a source income for small-scale farmers in the country. However, farmers are plagued by many factors that result in large crop losses, including pest attacks. Although agrochemicals are effective in preventing crop losses, they are expensive and harmful. Thus, research is now aimed at producing affordable biocontrol agents. To investigate the effects of biocontrol agents on crops, *in vitro* culture systems are useful tools.

In this study, two crops were used: maize and sorghum. The research was aimed at developing an *in vitro* culture system for the respective crops. As a result, this dissertation was divided into two parts, as follows:

Part A:

In this part of the dissertation, an *in vitro* culture system was developed and the effects of endophytic fungus: arbuscular mycorrhiza (*Glomus intraradices* strain MUCL 41833) on sorghum was investigated.

Plants were successfully introduced to *in vitro* HAM-P systems, with no possible culturable contamination. Under *in vitro* systems, our results indicated that sorghum comprised various growth stages, with different functioning and nutritional supply. Out of the eleven growth stages, only four growth stages were depicted under *in vitro* culture systems. These growth stages were the Emergence stage (GS-0) and 3-leaf stage (GS-1), for which our results indicated higher nutritional requirements for leaf and root formation, and maturity towards the next stage. Our results indicated that GS-1 allowed for root exudate formation, which was beneficial for plant development and protection. The 5-leaf stage (GS-2) allowed for further root and leaf maturity, as well as root exudate secretion. It was also indicated that at GS-2, plants required minimal nutrients, as this stage mainly involved root development and leaf maturation, as well as exudate secretion. Our results indicated that root exudate properties change when increased sources of macronutrients are supplied to the

plant at GS-2, which was detrimental for plant development. Death was noticed at growth point differentiation (GS-3), as plantlets required increased nutrient supplies and underwent stress due to space deprivation. The results indicated that plantlets could last for a maximum of eight weeks under *in vitro* culture systems, due to space constraints. After eight weeks, death of plant at GS-3, plant roots required more space for anchorage and completion of the sorghum life cycle. From the results obtained, new avenues can be researched in the future, such as the following:

- Certain non-culturable endophytes reside within the seed in certain plants. Molecular work can be done to investigate presence/absence of non-culturable endophytes that may reside within seeds or developed plantlets. This molecular work could involve DNA extraction, Polymerase chain reaction and sequencing. If necessary, denaturing gradient gel electrophoresis could be conducted, provided the DNA structure is too small in base pairs.

- As the sorghum plantlet has a short life span within *in vitro* culture systems, future work could focus on re-establishing plantlets either into fields or sterile pot cultures. This could involve testing the viability of *in vitro* cultured plantlets, as well as investigating the time taken for the plant to complete its growth stages to eventually form a grain crop.

It was established that under *in vitro* culture systems, phytochemicals can be exuded by plant roots. These phytochemicals are produced in the GS-2 and GS-3 of sorghum development. In GS-2, they acted as a chemoattractant for symbiotic organisms. This study focused on the symbiotic fungi *Glomus intraradices*. Under *in vitro* culture systems, our results indicated that upon association of *Glomus intraradices* to sorghum, plant roots exuded compounds concentrated around the vicinity of fungi. This exudate stimulated fungi spore germination. Upon High pressure liquid chromatography, mass spectrum (HPLC/MS) analysis, the results indicated that the exudate which allowed the germination of spores was sorgolactones. As the plant root matured, the sorgolactones were secreted throughout the plant root. However, due to its unstable nature, the plant roots modified the biochemical pathway, which produced the flavonoid Quercetin. At a low concentration, this flavonoid allowed for healthy plant and *Glomus intraradices* development. At this point, *Glomus intraradices* used the chemoattractant for the

stimulation of hyphal growth, in the search for roots. However, due to space deprivation, it was observed that plants produced these flavonoids at a higher concentration, which resulted in phytotoxic effects on fungi formation by the sixth week. The fungi hyphal network weakened and discontinued growth. In the eighth week of HAM-P development, the plants suffered from nutrient deficiencies and thus they weakened. It could be concluded from the results obtained that exudates excreted for symbiotic association with AMF resulted in the healthy growth of the plant. When plants underwent stress, they produced phytotoxic compounds that not only ceased AMF development, but had a negative effect on plant growth. It could be concluded that for AMF colonisation to occur, plants should not undergo stress. These results can be investigated further in new research, as follows:

- Creating a system similar to that of Voets *et al.* (2009), that involves fast mass production of AMF spores. With certain modifications, experiments can involve germinated sorghum plantlets aseptically transferred into pre-existing HAM-P culture systems that contains extensive extraradical mycelium network of AMF on Medicago. Thereafter, over a period sorghum plantlets will be removed after GS-1, 3, 9, and 12 days of GS-2, and GS-3; observation will be conducted for root exudate production and AMF colonization. Using Voets, *et al* (2009) system concept, we can observe which point of sorghum stage allows for positive AMF colonization. The positive sorghum plantlets associated with AMF can be run through various greenhouse trails to check for survival and resistance to fertilizer deficiencies and pests. This way, no plant stress would be encountered. At various points, AMF development within the plant under sterile conditions could be investigated and root exudates could be analysed using various HPLC techniques.

- Allowing plants to produce exudates at GS-1 and GS-2 under sterile conditions. These could be analysed for functioning and identified, and subsequently added to a database.

In sorghum, the main results observed in this study were the different nutrient requirements at various growth stages, followed by the exudate properties on plant development and lastly, stress factors that affected symbiotic association to the plant as well as plant development.

Part B:

This part focused on developing an *in vitro* culture system for maize. The first step in the development of the system was to produce completely sterile plantlets using optimal sterilisation methods. Our results indicated contamination within the kernel. When the tip cap was subsequently removed from the kernel and antibiotics were added to the sterilization techniques, this proved beneficial for eradicating culturable organisms within the kernel. Yet this also slowed the plant's development. The experiment indicated that maize was capable of developing under *in vitro* culture systems and clean plantlets were attainable. Future work could focus on the following:

- Kernels need to be further analysed using a molecular approach to identify the type of endophyte (if any) that resides within the plant.

- The development and observation of maize under *in vitro* culture systems could focus on nutrient capabilities and plant development.

- With knowledge of plant nutrient requirements and growth stages, the effects of indigenous epiphytes and endophytes on plants under *in vitro* culture systems could be analysed. This will form the framework of my PhD in the near future.

Our results indicated that *in vitro* culture systems provided new avenues for the observation of plant development. These systems could aid the study of plant development with the association of one or two organisms, thus the effects of microorganisms on plant development could be investigated. Obtaining the results of crop development could thus provide insight into possible plant requirements for symbiotic associations with other organisms and the deterrence of pathogenic attacks. Thus, research now focuses on creating biofertilizers and biocontrol agents to aid minimal crop loss for farmers.

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Appendices

Appendix 1:

Table 1: The Concentration and company supplier of stock solutions to prepare MSR-S-V medium, stock solutions stored at 4°C

Stock solutions	Concentration (g/l)	Suppliers
Macro-elements		
o KNO_3	7.6	Merck chemicals
o KCL	6.5	Merck chemicals
o $MgSO_4.7H_2O$	73.9	Merck chemicals
o KH_2PO_4	0.41	Merck chemicals
Calcium Nitrate	35.9	Merck chemicals
NaFeEDTA	1.6	Sigma Aldrich
Micro-elements		
o $MnSO_4.4H_2O$	2.45	Merck chemicals
o $ZnSO_4.7H_2O$	0.29	Merck chemicals
o H_3BO_3	1.86	Sigma Aldrich
o $CUSO_4.5H_2O$	0.24	Merck chemicals
o $(NH_4)_6Mo_7O_{24}.2H_2O$	0.035	Sigma Aldrich
o $Na_2Mo_7O_{24}.4H_2O$	0.0024	Sigma Aldrich

The table above depicts the chemical make-up of stock solutions required for MSR-S-V medium.

Appendix 2:

Table 2: Trials of sorghum development in HAM-P culture system small Petri plates, observed weekly and incubated in a phytotron

Sorghum Trials	Week 1		Week 2		Week 3		Week 4	
	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)
1	++	4.5	+++	14	++++	4.5	+++	14
2	+++	3.5	+++	13	+++++	3.5	+++	13
3	++	4.5	++	14	++	4.5	++	14
4	++	4.5	+++	13	+++++	4.5	+++	13
5	++	3.5	+++	10	++++	3.5	+++	10
6	++	5.5	++	12.5	+++	5.5	++	12.5
7	++	5	+++	13.5	++++	5	+++	13.5
8	++	5.5	+++	15	++++	5.5	+++	15
9	++	4.5	+++	13	++++	4.5	+++	13
10	+	4	+++	12	+++	4	+++	12
Average	++	4.35	+++	13	+++	21.25	+++++	26.11

Key: +, growth of primary root; ++, growth with new lateral roots; +++, growth with numerous lateral roots; and ++++, root system starting to discolour.

Appendix 3:

Table 3: Trials of sorghum plant development of HAM-P culture systems in large Petri plates, with single dose medium, observed weekly and incubated in phytotron

Sorghum Trials	Week 1		Week 2		Week 3		Week 4		Week 5	
	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)
1	+	4	++	8	+++	10	+++	15	+++++	16
2	++	3	+	7.5	++	9.5	+++	14	+++++	14
3	++	4.5	++	6	+++	12.5	+++	13.5	++++	13
4	+	5	++	8.5	+++	10	+++	14.5	+++++	13.5
5	+	3.5	++	8	++	11	+++	13.5	+++++	14
6	+	4	++	7.5	++	9.5	+++	12	++++	13
7	+	4.5	++	8	+++	10.5	+++	14	+++++	14.5
8	++	4.5	++	8	+++	10	+++	13.5	+++++	13.5
9	++	4	++	7.5	+++	10	+++	14	+++++	13.5
10	+	4	++	9	+++	9	+++	12.5	++++	10
Average	+	4.1	++	7.8	+++	10.2	+++	13.6	++++	13.5

Key: +, growth of primary root; ++, growth with new lateral roots; +++, growth with numerous lateral roots; discolouring of roots observed; and ++++, root system starting to discolour.

Appendix 4

Table 4: Trials of sorghum development of HAM-P culture systems in large Petri plates, with double dose medium, observed weekly and incubated in phytotron

Sorghum Trial	Week 1			Week 2			Week 3			Week 4			Week 5	
	Root	Shoot number	Shoot length (cm)	Root	Shoot number	Shoot length (cm)	Root	Shoot number	Shoot length (cm)	Root	Shoot number	Shoot length (cm)	Root	Shoot number	Shoot length (cm)
1	++	5	7	+++	5	12.5	++++	6	14	++++	5	18	++++	5	21
2	++	6	6	++	6	12	++++	7	13	++++	7	19.5	++++	5	21.5
3	++	6	7	+++	6	13.5	++++	7	16	++++	5	18.5	++++	4	22
4	++	6	7.5	+++	6	13	++++	6	15	++++	6	19	++++	4	20
5	++	5	6.5	+++	5	11.5	++++	6	14.5	++++	5	18.5	++++	4	21
6	++	5	7	+++	5	12	++++	6	13.5	++++	4	18	++++	4	22
7	++	5	6	++	5	11.5	++++	6	14.5	++++	7	17.5	++++	5	21.5
8	++	5	7.5	++	5	13.5	++++	7	15	++++	5	18	++++	5	20.5
9	++	5	6.5	+++	6	12.5	++++	7	15	++++	5	18.5	++++	5	22
10	++	7	7	+++	7	14	++++	7	17	++++	5	19	++++	5	22.5
Average	++	5.5	6.8	+++	5.6	12.6	++++	6.5	14.75	++++	5.4	18.45	++++	4.6	21.4

Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots and discolouring of roots observed; and ++++, root system starting to discolour and increase at a constant rate.

Appendix 5:

Table 5: Trials of sorghum plant development of HAM-P culture systems in large Petri plates, with double dose medium and maintained with single dose medium; plants were observed weekly and incubated in phytotron

ST	Week 0			Week 1			Week 2			Week 3			Week 4			Week 5			Week 6			Week 7			Week 8		
	R	Shoot		R	Shoot		R	Shoot		R	Shoot		R	Shoot		R	Shoot		R	Shoot		R	Shoot		R	Shoot	
		N	L		N	L		N	L		N	L		N	L		N	L		N	L		N	L		N	L
1	+	2	5	$+^2$	5	9.5	$+^4$	7	13	$+^4$	7	15.5	$+^4$	7	17	$+^4$	6	20.5	$+^4$	6	24	$+^4$	8	25.5	$+^4$	10	28
2	+	3	5	$+^2$	6	9.5	$+^4$	6	15	$+^4$	7	18	$+^4$	6	20.5	$+^4$	6	22	$+^4$	6	24.5	$+^4$	8	26	$+^4$	10	27.5
3	+	3	6	$+^2$	5	9	$+^4$	7	13.5	$+^4$	7	14	$+^4$	7	16.5	$+^4$	8	19	$+^4$	5	22.5	$+^4$	7	23	$+^4$	9	26
4	+	2	5	$+^2$	6	8	$+^4$	8	11	$+^4$	8	15	$+^4$	7	17	$+^4$	7	17.5	$+^4$	5	20	$+^4$	7	21.5	$+^4$	9	24
5	+	2	4	$+^2$	5	8	$+^4$	7	11.5	$+^4$	8	14	$+^4$	8	16	$+^4$	7	18	$+^4$	5	20.5	$+^4$	7	21	$+^4$	9	23.5
6	+	3	4	$+^2$	4	6.5	$+^4$	5	10	$+^4$	7	14.5	$+^4$	7	16	$+^4$	8	18.5	$+^4$	6	20	$+^4$	7	22	$+^4$	9	24
7	+	3	5	$+^2$	6	9	$+^4$	7	12	$+^4$	7	13	$+^4$	7	18	$+^4$	6	19	$+^4$	4	22.5	$+^4$	6	23	$+^4$	8	26
8	+	2	6	$+^2$	5	9	$+^4$	7	12.5	$+^4$	7	14	$+^4$	5	15.5	$+^4$	6	18	$+^4$	4	23	$+^4$	6	24.5	$+^4$	8	26.5
9	+	2	5	$+^2$	4	8	$+^4$	6	14	$+^4$	8	16.5	$+^4$	6	19	$+^4$	6	21.5	$+^4$	5	23	$+^4$	7	26	$+^4$	9	28.5
10	+	2	5	$+^2$	4	9	$+^4$	6	13	$+^4$	8	16	$+^4$	6	18	$+^4$	6	21	$+^4$	5	22.5	$+^4$	7	24	$+^4$	10	26.5
Av	+	2.4	5	$+^2$	5	8.55	$+^4$	6.6	12.55	$+^4$	7.4	15.05	$+^4$	6.6	17.35	$+^4$	6.6	19.5	$+^4$	5.1	22.25	$+^4$	7	23.65	$+^4$	9.1	26.05

Key: ST, sorghum Trials; R, root development; L, leaves length (cm); N, number of leaves; Av, Average; +, primary root; $+^2$, growth with new lateral roots; $+^3$, growth with numerous lateral roots and discolouring of roots observed; and $+^4$, root system discolouring and increase at a constant rate.

Appendix 6

Table 6: Trials of maize development in HAM-P culture systems, with whole seed surface sterilization, observed weekly and incubated at 22°C in phytotron

Maize Trials	Week 1			Week 2			Week 3		
	Roots	Shoots (cm)	Contam	Roots	Shoots (cm)	Contam	Roots	Shoots (cm)	Contam
1	+++	6	0	+++	11	1	*	9	1
2	++	7	0	+++	11.5	1	++++	8	1
3	+++	5	0	+++	10	0	++++	9	1
4	++	6.5	0	+++	11	0	++++	10	1
5	++	6.5	0	+++	11	0	*	9	1
6	++	6	0	+++	10	1	++++	9	1
7	+++	7	0	+++	11.5	0	++++	11	1
8	++	5.5	0	+++	10	0	*	8.5	1
9	++	6.5	0	+++	10	0	*	9	1
10	++	7	0	+++	9	0	*	8	1
Average	++	6.3	0	+++	10.5	0	++++	9.05	1

*Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots, discolouring of roots observed; ++++, root system starting to discolour and increase at a constant rate; 1, yes; 0, no; and *, contamination resulted in plant death.*

Appendix 7:

Table 7: Trials of maize plant development in HAM-P culture systems, with tip cap removed and developed onto non-supplemented MSR-S-V medium, observed weekly and incubated in phytotron

Maize	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6		Week 7		Week 8	
	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)
1	++	11	++	18	+++	22	++++	27.5	-	27	*	*	*	*	*	*
2	++	10	++	21	+++	24.5	++++	27.5	+++++	31.5	+++++	32	+++++	33.5	+++++	34
3	++	8	++	17.5	+++	21	++++	25.5	+++++	29	+++++	36	+++++	37	+++++	39
4	++	10	+++	16	++++	22	++++	26	+++++	28	+++++	33	+++++	35.5	+++++	37
5	++	10.5	++	17.5	+++	17	-	17	*	*	*	*	*	*	*	*
6	++	9.5	++	18	+++	24	++++	27.5	+++++	31	+++++	42	+++++	42	+++++	43
7	++	9	+++	17	+++	22	++++	26.5	+++++	30.5	-	30	*	*	*	*
8	++	10	++	17	+++	23	++++	27.5	+++++	31	+++++	36.5	+++++	39	+++++	40
9	++	11	++	18.5	+++	24.5	++++	29	+++++	32.5	+++++	37.5	+++++	39	+++++	41
10	++	8	++	15.5	+++	20.5	-	20	*	*	*	*	*	*	*	*
Average	++	9.7	++	17.6	+++	22.05	++++	25.4	+++++	30.0625	+++++	35.2857	+++++	37.6667	+++++	39

*Key: ++, growth with new lateral roots; +++, growth with numerous lateral roots; ++++, good thick root system; +++++, roots are thick and covering entire plate; -, endophyte emergence; and *, plant death.*

Appendix 8:

Table 8: Trials of maize plant development in HAM-P culture systems, with tip cap removed and developed onto antibiotic supplemented MSR-S-V medium, observed weekly and incubated in phytotron

Maize	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)	Root	Shoot (cm)
1	-	4	+	6.5	++	15	++	20	+++	29	++++	36.5
2	-	3.5	+	5	++	12	++	18.5	+++	26.5	++++	32.5
3	-	4.5	+	6	++	13.5	++	22.5	+++	32	++++	39
4	-	3.5	+	6.5	++	19	++	25	+++	35	++++	39.5
5	-	4.5	+	7	++	14.5	++	20	+++	28.5	++++	35.5
6	-	4	+	5	++	13	++	21	+++	30	++++	36.5
7	-	3.5	+	6	++	12.5	++	19.5	+++	27.5	++++	35
8	-	4	+	5.5	++	14	++	22	+++	30.5	++++	38
9	-	4	+	6	++	14.5	++	23	+++	32	++++	39.5
10	-	3.5	+	4.5	++	10	++	19.5	+++	29.5	++++	38
Average	-	3.9	+	5.8	++	13.8	++	21.1	+++	30.05	++++	37

Key: +, Primary growth beginning; ++, growth with new lateral roots; +++, growth with numerous lateral roots; ++++, good thick root system; and –, no.

Glossary

Abiotic stress: Stresses occurred due to non-living/no biological factors such as weather or drought

Adaptability: Ability to adjust and maintain effectiveness in new/changing environment

Arbuscules: Specialized hyphal branching of AMF formed intracellularly within living root cortical cells of host plant; serves as a nutrient and chemical exchange structure between plant and fungus

Appressorium: An enlargement on the hyphae or germ tube that attaches itself to the host before penetration takes place

Aseptic: A condition whereby sterile environment is attainable. Free from bacteria, viruses, fungi and other foreign DAN/RNA that might affect experiment

Autotrophic: Capable of growth independent of outside sources of nutrients or growth factors

Biotic stress: Stress that is caused by living/biological source such as pests

Biotroph: An organism that derives its nutrient source from its host

Chemoattractant: A chemical substance, secreted by the host, that triggers external factors to move within the host. Example: sorghum produces chemoattractants to stimulate AMF association

Coleoptile: a protective sheath that encloses shoot tip and embryonic leaves of grasses, usually present at seed germination stage of plants

Colonization: Species that invade a new habitat, usually within plant tissue. This invasion can allow for mass production of the species

Deficiency: A lack or shortage of a vital substance or chemical required for functioning of the host

Endomycorrhizae: Mycorrhizae that produce their hyphae within plant tissue

Endophyte: Plant organism that lives within plant tissue and which can be either fungus or bacterium. Some endophytes have no effect on plant development

Intercellular: Inside the cell

Intracellular: Between cells

In vitro: plant or organism cultivated within an artificial, non-living environment

Macronutrients: Essential chemical elements required by all life forms in large quantities. For a list of macronutrients, refer to Table 2.1

Micronutrients: Nutrients required in small quantities by all life forms. For a list of micronutrients, refer to Table 2.2

Obligate: Restricted to a particular set of environmental conditions, without which an organism cannot survive. Example: AMF cannot complete their life cycle without presence of plant host

Phytochemicals: Plant-derived chemical compounds

Phytotoxic: plant-derived chemicals that can be toxic to organisms surrounding their rhizophere

Rhizophere: The below-ground area that surrounds the root surface of plants, where root secretions occur, which can affect the environment surrounding the plant root

Sporocarps: Fruit body that produces spores

Sterile: Free from living organisms

Sterilization: Elimination of pathogens and/or other living organisms from environment of interest

Symbiotic: A mutualistic relationship that exists between two or more living organisms

Vesicles: Oil-filled organ of AMF that serves as a storage structure of nutrients for spores