The Production of Plant Growth Enhancers by *Trichoderma* Species

by

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A dissertation submitted in accordance with the requirements for the degree of *Magister Scientiae*

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Declaration

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Preface

This dissertation comprises of four chapters and a summary. The overall aim of this research study was to determine whether Southern African *Trichoderma* species could produce similar secondary metabolites to those found in literature from other countries, capable of enhancing plant growth in the absence of the living fungus. This could lead to a novel plant growth stimulating treatment for the agricultural sector. The first chapter is a literature review of the diversity of *Trichoderma* species, their unique features and roles in their ecological niche, their secondary metabolite formation, possible roles and uses thereof, and the possibility of their involvement in plant growth enhancement.

In Chapter 2, the diversity of *Trichoderma* species in the CGJM culture collection at the University of the Free State was determined. Isolations were performed from an array of sources with reference to the agricultural sector and from other non-agriculturally based substrates. A phylogenetic tree was assembled using the ITS gene region to indicate genetic relatedness and illustrate the diversity of the isolates collected.

In Chapter 3, *Trichoderma* isolates were cultivated to produce secondary metabolites. Extractions were performed on the culture filtrate and analysed using LC-MS/MS to identify possible growth promoting secondary metabolites from literature. The *Trichoderma* isolate, which produced the most secondary metabolites associated with plant growth promotion, was used to produce a metabolite cocktail in Chapter 4.

In Chapter 4, wheat and maize seeds were placed on metabolite infused agar and allowed to grow for 10 days. After this period the length of roots and shoots were measured before being dried for dry mass determinations. Data were analysed for significance and interactions.

The work presented in this dissertation will lead to a better understanding of plant growth enhancement attributable to *Trichoderma* species and may lead to the development of a novel growth enhancing biofertiliser using *Trichoderma* secondary metabolites as active ingredients. Future studies will include the relationship between secondary metabolites and increased yield in agriculture.

Chapter 1

The Production and Effect of Plant Beneficial Secondary Metabolites by *Trichoderma* Species

1 Introduction

The world population is growing at an exponential rate, placing extreme pressure on already diminishing natural resources. Ultimately, this could lead to dramatic changes in our environment with consequences affecting climate change on a global scale. It is envisaged that South Africa is changing towards a more semi-arid and dry climate, which threatens agricultural sustainability and puts even more pressure on the already scarce food supply (Musvoto *et al.*, 2015). This affords food producers two possible strategies to increase food production; either increase the arable land surface, or increase production yield on the existing land. With a very limited cultivatable land surface available due to extreme environmental conditions, food producers have no choice but to increase their yield by optimising the growth of their crops on currently available farmland.

Members of the genus *Trichoderma* include fungi that are prevalent in nearly all soil types. They possess the ability to endophytically colonise roots and provide a vast array of health and growth advantages to plants. The evolutionary development of life on earth has led to symbiotic relationships between many animal, plant, and microbial life forms, which hold health, growth and nutritional advantages for the organisms involved. One example is the beneficial relationship between plants and fungi. The relationships between microbes and plants are often neglected, and, in most cases, discouraged with the use of chemicals that are detrimental to microbial life. This can have a detrimental effect on growth due to micronutrient deficiencies and a reduced immune response in plants, thus decreasing the yield of the crop.

Research on the biotechnological applications of *Trichoderma* species is relatively new with regards to their metabolite effect on plants. It has only been studied in depth since the late seventies when this fungus was examined for its use as a cellulose degrader in the search for alternatives to fossil fuels.

US researchers at the Natick Massachusetts Research Institute discovered a green fungus with exceptional cellulolytic abilities that decayed and destroyed cotton derived clothing and equipment during the Second World War. It was first identified as *Trichoderma viride* and named as strain QM6a. Later on it was given a new species name of *T. reesei*, named after the Natick laboratory researcher Elwyn T. Reese (Simmons, 1977).

Trichoderma species have since proven to be much more than cellulose producing workhorses. They possess profound mycoparasitic abilities, allowing these fungi to not only parasitise, but also prey on other fungi (Kubicek *et al*., 2011). This made *Trichoderma* one of the leading biological control agents worldwide, prompting thousands of studies. It has been shown that *Trichoderma* species colonise roots of plants endophytically and improve plant growth and health; even helping to delay the onset of heat stresses and prevent the attack of pathogens (Harman *et al*., 2004).

Several species of *Trichoderma* have shown to be of substantial importance to humans on many levels, but as we further investigate the abilities of this group of fungi, many more potential applications are being discovered. This study focused on some plant growth promoting benefits and the possible use of this ability in plants to increase crop yield.

2 Biology and Biodiversity of *Trichoderma*

2.1 Introduction

Fungi belonging to the genus *Trichoderma* (syn. *Hypocrea*), are soil borne, green spored ascomycetes that are found in nearly all ecological niches. They have various characteristics and applications that allow them to be successful colonisers of their habitats, efficiently fighting their competitors and establishing a predominant role in their environment (Felix *et al.,* 2014).

The survival of *Trichoderma* species in diverse habitats can be attributed to their metabolic diversity, high reproductive capacity and their profound competitive capabilities in nature (Lopes *et al*., 2012). The purpose of this literature review is to elucidate the biodiversity of *Trichoderma* species in a wide variety of ecological habitats.

2.2 *Trichoderma* Species Diversity

The first description of a fungus under the genus *Trichoderma* dates back to 1794 (Persoon, 1794) and in 1865 a link to the sexual state, *Hypocrea,* was suggested by Tulasne and Tulasne (1865). This anamorph–teleomorph relationship was only confirmed more than 100 years later for *Trichoderma reesei* and *Hypocrea jecorina* (Kuhls *et al*., 1996). *Trichoderma* is a well-studied genus of fungi that currently comprises of more than 200 genetically defined species (Atanasova *et al*., 2013). *Trichoderma* taxa are morphologically very similar and for many years, they have been considered as a single species. In 1969, new species were discovered and Rifai (1969) proposed a consolidated taxonomical scheme and defined nine morphological species aggregates. Mycoparasitic species of *Trichoderma,* able to antagonise commercially important plant pathogens, were classified as *Trichoderma harzianum* (Druzhinina *et al*., 2006).

There has since been great genetic diversity found among previously described *Trichoderma harzianum* isolates, which now have their own species identifications such as *T. hamatum, T. harzianum, T. viride, T. aureoviride, T. virens, T. citrinoviride, T. roseeii, T. crissum, T. longibrachiatum, T. pseudokoningii, T. ovalisporum, T. koningii, T. asperellum, T. polysporum* and *T. saturnisporum.* There are four distinct species within the *Trichoderma harzianum* aggregate: *T. harzianum, T. atroviride, T. longibrachiatum,* and *T. asperellum* (Castro & Monte, 2000). There appears to be a consensus as to the most prevalent species isolated from the rhizosphere of crop plants with *T. harzianum, T. hamatum, T. atroviride,* and *T. viride* being the most isolated *Trichoderma* species (Kredics *et al*., 2014).

2.3 Methods of Studying *Trichoderma* Diversity

In former years, the identification of *Trichoderma* relied exclusively on morphological characteristics (Danielson and Davey, 1973; Summerbell, 2003). This was achieved by culturing *Trichoderma* isolates on a variety of media revealing their outstanding morphological characteristics. Different media elicited various changes in the culture, some allowing for conidiation and conidiophore branching, while others allowed the formation of pigment (Hoyos-Carvayal and Bissett, 2011). Taxonomic keys in literature were used for the identification of isolates based on conidiophore structure, culture morphology, and conidia morphology and size (Bissett, 1984; Gams and Bissett, 1998; Jaklitsch, 2009). This was, however, a questionable approach and often led to misidentification. Thus, the unreliability of earlier literature must be taken under consideration (Kubicek *et al*., 2008). It was, therefore logical that, to avoid inaccuracies, the use of biochemical as well as molecular methods was not only recommended, but also proved a necessity. DNA fingerprinting provided precise identifications (Arisan-Atac *et al*., 1995), the sequence analysis of the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA-ITS2), as well as genes coding for elongation factor 1-alpha (EF-1α), RNA polymerase II subunit (*RPB2*) and calmodulin (*CAL1*), were found to provide accurate identification to species level.

Following the successful identification of *Trichoderma* species using molecular techniques, an online ITS based barcoding program named TrichOKEY (Figure 1) was created and provided a useful tool for the identification of *Trichoderma*/ *Hypocrea* strains (International Subcommission on *Trichoderma* and *Hypocrea* Taxonomy, 2016; Druzhinina *et al*., 2005). In addition to this, species-specific primers for polymerase chain reactions were created for exact diagnosis, especially to identify *Trichoderma* pathogens on *Agaricus bisporus* for the mushroom industry (Kredics *et al*., 2014).

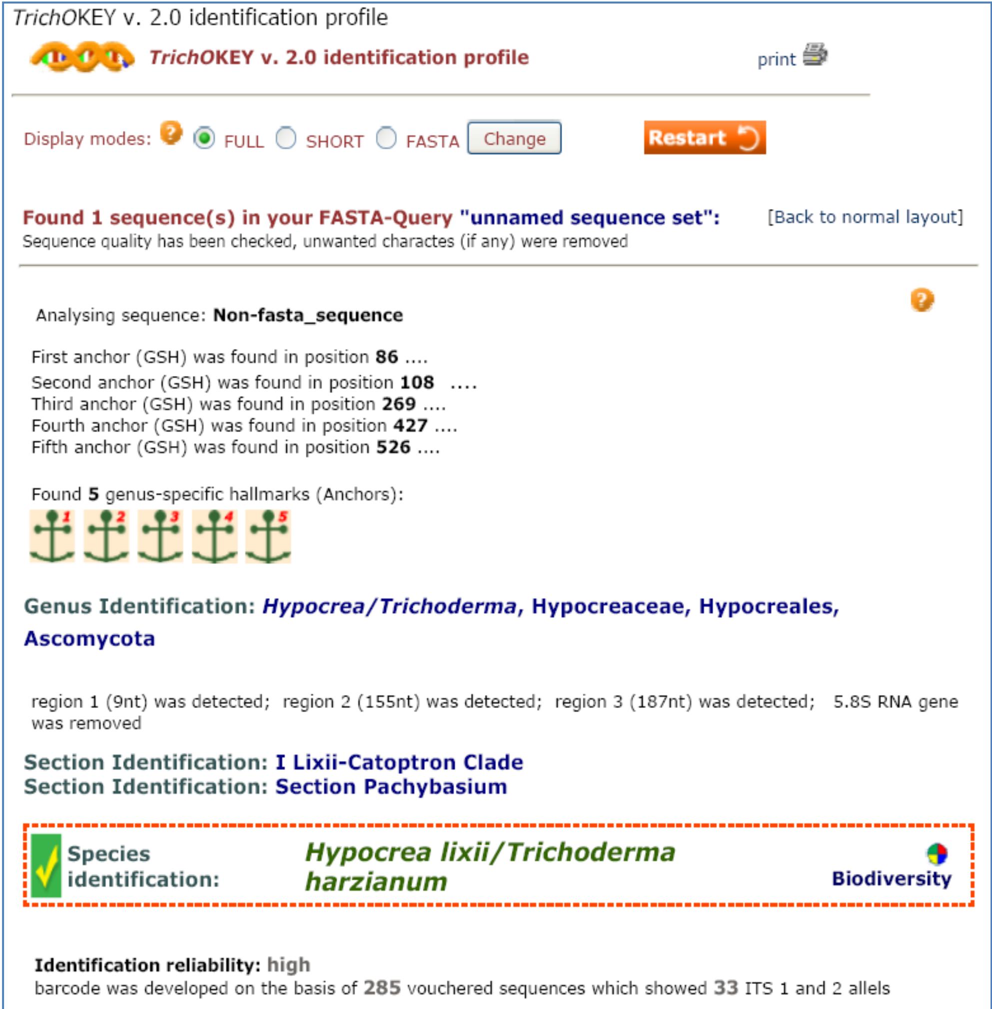

Figure 1
BLAST results using TrichOKEY indicating the genus and species name of the culture isolate based on five anchor positions located in the ITS gene region indicating the genus and species name as *Trichoderma harzianum*.

Biodiversity studies of *Trichoderma* species are routinely performed using the above-mentioned culture-based identification technique that relies on a series of steps, isolation and maintenance of cultures. These are performed on selective media as described in literature, and are followed by one or more of the popular molecular identification techniques. This is, however, not an accurate representation of biodiversity as some *Trichoderma* species and strains do not favour these culturing techniques, and are therefore not identified (Elad *et al*., 1981; Papavizas & Lumsden, 1982; Williams *et al*., 2003). This can cause an inaccurate analysis of the true *Trichoderma* species biodiversity in a given sample.

2.4 Habitat Diversity of *Trichoderma*

2.4.1 Agricultural Soil

Species belonging to *Trichoderma* have a strong capacity to mobilise and take up soil nutrients, making them more efficient and competitive than many other soil microbes (Vinale *et al*., 2008). *Trichoderma* species are found in nearly all soil types; interestingly the distribution of these species do not differ drastically from one area to another even though they may be separated by vast distances or have been isolated from foreign contamination for thousands of years (Kubicek, 2008). *Trichoderma* species have been regarded as the most prevalent fungi in soil borne habitats, yet in a metagenome study conducted by Friedl and Druzhinina (2012) it was found that only a small portion of these species were adapted to soil. Only certain adapted strains colonise roots, forming chemical communication with the plant host whereby alteration of plant physiology is systemically reached. Only roughly 20% of *Trichoderma* species have been detected in soil and rhizosphere environments with the remaining majority found in other ecological niches (Kredics *et al*., 2014).

Trichoderma species can, in theory, be isolated from all types of agricultural soil. These fungi have been used as biological control agents due to their extreme proliferation, root colonising ability, plant growth promotion and mycoparasitic abilities (Woo *et al*., 2006). *Trichoderma* species also possess the ability to degrade xenobiotic pesticides (Harman, 2006). This makes *Trichoderma* species excellent organisms to study and are widely available on a commercial scale for agricultural use, especially as biological control agents, biofertilisers and biofungicides (Vinale *et al*., 2008).

There are, however, certain factors, both biotic and abiotic, that influence *Trichoderma* populations in agricultural habitats. These include the plant species, microbial competitors, soil chemistry and physical attributes, xenobiotic substances and geographical locations (Kredics *et al*., 2014).

2.4.2 *Trichoderma* as Mycorrhizal Fungi

Mycorrhizal fungi play a very important role in the development and health of plants. Just as humans have evolved to form close synergistic relationships with gut-microbes such as bacteria, plants have evolved to form mutualistic relationships with certain fungi. These relationships promote the exchange of nutrients and minerals and provides plants with protection from possible pathogens (Harman *et al*., 2004). *Trichoderma* species have, in recent studies, been found to act as a mycorrhizal agent and colonise roots endophytically (Vinale *et al*., 2008). This, however, has raised many questions regarding the compatibility of *Trichoderma* with other mycorrhiza, seeing that *Trichoderma* species are prolific mycoparasites. These questions are, however, not that easy to address due to the complexity of studying these interactions *in vivo*.

3 Ecophysiology of *Trichoderma* Species

3.1 Ecological Niche and Role

Trichoderma species are among the most commonly isolated fungi on earth. They have been discovered on a wide variety of substrates and ecological habitats such as marine sponges, other fungi, herbaceous material, dead wood, bark, soil and as endophytes on living plants (Paz *et al*., 2010; Gal-Hemed *et al.,* 2011; Jacklitch, 2009; Zhang *et al*., 2007). These niches demonstrated their two major modes of action for acquiring nutrition: saprotrophy and biotrophy. A great number of these species live as parasites on living fungal hosts. *Trichoderma* species can live and flourish on a variety of other fungi without negatively affecting the host organism via necrotrophic hyperparasitism or mycotrophy (Kubicek *et al*., 2011).

3.2 Mycoparasitism

Necrotrophic hyperparasitism or mycoparasitism (Figure 2) refers to the ability of one fungus to feed on another (Kubicek *et al.*, 2011).

Trichoderma species possess the ability to grow within latent cells of a variety of plant pathogenic fungi such as in sclerotia through mycoparasitism, degrading these structures. *Trichoderma* species grow tropically towards hyphae of other fungi, coil around them in a lectin-mediated reaction, and degrade cell walls of the target fungi by the secretion of different lytic enzymes (Nagamani & Chakravarthy, 2006). This makes *Trichoderma* species exceptional fungi; leading to numerous possibilities for their agricultural application as plant pathogen control agents, especially for ascomycetous pathogens which comprise the most economically influential plant pathogens (Harman *et al.*, 2004).

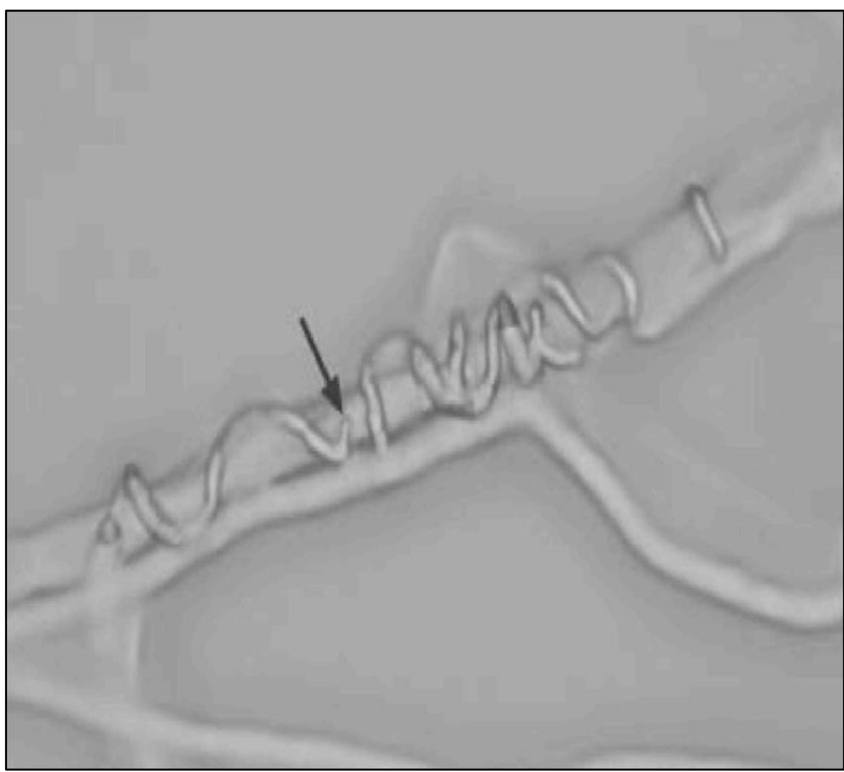

Figure 2
Mycoparasitic coiling of *Trichoderma atroviride* around *Botrytis cinerea* hyphae with the entry point of *Trichoderma* hyphae marked with an arrow (Steyaert *et al.*, 2010).

The majority of this research focused on the *Trichoderma harzianum sensu lato* complex, *T. atroviride, T. virens, T. asperellum* and *T. asperelloides* (Kubicek *et al*., 2011). A disadvantage of the strong mycoparasitic abilities of *Trichoderma* species is their ability to colonise and destroy cultivated edible mushrooms. This has led to extensive studies regarding the identification of the green mould disease for increased management of the mycoparasite (Muthumeenakshi *et al*., 1994; Castle *et al*., 1998; Park *et al.,* 2004, 2006; Hatvani *et al*., 2007; Komon-Zelazowska *et al*., 2007).

3.3 Saprophytic Capabilities of *Trichoderma* Species

Members of *Trichoderma* are also prolific saprophytes (Figure 3) and are often found on dead wood and the bark of trees and shrubs (Atanasova *et al*., 2013). The degradation pathways of lignocellulosic material by fungi are described in detail in Kubicek (2013). Perhaps the most well known *Trichoderma* species is *T. reesei* strain QM6a. This strain is widely used as a producer of cellulases and hemicellulases which are enzymes employed in degrading cellulose to glucose and xylose (Grigoriev *et al*., 2011).

Figure 3
Trichoderma growing saprophytically on wood pallet (Marais, G.)

The presence of cellulases during colonisation of plant roots by *Trichoderma* species is a key signalling enzyme for activation of the defence cascade of the plant host (Hermosa *et al.*, 2012). This cascade of defence measures employed by the plant leads to increased immunity and faster immune responses.

3.4 Rhizosphere Capabilities

In recent years, several *Trichoderma* strains have been patented as biological control agents, biofertilisers and plant growth promoters. These species improved the survival of their plant hosts by increasing tolerance to drought and high salinity (Harman, 2006). These benefits are closely associated with the profound ability of some *Trichoderma* species to grow in the rhizosphere and free soil, and establish associations with plants.

3.5 Endophytism

The mutualistic growth of a microorganism inside the tissue of a plant is called endophytic biotrophy and is very common among fungi. Very few *Trichoderma* species have thus far been isolated as true endophytes and none have been found to be obligate endophytes (Holmes *et al.*, 2004). In very few instances have there been reason to believe that *Trichoderma* species, occurring on living plants, act as parasites (Jaklitsch, 2009). The presence of *Trichoderma* species occurring as mycorrhizal fungi or as endophytes, afford them the opportunity to alter plant physiology through elicitor exchanges at various locations on the plant root (Vinale *et al.*, 2012), (Figure 4).

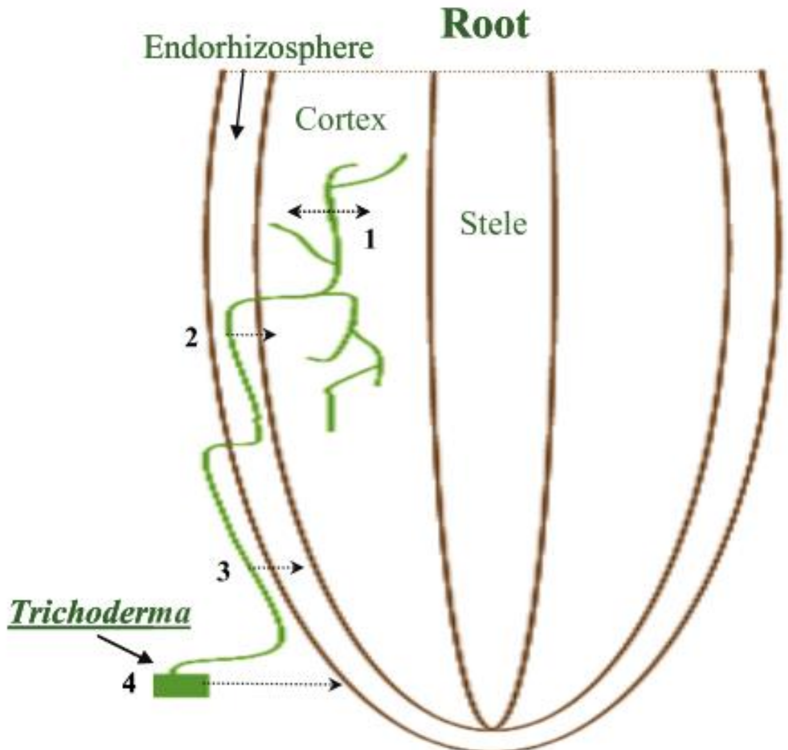

Figure 4
Potential sites for the production and exchange of *Trichoderma* metabolites that affect plant host physiology and metabolism. **1:** Metabolites produced within live cortical cells. **2:** Metabolites produced in or on the root surface. **3:** Metabolites produced in the immediate root region. **4:** Metabolites produced in the soil organic layer in low amounts are still adequate to function as elicitors of host defence (Vinale *et al.*, 2012).

4 Secondary Metabolism

Secondary metabolites are natural products that display strong biological activity and have had a tremendous impact on human society (Kredics *et al.*, 2014). Some of these metabolites are of great pharmaceutical benefit to mankind such as antibiotics, whereas others have shown to be detrimental to plants and animals (Fox & Howlett, 2008). For many years, it was assumed that these metabolites are mere by-products of other central metabolic pathways and served no particular purpose or function, and that they are only produced when active growth has ceased in the organism, or under specific circumstances (Keller *et al.*, 2005).

This assumption has since been found to be incorrect and the roles of these secondary metabolites are being elucidated with regards to their function and elaborate metabolic pathways (Osbourn, 2010). They play an extremely important role in the functioning of the producing organism, providing diversity and manipulation of other organisms in the environment (Bouhired *et al.*, 2007).

These secondary metabolites vary in structure and role, and provide the producing organism with advantages, both directly and indirectly. The vast majority of these metabolites are yet to be identified, and many of the identified metabolites have roles that are yet to be elucidated (Kredics *et al.*, 2014). It is, however, clear that these metabolites are not produced without reason, and provide the producing organism with an advantage over its ecological niche (Fox & Howlett, 2008).

Various factors influence the production of these metabolites such as environmental conditions, the genetic composition of the producing organism, and the function of the metabolites. These secondary metabolite genes are often arranged in clusters (Mukherjee et al., 2012), containing their own transcription factors that act on genes within the gene cluster, but which may also act on genes in other locations within the genome. The coding of some of these genes for secondary metabolite formation may also be regulated by global regulators. These allow gene expression to be controlled by environmental factors such as temperature or nutrition (Yu and Keller, 2005).

Members of *Trichoderma* (syn. *Hypocrea)*, are well adapted to exploit a wide range of ecological systems, especially due to the richness of the secondary metabolites they produce. This is also the reason why these fungi are good candidates as biological control agents and posess the ability to mycoparasitise a wide variety of bacterial and fungal plant pathogens (Harman *et al.*, 2004). Through interaction between cell wall degrading enzymes and secondary metabolites, some *Trichoderma* species have the ability to inhibit the colonisation of pathogens on the roots and in soil, thereby reducing the severity of the associated plant disease (Viterbo & Horwitz, 2010; Hermosa *et al.*, 2012).

Some *Trichoderma* strains have direct effects on their plant hosts. These include increased growth and yield, increased seed germination percentage, stimulation of plant immunity, defence against biotic and abiotic threats, and increased efficiency of fertilizer uptake and incorporation (Shoresh *et al*., 2010). Secondary metabolites have recently proven to be key facilitators in many of these positive effects through auxin-like analogues at low concentrations, or by acting as microbe-associated molecular patterns (MAMPs). MAMPs are motifs or domains within microbes or pathogens with conserved structural traits typical of entire classes of microbes, but not present in the host plant (Hermosa *et al.,* 2012).

Trichoderma species are prolific secondary metabolite producers with more of these metabolites being discovered on a continuous basis. Although these metabolites vary in function, many share close structural similarity. They have shown antimicrobial activity against bacteria, yeasts and other fungi involving metabolites such as peptaibols, gliotoxin and gliovirin, polyketides, pyrones, and terpenes (Vinale *et al*., 2006). From all these secondary metabolites, two main structures have been identified: (1) low-molecular weight and volatile metabolites, and (2) high-molecular weight polar metabolites (Kredics *et al*., 2014). Low molecular weight and volatile secondary metabolites are mostly non-polar with high vapour pressure. These metabolites include aromatic compounds such as polyketides, for example pyrones, butenolide and volatile terpenes. High molecular weight polar compounds include peptaibols and diketopiperazines such as gliotoxin and gliovirin, which show activity by direct contact or interaction with their antagonists (Mach & Zeilinger, 1998). More than 120 structurally distinct secondary metabolites from *Trichoderma* species have been described and determined analytically (Sivasithamparam & Ghisalberti, 1998). Detection and quantification of these vast numbers of secondary metabolites involve extensive studies with more than 1 000 compounds produced by this genus of fungi.

Recent studies on secondary metabolite formation have revealed regulatory impacts on genetic and genomic levels with several ecological factors influencing formation of necessary product biosynthesis (Lorito *et al*., 2010). These studies have greatly influenced our understanding of these secondary metabolites and the effects they elicit in the trifecta of plant, pathogen and *Trichoderma* species (Velazquez-Robledo *et al*., 2011; Mukherjee *et al*., 2012).

4.1 Peptaibols

Peptaibols are a large group of antibiotic peptides synthetised by non-ribosomal peptide synthetases (NRPSs) of fungi. NRPS enzymes are modular and assemble amino acid monomers in a stepwise fashion (Strieker *et al.*, 2010). These peptides contain 7-20 amino acids with a high 2-amin-isobutyric acid, a C-terminal hydroxyl group and typically an alkyl-N-terminal amino acid. The first characterised peptaibol was termed almathicin F30 by Brewer and co-workers (1987). Today it is known that almathicin consists of a group of 12 compounds (Kredics *et al.*, 2014). Over 300 peptaibols are known today, grouped into nine distinct sub-families, and over 190 of these known compounds are produced by *Trichoderma* (Mukherjee *et al.*, 2012). The majority of research efforts have gone into the isolation, biosynthetic pathway elucidations, amino acid content and conformational properties of this unique group. Peptaibols have been shown to have antifungal, antibacterial and anticancer properties (Schuhmacher *et al.*, 2007). Literature has also revealed that some of these peptaibols may be involved with biological control and plant growth stimulation; although this, however, still needs to be confirmed.

4.2 Diketopiperazine Compounds

Trichoderma species also have NRPSs involved in the synthesis of secondary metabolites other than peptaibols. An example is gliotoxin, a substance known since 1944 to be produced by *T. viride* (formerly *Gliocladium virens*) (Brian, 1944). Gliotoxin has a wide range of applications including antiviral, antibacterial and immunosuppressive properties (Hebbar & Lumsden, 1998). Bezuidenhout an co workers (2012), whose investigation showed that gliotoxin acts as a growth hormone analogous to gibberelic acid, has linked this metabolite to increased height in maize seedlings.

4.3 Pyrones

One of the first and most notable volatile compounds isolated from *Trichoderma* species was 6-pentyl2H-pyran-2-one (6PP) (Collins and Halim, 1972). This compound, with antifungal and growth promoting benefits, has since been isolated from several *Trichoderma* species (Vinale *et al*., 2008) and is the major contributing compound to the coconut aroma experienced when smelling vegetative growth of some isolates (Bisby, 1939). This volatile compound exhibits inhibitory properties against plant pathogenic fungi such as *Rhizoctonia solani* and *Bortrytis cinerea* (Cooney & Lauren, 1999). In a study by Vinale and co-workers (2008), it was found that 6PP reduced disease severity and significantly increased plant height and leaf area on 10^{-6}M (0.166mg.L^{-1}) 6PP-treated plants when compared to controls.

4.4 Terpenes

One of the largest groups of natural products from fungi is terpenes. These secondary metabolites comprise one of the most important groups with a very wide range of pharmacological application. Some of the effects of terpenes are antiviral, antibacterial, antimalarial, anticancer, anti-inflammatory and they exhibit an inhibition of cholesterol synthesis (Sivasithamparam & Ghisalberti, 1998).

The most important effects regarding this class of compounds are their antifungal ability and their essential role as constituents of cell membranes (ergosterol) (Sivasithamparam & Ghisalberti, 1998). Trichothecenes are among the most well known mycotoxins due to their toxic effects on humans and animals. Recent studies have also found that trichothecenes increase plant yield and growth, and inhibit growth of phytopathogenic fungi (Malmierca *et al*., 2012).

4.5 Anthraquinones

These well-known metabolite compounds function as pigments, but also act as laxatives, diuretics, phytoestrogens, immune stimulators, antifungal agents, antiviral agents and anticancer agents (Liu *et al.*, 2009). Some of the more well known anthraquinones, such as pachybasin and emodin, increased the number of coils formed by *T. harzianum* during mycoparasitism of *Rhizoctonia solani*, a phytopathogen, aiding in biological control efficiency (Lin *et al.*, 2012). Although no solid evidence yet exists for growth promotion by anthraquinone metabolites, it is strongly believed that this class of compounds would yield enhanced growth in plants (Vinale *et al.*, 2008).

5 Biological Control and Plant Growth Promotion

The close association of *Trichoderma* species and other microbes with plant roots has been well established as having direct and indirect influences on the growth of host plants. Lindsey and Baker (1967) reported nearly 50 years ago that significant increases in plant height and weight under sterile growth conditions were observed for dwarf tomato plants using *Trichoderma viride*. Many other reports have since shown similar growth promotion on a wide variety of commercially significant plants. These growth enhancements include increased germination rates, more rapid and increased flowering, increased height and weight of plants with more developed root systems, and increased yield of both grain and fruit crops (Chang *et al.*, 1986).

Over the years, most of these articles have focused on plant growth promotion due to the effect of *Trichoderma* species on plant pathogens as biological control agents rather than on the direct influence on the plant itself.

The major focus of the following section is devoted to the effects that *Trichoderma* species elicit directly on the plant in the absence of pathogens.

5.1 Plant Growth Promotion by *Trichoderma* Species

Large varieties of plants have shown growth promotion by *Trichoderma* species including vegetables, grains, flowers, and forest trees. The majority of research done in this regard has been on glasshouse grown vegetable crops, especially cucumber, lettuce and green pepper. The use of soil beneficial microbes such as *Trichoderma* species and bacteria for live symbiotic relationships with plants to improve nutrition or growth, is known as biofertilisation (Vinale *et al*., 2008a).

The goal of a biofertiliser is to improve soil quality and, in this sense, some *Trichoderma* species may be considered as biofertilisers as they possess the ability to rehabilitate soil from xenobiotic substances and alter soil pH (Vinale *et al*., 2013). *Trichoderma* species also have the ability to colonise roots and thereby improve plant nutrition, growth and provide abiotic stress relief. There are several conflicting reports in literature as to the first report concerning growth promotion, yet all of these 'first reports' demonstrate enhanced development in the plants tested. It was, however, an article by Baker and co-workers (1984) that concluded that these growth promoting factors were caused by something other than direct contact between the plant host and *Trichoderma*.

In vitro experiments on pepper seedlings, using cell-free culture broths from *Trichoderma* species, showed that high concentrations influenced the growth negatively whereas a 1/16 dilution of the same culture broth exhibited significant increases in both dry weight and height of the plants tested.

These results demonstrated that *Trichoderma* species produce substances in the cell-free culture filtrate responsible for these effects and that they have a phytohormonal effect on pepper seedlings, exhibiting the same growth enhancement as natural plant hormones (Monfil & Casas-Flores, 2014). It was further demonstrated in later studies that the volatile compounds produced by *Trichoderma atroviride* stimulated lateral root formation in *Arabidopsis thaliana* without influencing root length (Vinale *et al*., 2008b).

5.2 Enhanced Plant Defence and Immune Stimulation

5.2.1 Abiotic Stress Relief

One of the most important challenges facing crop protection is abiotic stress relief (Felix *et al*., 2014). Some of the key factors influencing this challenge include shortages of essential resources, excess amounts of toxic substances and environmental changes. These are major yield limiting factors in plants and such challenges are rapidly increasing due to climate change causing more extreme droughts or flooding. Salt and phosphate build-up is an additional problem in the majority of agricultural soils, especially on irrigated land. It has thus become of considerable importance to implement a mitigation strategy to combat the loss in yield in a cost effective and sustainable way (Felix *et al*., 2014).

Traditionally, *Trichoderma* species were regarded as agents of biological control alleviating biotic stress and inhibiting plant pathogenic microorganisms. New evidence has also indicated that *Trichoderma* can be used as growth promoters, biofertilisers and avirulent plant simbionts (Harman *et al*., 2004). Harman (2000) indicated that the advantages of *Trichoderma* species for enhanced plant growth are especially prominent during extreme stress periods, alleviating symptoms of drought pressure, high salinity, and temperature extremes in plants.

Members of *Trichoderma* invade and colonise plant roots. This creates a localised symbiotic relationship with chemical communication, altering plant gene expression and thereby changing the plant physiology (Shoresh *et al*., 2010). Studies have concluded that *Trichoderma* species colonise plant roots and increase the levels of plant enzymes such as peroxidases, chitinases, glucanases, lypoxygenases and hydroperoxide lyases. These compounds have the ability to change plant metabolism and lead to increases in plant derived compounds such as phytoalexins and phenols, leading to increased immuninty (Harman, 2006)

5.3 Mechanism for Immune Stimulation

Species of *Trichoderma* develop and colonise to interact with the host plant roots (Figure 5). By establishing this interaction, the fungus and the plant exchange chemical signals and alter gene expression in both parties. *Trichoderma* species release elicitors into the zone of chemical communication, both outside and inside root tissue that activates a mitogen-activated protein kinase (MAPK) cascade in the plant (Figure 5). The jasmonic acid (JA)/ ethylene signalling pathway is activated next, resulting in priming or increased activation of plant defence genes that increase plant resistance against pathogens. In addition, increases of carbohydrate metabolism and photosynthesis result in more energy and carbon for the growing plant. For *Trichoderma* species, it is known that there are strains that induce defence against abiotic and biotic stress but not necessarily growth. This suggests that in the presence of *Trichoderma*, there are different signalling pathways that lead to plant responses, either directly or indirectly. Whether these signalling pathways also differ from those leading to abiotic stress responses still needs to be determined (Shoresh *et al*., 2010).

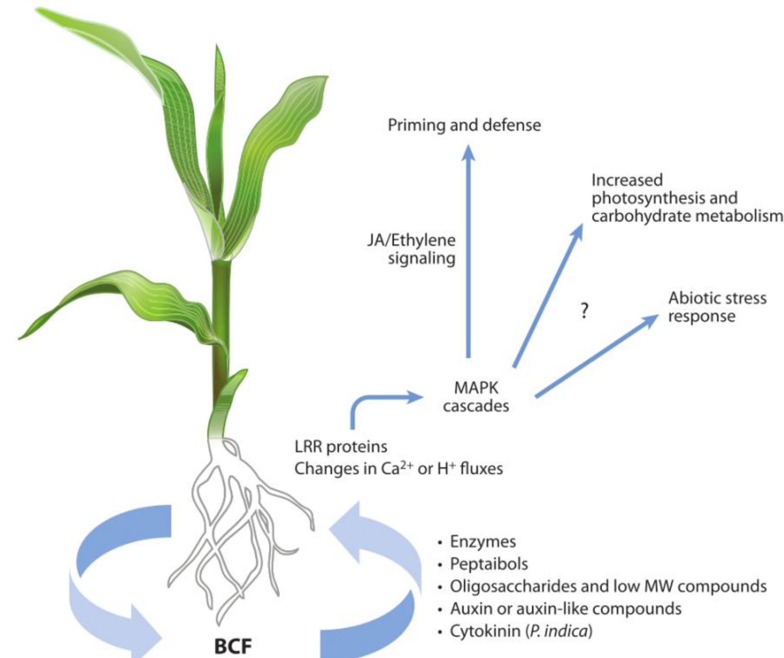

Figure 5
Immune stimulation and growth enhancement as elicited by biological control fungi such as *Trichoderma* species (Shoresh *et al*., 2010).

5.3.1 Biological Control

The use of *Trichoderma* species as biological control agents have been under investigation for over 70 years, but only since 1998 have commercial strains become available for this purpose (Limon *et al.*, 1998). The most prevalent of *Trichoderma* candidates for biological control are *T. harzianum, T. viride* and *T. virens* (formerly known as *Gliocladium virens*). These species have shown sustainable potential in an array of studies through the following modes of action: (1) colonising the soil and/ or parts of the plant thereby occupying physical space that prevents increases of pathogens, (2) producing cell wall degrading enzymes against pathogens, (3) directly and indirectly stimulating plant defence cascade systems, (4) producing antibiotics that inhibit or kill competing pathogens and (5) promotes plant development and growth that leads to increased yields (Harman, 2006).

6 *Trichoderma*, the Unsung Hero of the Rhizosphere

There is a changing perspective by the public regarding fertilisation in the agricultural sector and the era of using only chemical supplementation is coming to an end. Organically acceptable biopesticides and biofertilisers are increasing in popularity and the availability of such products has increased dramatically over the past few years. Organic producers rely on such measures as their primary form of disease management. This form of management, however, relies on the knowledge of what these measurements are and how they work to ensure sustainable and efficient mitigation.

In recent years, *Trichoderma* has become a buzzword and champion of the agricultural sector due to its high activity in root, soil and foliar environments. It has been shown that *Trichoderma* species produce a wide range of antibiotic substances (Vinale *et al.*, 2009), enabling parasitism of other fungi. They also have direct effects such as competing for exudates from germinating seeds that stimulate the growth of phytopathogens (Howell & Stipanovic, 1995). Because of these abilities of *Trichoderma* species a large variety of applications and strains are commercially available. It has also become evident that our understanding of the mechanisms for biological control is still limited.

However, some factors can limit the use of *Trichoderma* species as biofertilisers, biological control agents and plant growth enhancers. Effective and sustainable control relies on a living organism that can only provide these benefits if it colonises successfully, grows in an environment conducive to its requirements and the host-plant specificity is met for the particular strain used. Therefore, the aim of this study was to evaluate the use of secondary metabolites of *Trichoderma* species rather than the fungi itself as elicitors of increased plant growth. In this study, several *Trichoderma* species were isolated and identified from Southern African environments to determine whether these strains produce secondary metabolites which may increase plant growth.

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Chapter 2

Trichoderma Species Diversity and Phylogeny of the CGJM Culture Collection

Abstract

Members of the genus, *Trichoderma*, are cosmopolitan ascomycetes that are prevalent species in different ecosystems across a wide range of climatic zones. *Trichoderma* was first described in 1794 and in 1865 a link to the sexual state *Hypocrea* was suggested. This anamorph–teleomorph relation was later confirmed for *Trichoderma reesei* and *Hypocrea jecorina*. *Trichoderma* is a well-studied, ubiquitous genus of fungi that currently comprises of more than 200 genetically defined species. The survival of *Trichoderma* species in diverse habitats can be attributed to their metabolic diversity, high reproductive capacity and their profound competitive capabilities in nature. In former years, the identification of *Trichoderma* relied exclusively on morphological characteristics. This was, however, a questionable approach and often led to misidentification. The use of biochemical, as well as molecular methods, has since proven to be significantly more reliable. Following the successful identification of *Trichoderma* species using molecular techniques, an online ITS based barcoding program named TrichOKEY was created and provided a useful tool for the identification of *Trichoderma* and *Hypocrea* strains.
In the present chapter of this study, *Trichoderma* diversity was studied on the strains available in the CGJM culture collection at the University of the Free State in order to establish a base for future selection and screening of secondary metabolite formation for enhanced plant growth. All strains studied were isolated from Southern African environments. With literature concerning the diversity of South African *Trichoderma* species severely lacking, this contributed to the elucidation of this genus and its presence in Southern African environments. In total, 54 isolates of *Trichoderma* were genetically identified using the Internal Transcribed Spacer (ITS) region and a phylogenetic tree was constructed. Results indicated that three of the four sections of *Trichoderma* were represented in the CGJM culture collection with the most prominent fungus *Trichoderma atroviride*. In total, 11 different species were identified including *T. asperellum*, *T. atroviride*, *T. citrinoviride*, *T. gamsii*, *T. hamatum, T. harzianum, T. longibrachiatum*, *T. reesei*, *T. spirale, T. virens,* and *T. viride.*

1 Introduction

The genus *Trichoderma* was first described in 1794 (Persoon 1794), and in 1865 a link to the sexual state *Hypocrea* was suggested (Tulasne and Tulasne, 1865). This anamorph–teleomorph relation was only confirmed more than 100 years later for *Trichoderma reesei* and *Hypocrea jecorina* (Kuhls *et al*., 1996). *Trichoderma* is a well-studied, ubiquitous genus of fungi that currently comprises of more than 200 genetically defined species (Atanasova *et al*., 2013).

New *Trichoderma* species have been found among isolates previously described by morphological characteristics as *Trichoderma harzianum. Trichoderma* species such as *T. hamatum, T. harzianum, T. viride, T. aureoviride, T. virens, T. citrinoviride, T. roseeii, T. crissum, T. longibrachiatum, T. pseudokoningii, T. ovalisporum, T. koningii, T. asperellum, T. polysporum,* and *T. saturnisporum* have been defined by molecular techniques as individual species. Out of these there are four distinct species that fall within the *Trichoderma harzianum* aggregate: *T. harzianum, T. atroviride, T. longibrachiatum,* and *T. asperellum* (Castro & Monte, 2000). There appears to be a consensus as to the most prevalent species isolated from the rhizosphere of crop plants with *T. harzianum, T. hamatum, T atroviride,* and *T. viride* being the most isolated *Trichoderma* species (Atanasova *et al*., 2013).

Members of *Trichoderma* are prevalent in nearly all soil types from around the world (Vinale *et al*., 2008). In fact, it would be a surprise, if, when a diversity study of soil in any region is conducted, *Trichoderma* species are not found. These fungi are also some of the most prolific decomposers of cellulosic material and are prevalent on agricultural by-products and fallen timber (Carreras-Villaseñor *et al*., 2012).

Some *Trichoderma* species can be found on living plants as endophytes, but this characteristic is sometimes only restricted to certain strains within a species (Kredics *et al*., 2014). Members of *Trichoderma* were also found to be abundant in water-related environments including marine and fresh water habitats (Kredics *et al*., 2014).

Previously, the identification of *Trichoderma* species relied exclusively on morphological characteristics (Danielson and Davey, 1973, Summerbell, 2003). For this purpose, a variety of media was used to cultivate *Trichoderma* isolates to determine their culture characteristics and morphology. Different media elicited various changes in the culture, some allowing for conidiation and conidiophore branching while others allowed the formation of pigment production (Hoyos-Carvajal and Bissett, 2011). Taxonomic keys in literature allowed for the identification of isolates based on conidiophore structure, morphology and conidia morphology and size (Bissett, 1984; Gams and Bissett, 1998; Jaklitsch, 2009). This was, however, an inadequate approach and often led to misidentification and thus the unreliability of earlier literature must be taken into consideration (Kubicek *et al*., 2008). It was thus logical that, to avoid inaccuracies, the use of biochemical, as well as molecular methods was not only recommended but proved a necessity. DNA fingerprinting provided precise identifications (Arisan-Atac *et al*., 1995) while the sequence analysis of the internal transcribed spacer (ITS) region (ITS1-5.8S rDNA –ITS2), as well as gene coding for elongation factor 1-alpha (tef1), RNA polymerase II subunit (RPB2), and calmodulin (cal1) were found to provide accurate identification to species level.

Following the successful identification of *Trichoderma* species using molecular techniques, an online ITS based barcoding program named TrichOKEY was created and provides a useful tool for the identification of *Trichoderma*/ *Hypocrea* strains (Druzhinina *et al*., 2005).

In the present chapter of this study, *Trichoderma* diversity was studied on the strains available in the CGJM culture collection at the University of the Free State to establish a base for future selection and screening of secondary metabolite formation for enhanced plant growth. All strains studied were isolated from South African environments. With literature concerning the diversity of South African *Trichoderma* species severely lacking, this was a crucial first step to elucidation of this genus and its presence in Southern Africa.

Isolates were collected from an array of locations and substrates ranging from the most southern province of South Africa to the north as far as Namibia and Zimbabwe. By no means were sufficient numbers of areas and strains tested to establish a profound understanding of *Trichoderma* diversity, yet sufficient strains and data were collected to support a base from which selection could be performed. The diversity of substrates, although some were far removed from agriculture or plant hosts, were selected to determine metabolite formation prejudice due to host substrate. In total, 54 isolates of *Trichoderma* were genetically identified using the internal transcribed spacer (ITS) region and a phylogenetic tree was constructed. Of the 54 isolates analysed, results yielded 11 distinct *Trichoderma* species. *Trichoderma atroviride* was the most prominent isolate with 24 strains isolated, and *Trichoderma harzianum* constituted 13 of the total isolates from mainly agricultural niches. Other isolates identified include *T. asperellum*, *T. gamsii*, *T. citrinoviride*, *T. viride*, *T. longibrachiatum*, *T. reesei*, *T. virens, T. spirale* and *T. hamatum*. Phylogenetic studies were performed and a tree assembled to establish relatedness, which revealed that three of the major taxonomic clades were represented by isolates analysed in this study. This study represented only a small portion of the diversity of *Trichoderma* species in Southern Africa, and further studies are necessary to realise the full extent of their distribution and diversity.

2 Methods and Materials

2.1 *Trichoderma* Strains Used

Trichoderma isolates used were obtained from the culture collection of CGJM (Culture collection of Gert. J. Marais). Strains 31-39 were isolated from an array of different sources (see Table 1). Samples 40-52 were isolated from sorghum and soya bean roots from different locations across South Africa and samples 53-54 were commercial *Trichoderma* species. The reason behind choosing such a wide and unrelated range of host-substrates was to attempt to determine whether the *Trichoderma* species differ in metabolite formation due to host-substrate discretion.

Table 1
Trichoderma isolates indicating isolate number, culture collection number, locality, substrate, and preliminary identification.

Isolate #	**Isolate locality**	**Culture collection**	**Culture collection #**	**Substrate**	**Preliminary identification**
1	Harare, Zimbabwe	CGJM	2308	Maize seeds	*Trichoderma* sp.
2	Harare, Zimbabwe	CGJM	2309	Maize seeds	*Trichoderma* sp.
3	Zimbabwe	CGJM	2310	Maize seeds	*Trichoderma* sp.
4	Zimbabwe	CGJM	2311	Maize seeds	*Trichoderma* sp.
5	Zimbabwe	CGJM	2312	Maize seeds	*Trichoderma* sp.
6	Zimbabwe	CGJM	2313	Maize seeds	*Trichoderma* sp.
7	Zimbabwe	CGJM	2314	Maize seeds	*Trichoderma* sp.
8	Zimbabwe	CGJM	2315	Maize seeds	*Trichoderma* sp.
9	Zimbabwe	CGJM	2316	Maize seeds	*Trichoderma* sp.
10	Zimbabwe	CGJM	2317	Maize seeds	*Trichoderma* sp.
11	Zimbabwe	CGJM	2318	Maize seeds	*Trichoderma* sp.
12	Zimbabwe	CGJM	2319	Maize seeds	*Trichoderma* sp.
13	Zimbabwe	CGJM	2320	Maize seeds	*Trichoderma* sp.
14	Kwazulu-Natal	CGJM	1228	Unknown	*Trichoderma reesei*
15	Kwazulu-Natal	CGJM	1229	Unknown	*Trichoderma harzianum*
16	Port Elizabeth	CGJM	1230	Air in a leather tannery	*Trichoderma viride*
17	Port Elizabeth	CGJM	1231	Air in a leather tannery	*Trichoderma* sp.
18	Port Elizabeth	CGJM	1232	Air in a leather tannery	*Trichoderma viride*
19	Vaalpark	CGJM	1233	Air in a leather tannery	*Trichoderma* sp.
20	Port Elizabeth	CGJM	1234	Air in a leather tannery	*Trichoderma* sp.
21	Port Elizabeth	CGJM	1235	Air in a leather tannery	*Trichoderma viride*
22	Port Elizabeth	CGJM	1236	Air in a leather tannery	*Trichoderma* sp.
23	Port Elizabeth	CGJM	1237	Leather	*Trichoderma viride*
24	Port Elizabeth	CGJM	1238	Leather	*Trichoderma harzianum*
25	Port Elizabeth	CGJM	1239	Leather	*Trichoderma harzianum*
26	Johannesburg	CGJM	1240	Cardboard Box	*Trichoderma viride*

Isolate #	**Isolate locality**	**Culture collection**	**Culture collection #**	**Substrate**	**Preliminary identification**
27	Johannesburg	CGJM	1241	Cardboard Box	*Trichoderma pseudokoningii*
28	Grootfontein	CGJM	1242	*Acacia mellifera*	*Trichoderma aureoviride*
29	Windhoek	CGJM	1243A	*Acacia karoo*	*Trichoderma koningii*
30	Windhoek	CGJM	1243B	*Acacia karoo*	*Trichoderma koningii*
31	Hartswater	CGJM	2321	Pecan Roots	*Trichoderma* sp.
32	Western Cape	CGJM	2322	Unknown	*Trichoderma* sp.
33	Western Cape	CGJM	2323	Unknown	*Trichoderma* sp.
34	Western Cape	CGJM	2324	Unknown	*Trichoderma* sp.
35	Gauteng	CGJM	2325	Hydrating serum	*Trichoderma* sp.
36	Gauteng	CGJM	2326	Bottling plant	*Trichoderma* sp.
37	Gauteng	CGJM	2327	Bottling plant	*Trichoderma* sp.
38	Gauteng	CGJM	2328	Bottling plant	*Trichoderma* sp.
39	Western Cape	CGJM	2329	Unknown	*Trichoderma* sp.
40	Senekal	CGJM	2298	Soil	*Trichoderma harzianum*
41	Senekal	CGJM	2299	Sorghum Roots	*Trichoderma harzianum*
42	Senekal	CGJM	2300	Soyabean Roots	*Trichoderma harzianum*
43	Cedara	CGJM	2301	Sorghum Roots	*Trichoderma hamatum*
44	Bethlehem	CGJM	2302	Soil	*Trichoderma* sp.
45	Bethlehem	CGJM	2303	Soyabean Roots	*Trichoderma* sp.
46	Winterton	CGJM	2034	Maize Roots	*Trichoderma harzianum*
47	Winterton	CGJM	2305	Soil	*Trichoderma harzianum*
48	Greytown	CGJM	2306	Soyabean Roots	*Trichoderma spirale*
49	Johannesburg	CGJM	2294	Thatch Roof	*Trichoderma* sp.
50	KwaZulu-Natal	CGJM	2295	Plant material	*Trichoderma reesei*
51	Gauteng	CGJM	2330	Bottling plant	*Trichoderma* sp.
52	Cedara	CGJM	2307	Soil	*Trichoderma virens*
53	Western Cape	CGJM	2296	Soil	*Trichoderma virens*
54	Western Cape	CGJM	2297	Soil	*Trichoderma viride*

2.2 *Trichoderma* Soil Isolations

Isolations from soil were performed using a modified method described by Warcup (1950). One gram of soil was subjected to a serial dilution up to 10^{-6} and plated onto Streptomycin Potato Dextrose Agar (SPDA). Fungal colonies were allowed to grow for seven days until *Trichoderma* colonies were distinguishable from other fungal colonies. An isolation needle was then used under a stereo microscope to carry a single conidiophore to a new SPDA plate for hyphal expansion prior to DNA extraction.

2.3 *Trichoderma* Isolations from Plant Roots

Isolations from roots were performed using a modified method of Bills (1996). Roots were cut into sections and surface sterilised for 1 minute in 76% ethanol. Root sections were then rinsed with sterile distilled water and air-dried in a laminar flow hood, followed by plating onto SPDA. Fungal colonies were allowed to grow for seven days and *Trichoderma* colonies were transferred using the same methods as described in 2.2 for soil isolations of *Trichoderma*.

2.4 Single Spore Isolation and Cultivation

In order to obtain pure cultures, single spore isolation was performed using a modified protocol from Choi *et al*. (1999). A small amount of fungal material was aseptically removed from the original seven-day old culture grown on Potato Dextrose Agar (PDA), and suspended in 100 µl sterile water containing 10% Tween 80. The contents were vortexed to obtain a homogeneous spore suspension. The suspension was then plated on water agar plates and spread using a sterilised glass rod. After 18 hours incubation at 25°C, the spores were inspected under a stereo microscope for germination.

Single germinated spores were separately removed with a sterile hypodermic needle and placed on a PDA plate for further growth. Upon seven days incubation at 25°C, spores were placed on PDA slants, allowed to grow and cover the agar slant, then refrigerated at 4°C as stock cultures.

2.5 Morphological Identification of *Trichoderma* Species

Morphological identification of each isolate was carried out to confirm their association to the genus *Trichoderma.* Characteristics such as conidial shape and texture, colony morphology, conidiophore arrangement and shape of conidiophores according to the methods and dichotomous key by Bissett (1984) were analysed.

2.6 Genomic DNA Purification

An adaptation of the method from Doyle & Doyle (1987) was used for DNA isolation. Freeze dried fungal hyphae were ground into a fine powder using a Tissue-lyzer (TissueLyserII). Pre-warmed CTAB/ PVP buffer (500 µl) was added to each sample and tubes were incubated for 30 min at 70°C. Chloroform/ isoamylalcohol (24:1 v/v) (500 µl) was added and the suspension was mixed well. The samples were centrifuged at 12 000 x *g* for 5 min at room temperature (RT) and the aqueous phase was removed and transferred to a clean, 1.5 ml eppendorf. A measure of 1 ml of CTAB precipitation buffer was added and mixed by inversion for 2 min and centrifuged at 12 000 x *g* for 15 min at RT. The pellet was re-suspended in 350 µl NaCl. RNase A (10 mg/ml) (2 µl) was added to each sample and incubated at 37°C for one hour. Chloroform/ isoamylalcohol (24:1 v/v) (500 µl) was added and the suspension was mixed well and centrifuged at 12 000 x *g* for 5 min at room temperature (RT). The aqueous phase was removed and transferred to a clean 1.5 ml eppendorf. DNA was precipitated from the aqueous phase by adding 210 µl isopropanol and incubating at -20°C for 15 min. The supernatant was discarded using a water jet-pump. The pellet was washed by the addition of 500 µl ice-cold ethanol/water 70% (v/v). The samples were centrifuged at 12 000 x *g* for 5 min at 4°C, all liquid was removed and the pellets were air-dried for one hour at RT. The pellets were re-suspended in 200 µl TE buffer and left overnight at 4°C.

2.7 Genetic Identification

Polymerase chain reaction (PCR) was performed for the ITS regions (Figure 5) using fungal primers ITS-1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and ITS 4 (5' GGTCCGTGTTTCAAGACGG-3') (White *et al.*, 1990). Sequence reactions were performed with an ABI BigDye Terminator Cycle sequencing kit and sequence data was collected on an ABI PRISM 3130XL Genetic Analyser (Applied Biosystems®). Resulting sequence data of the ITS region were analysed using GeneiousPro 5.6.5 (Drummond *et al.*, 2011) and searched against the available fungal sequences in GenBank using BLAST (Altschul *et al.,* 1990).

The sequence was also aligned using TrichOKEY version 2 as described by Druzhinina *et al.,* (2005). This is a website that contains a BLAST database dedicated to the identification of *Trichoderma* species by using the ITS region.

For phylogenetic analyses, sequences were aligned using CLUSTAL_X version 2.0 (Larkin *et al.*, 2007). Analyses were conducted with MEGA version 5 (Tamura *et al.*, 2011) using the neighbour-joining method with the Kimura two parameter distance measure. Confidence values were estimated from bootstrap analysis of 1000 replicates.

2.8 Storage and Maintenance of *Trichoderma* Isolates

Following genetic identification, *Trichoderma* isolates were transferred to Potato Dextrose Agar (PDA) slants and stored at 4°C. Working cultures were maintained on 90 mm PDA Petri plates kept at 4°C. Cultures were also cryo-preserved to extend viability and impede gene expression deterioration. Cultures were freeze dried by using a Virtis Advantage freeze dryer. Cultures were grown on PDA plates for two weeks and spore suspension was prepared by placing 6 ml of a lyophilization buffer solution on top of the culture. The culture surface was scratched with a blunt pinsette until the spores were in suspension.

The spore suspension was placed in a 2 ml freeze dried vial, and freeze dried under vacuum according to the specifications of the manufacturer. The lyophilization buffer solution was prepared in three separate containers. The first container contained 3.75 g $Na_2HPO_4 \cdot H_2O$, 0.22 g KH_2PO_4, and 50 ml distilled H_2O. The second contained 25 g lactose and 100 ml distilled H_2O. The third contained 5 g Difco peptone (proteose) and 100 ml distilled H_2O. After autoclavation and cooling, the ingredients of all three containers were aseptically mixed together to make up 250 ml of buffer solution. Freeze dried cultures were deposited in the CGJM culture collection at the University of the Free State.

3 Results

3.1 *Trichoderma* Species Identification and Diversity

Following the isolation of all *Trichoderma* strains, DNA extraction was performed using a modified version of the CTAB method by Doyle & Doyle (1987). This method of DNA extraction has shown to produce large quantities of quality DNA with minimal contaminants. PCR amplification was performed to amplify the ITS gene region and sequence analysis was done to determine base pair composition. Gene sequences were edited using GeneiousPro 5.6.5 (Drummond *et al.*, 2011) and a BLAST analysis was performed using TrichOKEY. In total 54 isolates of *Trichoderma* were identified using molecular methods (as described, to be the most reliable). The 54 isolates comprised of 11 different species representing three major clades as described by Błaszczyk and co-workers (2011).

Trichoderma atroviride was the species most commonly isolated with 24 of the 54 isolates representing this species. Twelve isolates of *Trichoderma harzianum* were identified with soil and roots being the preferred substrate. Other isolates identified were *T. asperellum* (3 isolates), *T. gamsii* (3), *T. citrinoviride* (1), *T. viride* (3), *T. longibrachiatum* (3), *T. reesei* (2), *T. virens* (2), *T. spirale* (1) and *T. hamatum* (1).

Table 2

Preliminary morphological and molecular identifications of *Trichoderma* isolates

Isolate #	Preliminary identification	Genetic identification
1	*Trichoderma* sp.	*Trichoderma atroviride*
2	*Trichoderma* sp.	*Trichoderma asperellum*
3	*Trichoderma* sp.	*Trichoderma asperellum*
4	*Trichoderma* sp.	*Trichoderma atroviride*
5	*Trichoderma* sp.	*Trichoderma atroviride*
6	*Trichoderma* sp.	*Trichoderma gamsii*
7	*Trichoderma* sp.	*Trichoderma atroviride*
8	*Trichoderma* sp.	*Trichoderma atroviride*
9	*Trichoderma* sp.	*Trichoderma harzianum*
10	*Trichoderma* sp.	*Trichoderma atroviride*
11	*Trichoderma* sp.	*Trichoderma atroviride*
12	*Trichoderma* sp.	*Trichoderma asperellum*
13	*Trichoderma* sp.	*Trichoderma citrinoviride*
14	*Trichoderma reesei*	*Trichoderma reesei*
15	*Trichoderma harzianum*	*Trichoderma harzianum*
16	*Trichoderma viride*	*Trichoderma atroviride*
17	*Trichoderma* spp.	*Trichoderma atroviride*
18	*Trichoderma viride*	*Trichoderma atroviride*
19	*Trichoderma* sp.	*Trichoderma atroviride*
20	*Trichoderma* sp.	*Trichoderma atroviride*
21	*Trichoderma viride*	*Trichoderma atroviride*
22	*Trichoderma* sp.	*Trichoderma atroviride*
23	*Trichoderma viride*	*Trichoderma atroviride*
24	*Trichoderma harzianum*	*Trichoderma viride*
25	*Trichoderma harzianum*	*Trichoderma viride*
26	*Trichoderma viride*	*Trichoderma atroviride*
27	*Trichoderma pseudokoningii*	*Trichoderma longibrachiatum*
28	*Trichoderma aureoviride*	*Trichoderma harzianum*
29	*Trichoderma koningii*	*Trichoderma reesei*
30	*Trichoderma koningii*	*Trichoderma harzianum*
31	*Trichoderma* sp.	*Trichoderma atroviride*
32	*Trichoderma* sp.	*Trichoderma atroviride*
33	*Trichoderma* sp.	*Trichoderma atroviride*
34	*Trichoderma* sp.	*Trichoderma atroviride*
35	*Trichoderma* sp.	*Trichoderma atroviride*
36	*Trichoderma* sp.	*Trichoderma longibrachiatum*
37	*Trichoderma* sp.	*Trichoderma atroviride*
38	*Trichoderma* sp.	*Trichoderma atroviride*
39	*Trichoderma* sp.	*Trichoderma atroviride*

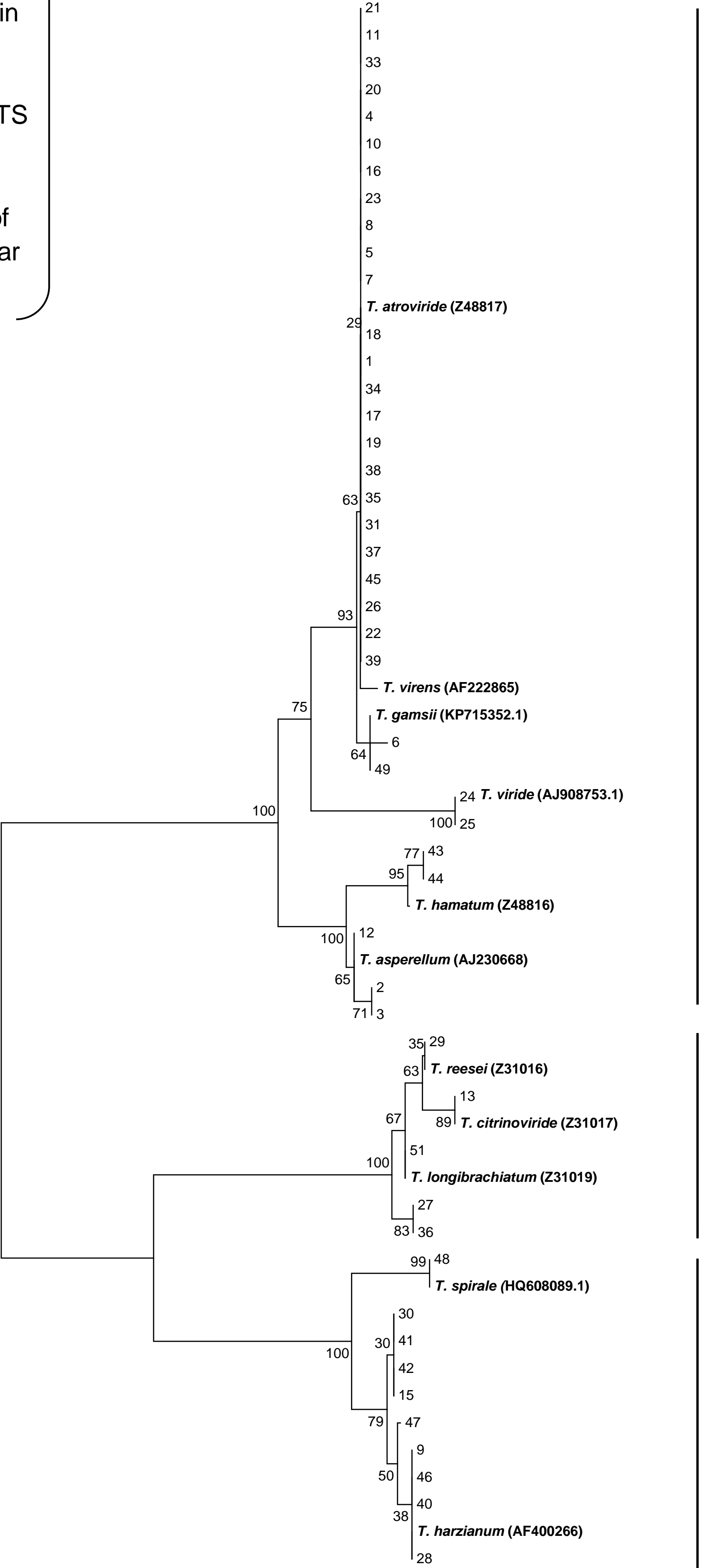
Isolate #	Preliminary identification	Genetic identification
40	*Trichoderma harzianum*	*Trichoderma harzianum*
41	*Trichoderma harzianum*	*Trichoderma harzianum*
42	*Trichoderma harzianum*	*Trichoderma harzianum*
43	*Trichoderma hamatum*	*Trichoderma harzianum*
44	*Trichoderma* sp.	*Trichoderma hamatum*
45	*Trichoderma* sp.	*Trichoderma gamsii*
46	*Trichoderma harzianum*	*Trichoderma harzianum*
47	*Trichoderma harzianum*	*Trichoderma harzianum*
48	*Trichoderma spirale*	*Trichoderma spirale*
49	*Trichoderma* sp.	*Trichoderma gamsii*
50	*Trichoderma reesei*	*Trichoderma harzianum*
51	*Trichoderma* sp.	*Trichoderma longibrachiatum*
52	*Trichoderma virens*	*Trichoderma virens*
53	*Trichoderma virens*	*Trichoderma virens*
54	*Trichoderma viride*	*Trichoderma viride*

3.2 Phylogenetic Analysis

Genetic results indicated that *Trichoderma* species initially identified from the CGJM culture collection, based on morphology, was not always accurate (Table 2). This confirms the findings of previous authors (Kubicek *et al.*, 2008) that morphological identification is not always reliable (Figure 1).

3.3 Culture Preservation and Maintenance

Following successful identification, all cultures were stored on PDA slants at 4°C for preservation of original samples and working cultures were maintained on PDA plates at 25°C.



Trichoderma

Longibrachiatum

Pachybasium

0.01

(**Figure 1**) Phylogenetic tree derived from neighbour-joining analysis based on the ITS domain showing the placement of *Trichoderma* species to related taxa. Accession numbers of the ITS sequences are given after the species names. Bootstrap percentages (1000 replicates) of 50% or greater are shown, the bar represents 0.1% nucleotide

0.01

4 Discussion

Trichoderma is a ubiquitous genus of soil fungi and nearly all soil and root samples derived from agricultural environments contained *Trichoderma.* Results indicated that *Trichoderma atroviride* was the most commonly isolated *Trichoderma* species, with 24 of the 54 isolates comprising of this species. *Trichoderma harzianum* were mainly isolated from soil and roots. This might be due to the observations made in many publications that *Trichoderma harzianum* is the most successful agricultural coloniser, or likely, that the soils might have been treated with *Trichoderma* based products for biological control, as the majority of such products use *Trichoderma harzianum* (Vinale *et al.*, 2008).

It became clear from the findings that Southern Africa has a wide variety of *Trichoderma* species associated with both natural and man-made environments. However, this study is not sufficient to capture the full extent of the diversity in Southern Africa, and it was not the purpose. It is evident that the CGJM culture collection represents 11 different species of *Trichoderma* from diverse environments. For example, *Trichoderma atroviride* was isolated from the air, leather, cardboard, pecan trees, a variety of food samples, a bottling plant, and maize. The varied nature of these items is further proof of how well *Trichoderma* species are adapted to colonise diverse environments.

Two species, *Trichoderma asperellum* and *T. citrinoviride*, were only found in maize samples from Zimbabwe and not from any samples from South Africa. Whether this is significant is yet to be established.

Due to its high mycoparasitic capabilities, *T. viride* has previously been used as such in commercial product formulations (Harman *et al*., 2012). Species from the CGJM culture collection represent three of the major genetic clades in the genus, *Trichoderma*. This was determined by using only one gene (ITS), which gave sufficient resolution for species differentiation.

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Chapter 3
The Production of Growth Promoting Secondary Metabolites by *Trichoderma* Species

Abstract

Members of the genus *Trichoderma* are prolific producers of secondary metabolites. More than 120 structurally distinct secondary metabolites from *Trichoderma* species have been determined and described analytically. Metabolites are natural products that are biologically active and are of great use to society. Some of these metabolites are of great pharmaceutical benefit to mankind such as antibiotics, whereas others are detrimental to plants and animals. The vast majority of these metabolites are yet to be identified, and the roles of many of the identified metabolites have yet to be explained. It is, however, clear that these metabolites provide the producing organism with an advantage over its ecological niche. Numerous factors influence the production of these metabolites such as environmental conditions, the genetic composition of the producing organism and the function of the metabolites. Secondary metabolites produced by *Trichoderma* species have recently been shown to be key facilitators of positive effects on plant physiology and metabolism by acting as microbe-associated molecular patterns (MAMPS) and as auxin-like analogues at low concentrations. In this study, *Trichoderma harzianum* and *T. virens* were cultivated in a culture broth to produce possible plant growth promoting secondary metabolites. The culture broth was analysed for secondary metabolites and T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone, T39butenolide, and a metabolite structurally related to gliotoxin were qualitatively detected and identified using liquid mass spectrometry with transition monitoring. Metabolites from *Trichoderma* species could likely be used as biopesticides and biofertilisers based on their active compounds instead of the living organism itself. This could have a significant beneficial impact, increasing crop yields and improving crop health.

1 Introduction

The fungal kingdom is diverse, with approximately 100 000 identified species (Kirk *et al.*, 2008) of an estimated total fungi on earth between of 1.5 and 5.1 million, most of which are filamentous (Blackwell, 2011). Filamentous fungi like *Trichoderma* are prolific producers of secondary metabolites (SMs). These are natural products that display biological activity and are of great use to society. Only one percent of the total number of fungi been discovered has been examined for the spectrum of compounds they produce (Siddiquee, 2012). Many of these compounds are of use to the pharmaceutical industry like penicillin (antibiotics), while others, like mycotoxins, display harmful effects on plants, humans and animals (Fox and Howlett, 2008). The vast majority of these compounds have obscure or unknown functions, yet they are a crucial element of their diversity (Keller *et al.*, 2005). Most interactions with other fungi are also influenced or controlled by secondary metabolites, but the most likely role of these diverse compounds is to provide the fungus with an advantage in its ecological niche (Fox and Howlett, 2008).

The publication of the genome sequences of *T. atroviride*, *T. reesei* and *T. harzianum* have revealed a vast repertoire of genes putatively involved in the biosynthesis of secondary metabolites, such as non-ribosomal peptides, polyketides, terpenoids and pyrones (Osbourn, 2010). These secondary metabolites vary in structure and role, and provide the producing organism with advantages both directly and indirectly. The vast majority of these metabolites are yet to be identified, and many of the identified metabolites have roles yet to be elucidated. Various factors influence the production of these compounds such as environmental conditions, the genetic composition of the producing organism, and the function of the metabolites.

Genes involved in the production of secondary metabolites are often arranged in clusters (Mukherjee *et al*., 2012). They contain their own transcription factors, which act on genes within the gene cluster, but may also act on genes in other locations within the genome. Some of the genes coding for secondary metabolite formation may also be regulated by global regulators that allow the expression of these genes to be controlled by environmental factors such as temperature or nutrition (Yu and Keller, 2005).

Members of *Trichoderma,* and its teleomorph *Hypocrea,* are fungal genera that are well adapted to a wide variety of ecological systems; some of these species are currently being used as biological control agents due to their ability to antagonise and mycoparasitise a wide variety of plant pathogens, both bacterial and fungal (Harman *et al*., 2004). Through interaction between *Trichoderma* cell-wall degrading enzymes and secondary metabolites, *Trichoderma* species have the ability to inhibit colonisation of pathogens on the roots and in soil, thereby reducing the severity of the associated plant disease (Viterbo & Horwitz, 2010; Hermosa *et al*., 2012). *Trichoderma* species have been shown to have direct effects on their plant hosts. These effects include increased growth and yield, nutrient uptake, germination percentage, stimulation of plant immunity, defence against biotic and abiotic threats, and increased efficiency of fertilizer uptake (Harman *et al*., 2004). Secondary metabolites of *Trichoderma* species have been shown to be key facilitators to many of these positive effects by acting as microbe-associated molecular patterns (MAMPS) and as auxin-like analogues at low concentrations (Vinale *et al*., 2008). Secondary metabolites from the *Trichoderma* species that may act as auxin-like compounds typically have an optimum activity at low concentrations (10^{-5} and 10^{-6} M) while having an inhibitory effect at higher doses (Brenner, 1981; Cleland, 1972).

More than 120 structurally distinct secondary metabolites produced by *Trichoderma* species have been described analytically, with more being discovered continually (Sivaisithamparam and Ghisalberti, 1998; Reino *et al*., 2008).

Detection and quantification of these metabolites from *Trichoderma* species involve extensive studies with more than 1 000 compounds being produced, depending on the strain of *Trichoderma*, the environmental factors, and the sensitivity of the detection method or instruments used.

Studies regarding secondary metabolite formation have revealed ecological impacts on genetic and genomic levels with several factors regulating formation of necessary product biosynthesis (Lorito, Woo, Harman, & Monte, 2010). These studies have significantly influenced our understanding with regards to the role of some secondary metabolites and the effects they elicit in the trifecta of plant, pathogen and *Trichoderma* (Velazquez-Robledo *et al*., 2011; Mukherjee *et al*., 2012).

Production of fungal secondary metabolites is a multifaceted process associated with morphological development (Calvo *et al*., 2002). Secondary metabolites are of remarkable importance in biotechnological applications. In previous work by Vinale and co-workers (2006) the major secondary metabolites produced by two commercial strains of *T. harzianum* (T22 and T39) were analysed. Six compounds, including T22azaphilone, 1-hydroxy-3-methyl-anthraquinone, 1-, 8-dihydroxy-3-methyl-anthraquinone, T39butenolide, harzianolide, harzianopyridone, obtained from fungal culture filtrates, were isolated and characterised. Six-day-old wheat seedlings were grown in liquid suspensions of these secondary metabolites at different concentrations ranging from 1 to 10 mg.L^{-1}. T39butenolide (1 mg.L^{-1}), anthraquinone (10 mg.L^{-1}) and harzianolide (1 mg.L^{-1}) significantly increased stem and leaf length compared with the control (range 23–24 cm compared with 18.6 cm) and harzianolide (1 mg.L^{-1}) also significantly increased dry weight (43 mg) compared to untreated plants (34 mg). These metabolites also showed different levels of antibiotic activity against the fungal pathogens *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani* and *Pythium ultimum* (Vinale *et al*., 2006). *Trichoderma* species also have NRPSs involved in the synthesis of secondary metabolites other than peptaibols.

Gliotoxin is an important member of the peptaibols class and its production by *T. virens* (formerly *Gliocladium virens*) has been known since 1944 (Brian, 1944). Gliotoxin has a wide range of properties including antiviral, antibacterial and immunosuppressive (Hebbar and Lumsden, 1998). This metabolite has been linked to increased height in maize seedlings by Bezuidenhout (2012) and studies showed that gliotoxin acts as a growth hormone analogue to gibberelic acid.

In this study, *Trichoderma harzianum* (CGJM 2295) was cultivated in a culture broth to produce possible plant growth promoting secondary metabolites. The purpose was to determine the secondary metabolites produced by *T. harzianum* (CGJM 2295) and whether this correlates with what is published in literature regarding other isolates of *T. harzianum*.

2 Materials and Methods

2.1 Preparation of Spore Suspension and Pre-inoculum

Spores were harvested by adding 10 ml sterile distilled water to the surface of four seven-day-old *Trichoderma harzianum* (CGJM 2295) plates. The water was removed, pooled, and the spore count was determined using a haemocytometer (Bright-Line™).

A pre-inoculum was prepared by diluting the spores to a concentration of 10^8 spores per ml in a final volume of 100 ml PDB and incubated at 25°C in 250 ml Erlenmeyer flasks.

2.2 Liquid Cultivation for Growth Curve and Metabolite Production

Three five-litre Erlenmeyer flasks were used to conduct a growth study in triplicate. Each flask contained 1.5 litres of PDB (Potato Dextrose Broth; Difco™) and was inoculated using 3 ml of the pre-inoculum. Flasks were incubated on a shaker at 27°C for 260 hours at 220 rpm.

Samples of 5 ml were taken at 12 hour intervals. The samples were filtered using 25mm Whatman® glass fibre filters. The filters were dried for 12 hours at 100°C, then cooled down for 12 hours in a desiccator. The dried filters were then weighed for biomass determinations.

2.3 Liquid Cultivation for Gliotoxin Production

A *Trichoderma virens* (CGJM 2307) spore suspension was prepared using the method as described in section 2.1. One five-litre Erlenmeyer flask, containing 1.5 litres of PDB, was inoculated using the spore suspension and placed on a rotary shaker for 260 hours at 220 rpm. Gliotoxin was extracted from samples as described by Kosalec and co-workers (2004) and a pure standard was obtained from Sigma®. Extraction was performed using a 1:3 (sample to solvent) volume. Dichloromethane was added to the sample and vortexed for five minutes then placed on a shaker overnight to allow for extended extraction. The dichloromethane fraction was pooled and evaporated using nitrogen to avoid oxidation of the gliotoxin moiety.

2.4 LC-MS/MS Instrumentation Setup for Secondary Metabolite Detection

Samples were analysed using an ABSCIEX 4000 QTRAP hybrid triple quadrupole ion trap mass spectrometer with a Shimadzu HPLC stack as a front end. All data acquisition and processing was performed using Analyst 1.5 (AB SCIEX) software.

Twenty microliters of each extracted sample was separated on a C18 (150 mm x 3mm, Luna 3µ, Phenomenex) column at a flow rate of 300 µL/min starting at 60% mobile phase B (MeOH/0.1% formic acid) at 1 minute ramping up to 98% B and kept for an 8 minute period, before re-equilibrating the column at 60% B. Eluting analytes were analysed in positive ionization mode in the TurboV ion source using an ion spray voltage setting of 5 500 V, 500°C heater temperature to evaporate excess solvent, 50 psi nebuliser gas, 50 psi heater gas and 25 psi curtain gas.

Table 1

LC-MS/MS transitions monitored for each metabolite by multiple reaction monitoring (Vinale *et al.*, 2009).

Metabolite name	**Precursor ion $(M+H)^+$**	**Product ions $(M+H)^+$**
T22azaphilone	345	259,241
1-, 8-dihydroxy-3-methyl-anthraquinone	255	209
T39butenolide	221	203, 157, 133
Harzianolide	222	204, 158, 134
Gliotoxin	327	263.3, 245.2, 227.2,111.1, 56.1

A triple quadrupole mass spectrometer allows only the precursor ionised mass into the collision cell where it is fragmented into product ions. These fragments are then recorded in the third quadrupole. A precursor-fragment mass combination is called a transition. Whenever a transition is detected the instrument determines the intensity relative to the transition. When used as a screening tool, the more transitions per analyte recorded the higher the certainty of the analyte's presence.

3 Results

3.1 Growth Curve of *Trichoderma harzianum*

To complete a growth curve, a pre-inoculum was used to inoculate potato dextrose broth. The spore suspension used was deliberately dense and each spore resulted in its own propagule. By incubating on a shaker, the mechanical force exerted on the hyphal structure resulted in some tearing and a shift was observed from conidiation to the formation of chlamydospores for propagation (Figure 1). Chlamydospores are survival structures, which are hyphal segments that have thick cell walls. This could have been caused by the liquid cultivation paired with the sheer force of the shaker. The cultivation was performed in PDB (Potato Dextrose Broth) as it was suspected that the production of certain growth promoting metabolites might be induced by the plant-derived sucrose present in the PDB and that these metabolites would only be formed in the late-exponential phase of growth.

Each sample taken was used for dry mass determinations. The growth curve (Figure 2) demonstrates a relatively fast specific growth rate of 0.037 h^{-1} for a fungus and stationary phase was reached within 94 hours.

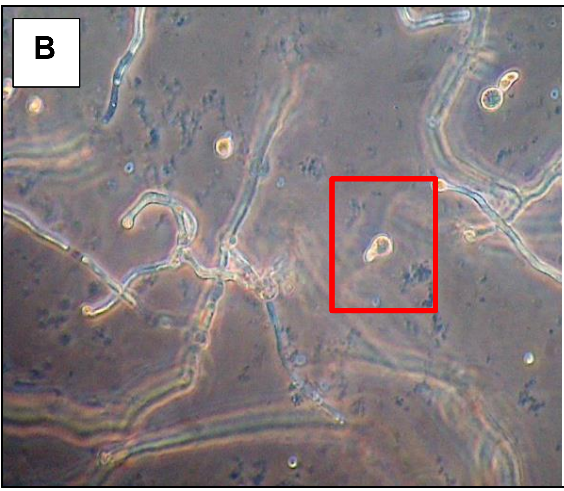

Figure 1
(A) Light micrograph of chlamydospore formation by *Trichoderma harzianum* in liquid cultivation and (B) Germinating chlamydospore indicated by the box.

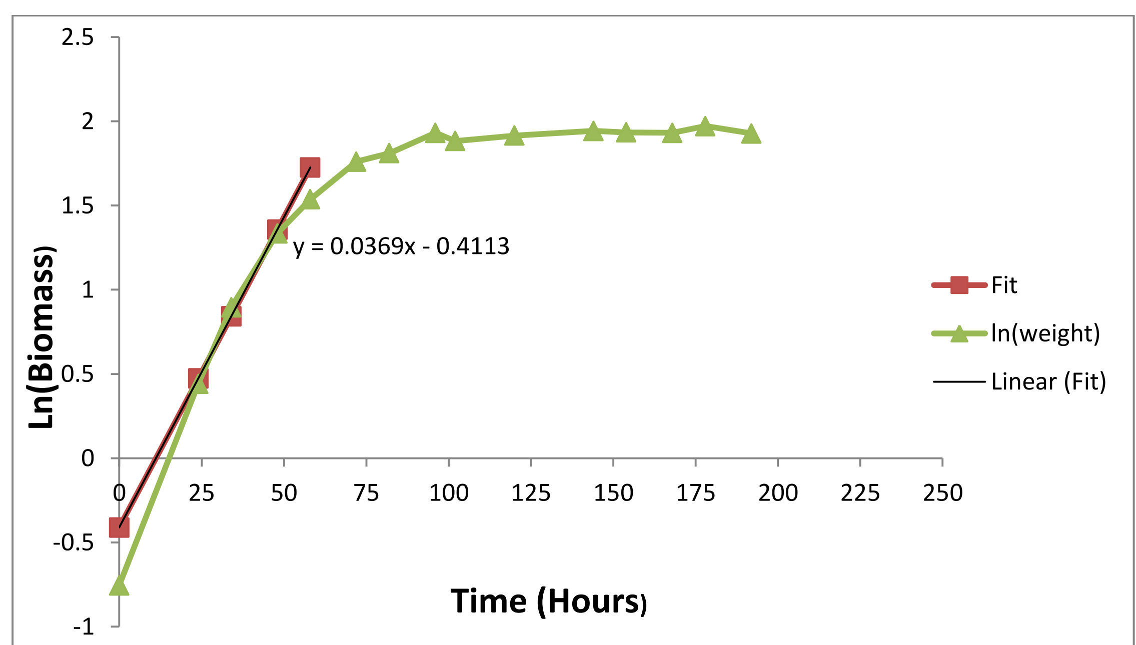

Figure 2
Growth curve of *Trichoderma harzianum* liquid cultivation in PDB. The stationary phase was reached after approximately 94 hours with a specific growth rate of 0.037 h^{-1}.

3.2 Production of Secondary Metabolites by *T. harzianum*

Four metabolites could be identified from the culture broth of *Trichoderma harzianum*: T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone, and T39butenolide.

3.2.1 T22azaphilone

Both transitions selected from literature (345>259; 345>241) were detected with a retention time of 9.56 minutes.

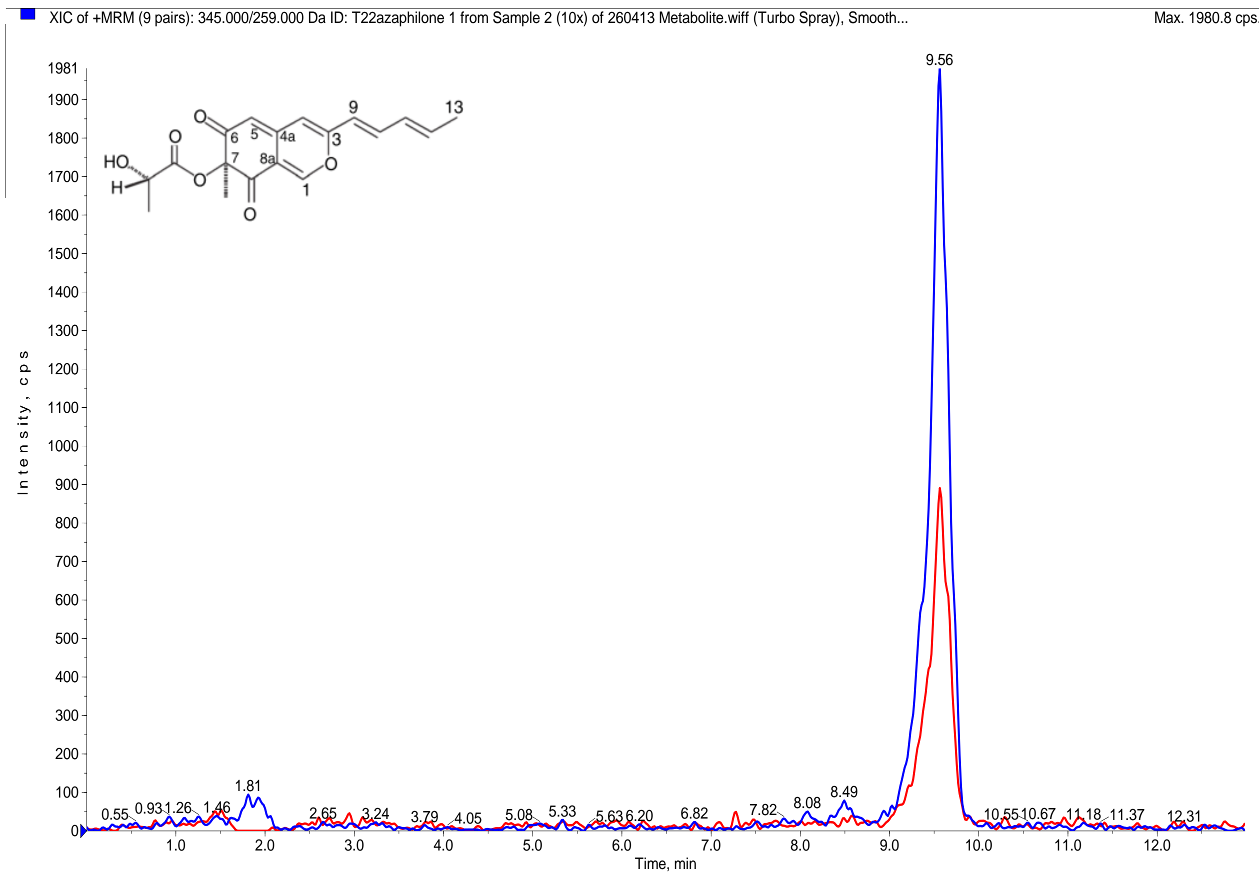

Figure 3
Chromatogram obtained by LC-MS/MS of the two transitions for T22azaphilone from the culture broth extract of *Trichoderma harzianum* (CGJM 2294). Transition 345>259 is represented by the blue trace line and 345>241 is represented by the red trace line.

3.2.2 1-, 8-dihydroxy-3-methyl-anthraquinone

Only one transition (255>209) was available from literature and a peak at retention time 9.51 minutes was recorded from the extract.

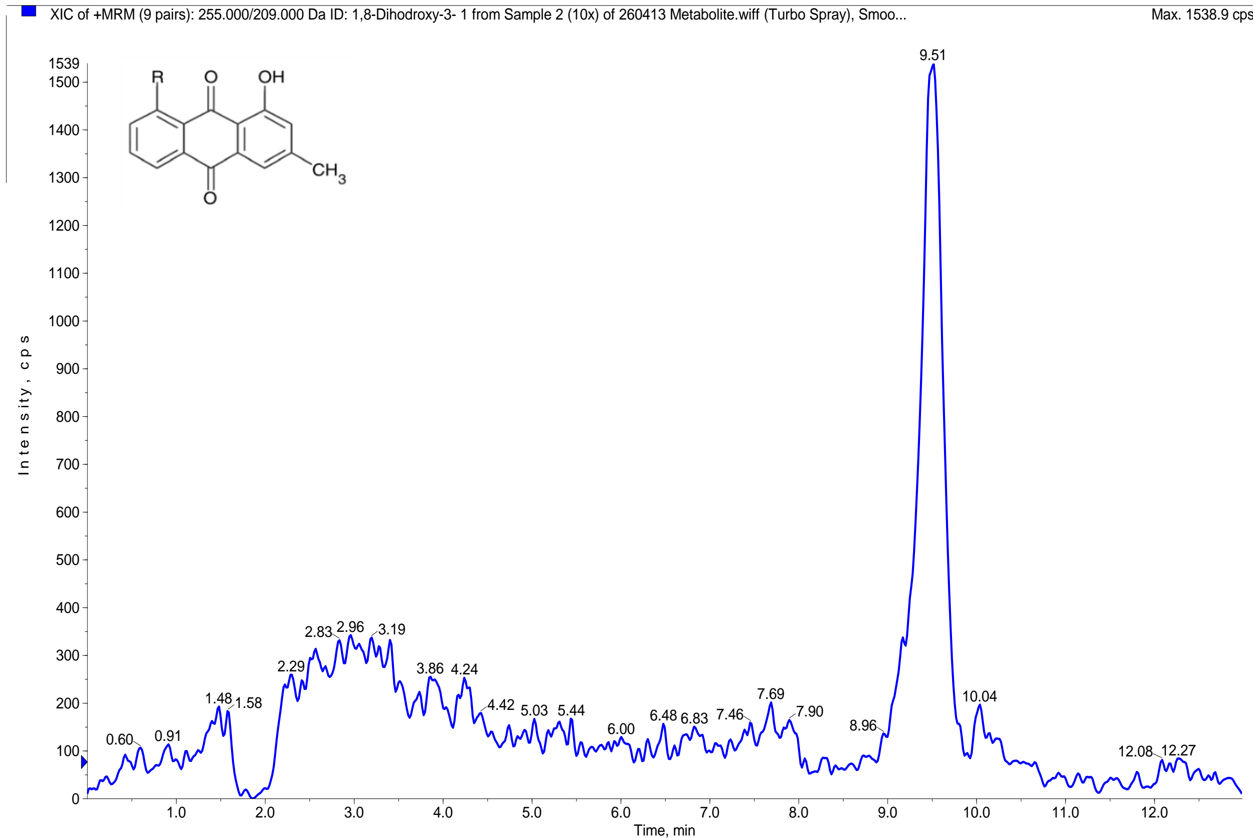

Figure 4

Chromatogram obtained by LC-MS/MS of the single available transition (255>209) for 1-, 8-dihydroxy-3-methyl-anthraquinone was observed at a retention time of 9.51 min and is represented by the blue trace.

3.2.3 T39butenolide

Two transitions (221>133; 221>203) were selected from literature for T39butenolide analysis and recorded a peak for both transitions at a retention time of 9.76 minutes.

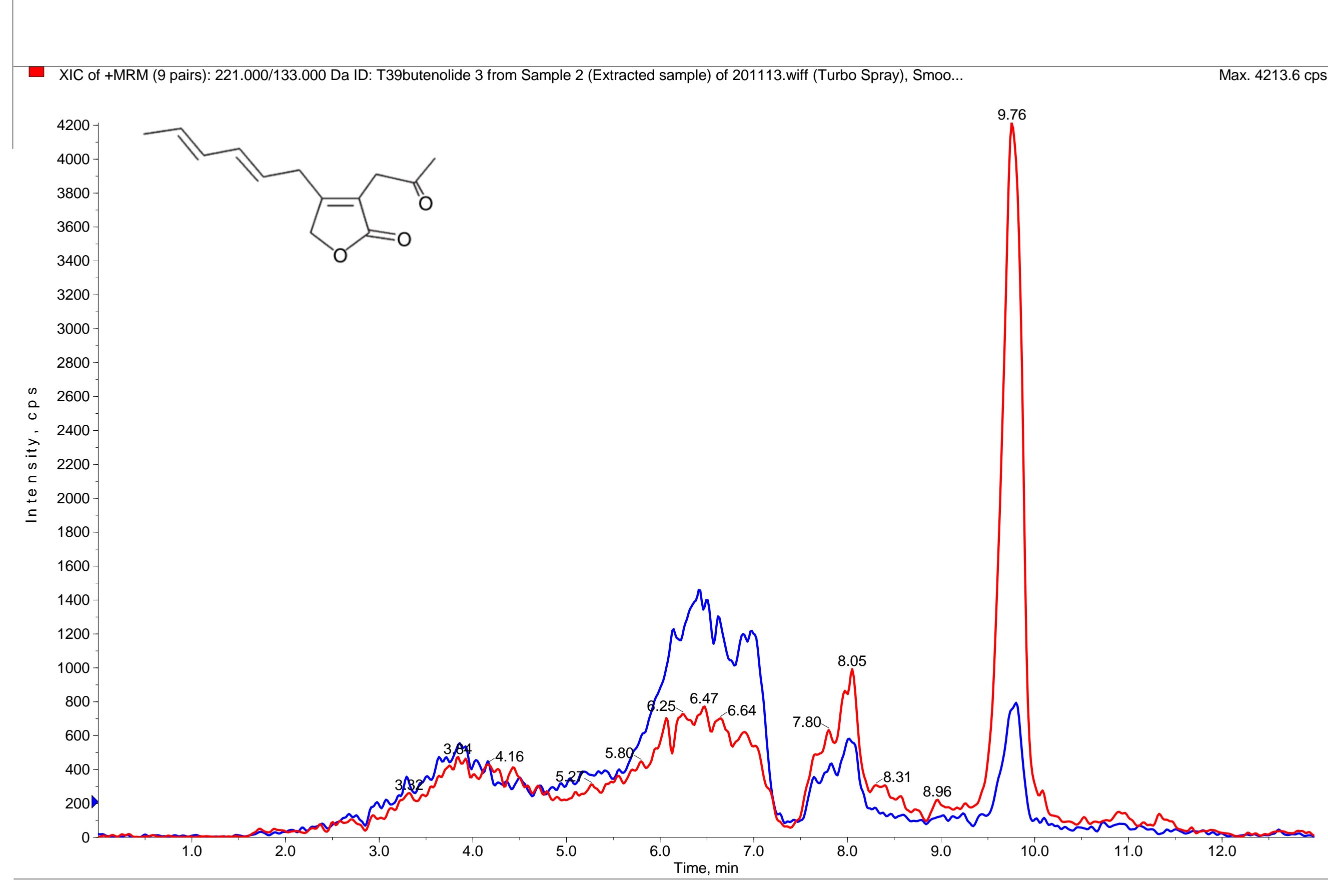

Figure 5

Chromatogram obtained by LC-MS/MS of the two transitions for T39butenolide from the culture broth extract of *Trichoderma harzianum* (CGJM 2295). Transition 221>203 is represented by the blue trace line and 221>133 is represented by the red trace line.

3.3 Production of Gliotoxin by *T. virens*

Five transitions (327>263>245>227>111>56) were generated from a pure standard for gliotoxin analysis. A peak for three of the transitions was recorded at 7.82 minutes from the extract. When the sample was spiked with the standard a peak with all five transitions was recorded at a retention time of 3.92 minutes.

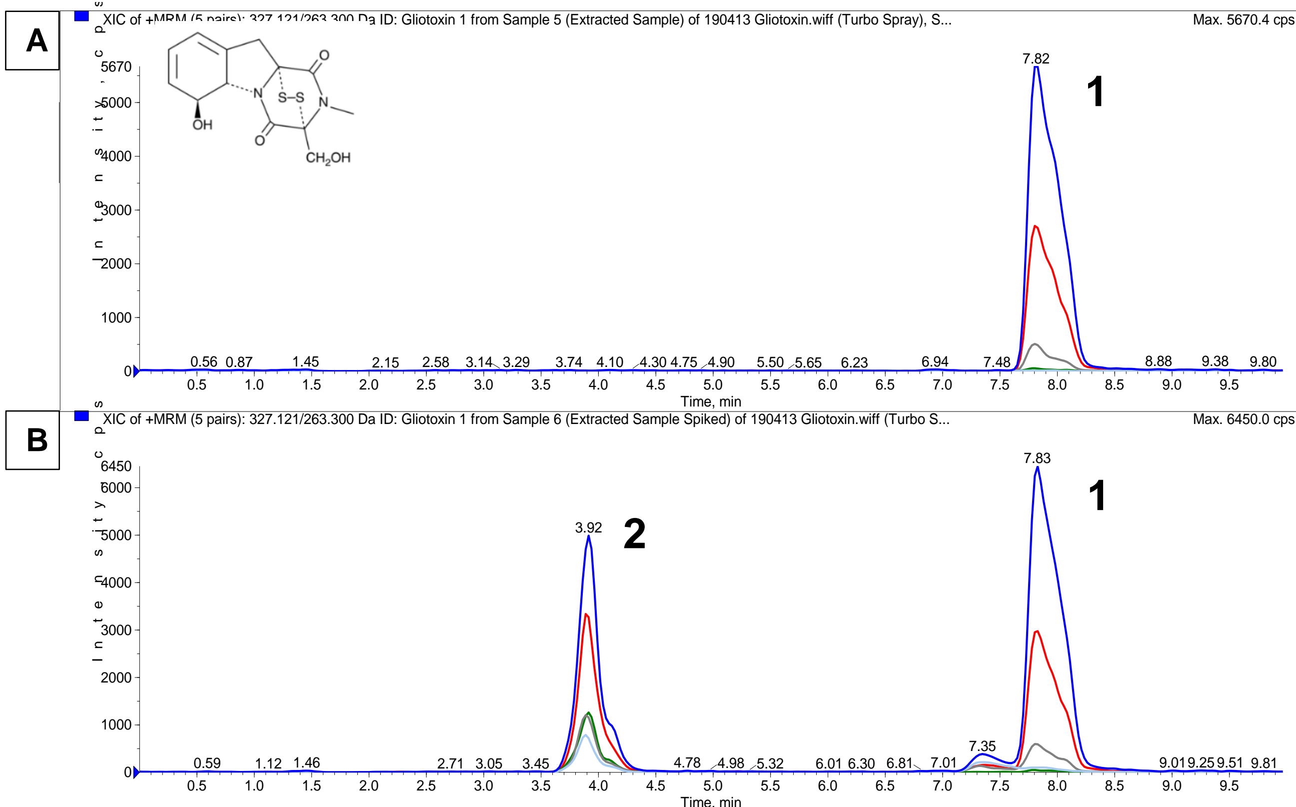

Figure 6

Chromatogram obtained by LC-MS/MS of the generated transitions for gliotoxin. Peak 1 is the analyte in the culture broth extract and peak 2 represents a pure gliotoxin standard spiked into the extract. Chromatogram A represents the unknown metabolite from the culture broth and extract chromatogram B represents the culture broth extract that was spiked with a pure standard gliotoxin.

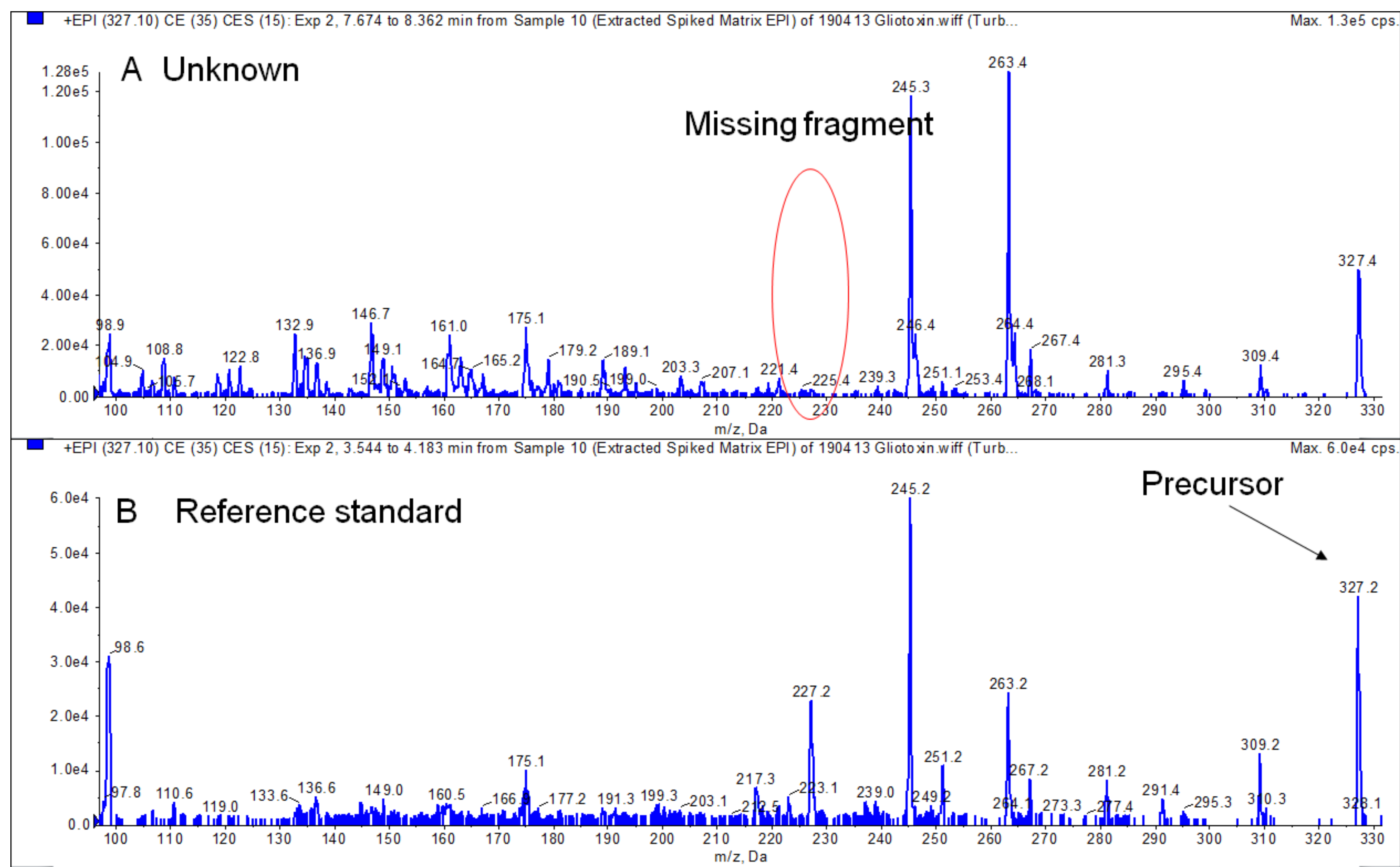

Figure 7

Product ion scan spectra showing the 327 Da precursor and its subsequent fragments. Chromatogram A represents the unknown metabolite from the culture broth extract and chromatogram B represents the pure standard gliotoxin.

4 Discussion

Four compounds namely; T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone, T39butenolide, and a metabolite structurally related to gliotoxin obtained from fungal culture filtrates were isolated and characterised. All of these have previously been found in *Trichoderma* species, and have been studied for their beneficial role in plants (Vinale *et al.,* 2006).

The aim of this study was to establish whether Southern African *Trichoderma* species possess the ability to produce the same secondary metabolites as reported by Vinale and co-workers (2006). One *Trichoderma harzianum* strain was selected as the preliminary candidate for screening of the six major metabolites found by Vinale and co-workers (2006). Due to lack of metabolite standards available for reference the only method for qualification of these metabolites is LC-MS/MS transition monitoring. This detection method relies on the fragmentation of a molecule with known precursor weight and the consequent detection of fragmentation ion numbers and weights.

The targeted analyses for these metabolites were performed using a multiple reaction monitoring (MRM) experimental approach. During an MRM experiment the instrument is used in triple quadrupole mode where every ionised analyte (the precursor) eluting off the column is fragmented in the collision cell to produce fragment masses. A set of masses, the precursor mass and one fragment mass, constitutes a transition. The instrument jumps between different transitions in an MRM transition list during an analysis cycle, each cycle typically lasting a few seconds. If a transition is detected the instrument's response is registered and a chromatogram is generated. The peak area on the chromatogram generated from the first and most sensitive transition was used as the quantifier while the rest of the transitions (if present) were used as a qualifier. The qualifier serves as an additional level of confirmation for the presence of the analyte, the retention time for these transitions needs to be the same and correspond to the retention time of the reference standard.

The transitions used for the metabolites were obtained from Vinale and co-workers (2009).

Due to lack of available reference standards for the metabolites, only the mass transitions from literature was used to establish metabolite presence. This approach is less than accurate for determination of the presence of a metabolite and thus the results obtained are open for future study.
The more transitions available per metabolite, the greater the chance that the specific metabolite is indeed present.

Only one transition from literature was available for 1-, 8-dihydroxy-3-methyl-anthraquinone to determine its presence and no reference standard. With these parameters a chromatographic peak was recorded at 9.57 minutes. This should be kept in mind when the accuracy of the assumption of its presence is called into question. Two transitions were recorded for T22azaphilone and they co-eluted a retention time of 9.56 minutes. Although double the number of transitions was available for identification compared to 1-, 8-dihydroxy-3-methyl-anthraquinone, the absolute identification can only be verified with a pure standard. The same results were recorded for T39butenolide, which yielded two transitions at a retention time of 9.76 minutes.

According to literature, gliotoxin is only produced by *Trichoderma virens* (formerly known as *Gliocladium virens*). Gliotoxin has been shown by Bezuidenhout and co-workers (2012) to mimic the gibberellic acid growth hormone in *Phaseolus vulgaris*, increasing germination rates, root formation and photosynthetic ability. Since a pure standard was available for gliotoxin, a method using five transitions could be generated and compared to a reference standard. The sample tested revealed a three-transition peak at a retention time of 7.82 minutes. When the standard was spiked into the extract a peak consisting of five transitions at a retention time of 3.92 minutes was observed.

These results indicated that a compound with the same molecular weight as gliotoxin was present in the extract of the culture broth. When fragmented, this analyte produced similar fragments to gliotoxin. The compound was, however, much more non-polar and thus separated differently to gliotoxin on a C18 reverse phase chromatograph (Figure 7). This indicated that the two precursor fragments share molecular masses. More importantly a 227 Da mass is missing in the extracted compound although it does contain masses at 133; 146; 161 and 179 Da, which are absent from gliotoxin. This indicates that the analyte in the extract is not gliotoxin, but could be structurally related and warrants further investigation.

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Chapter 4

Growth Promotion by *Trichoderma harzianum* Secondary Metabolites in Wheat and Maize

Abstract

Microorganisms associated with plants serve important functions in plant growth and health stimulation. Direct plant growth promotion by some microbes is based on improved nutrient uptake and hormonal stimulation due to interactive exchanges of secondary metabolites by both the plant and associated microbe. Diverse mechanisms are employed in the suppression and inhibition of plant pathogens, which is often indirectly connected to plant growth due to deterioration of immune functions. Members of the bacterial genera, *Azospirillum* and *Rhizobium,* are well-studied examples for plant growth promotion. *Bacillus*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Streptomyces* as well as the fungal genera, *Ampelomyces*, *Coniothyrium*, and *Trichoderma* are model organisms to demonstrate influence on plant health. By utilising the beneficial features found in these organisms, it has become possible to manipulate and develop these microorganisms for the agricultural-biotechnology industry. In recent years, *Trichoderma* has been a hot topic of study and new information surrounding its beneficial impact has changed the way microbe-plant interactions are viewed. A number of *Trichoderma* species have been shown to act as biofertilisers, biopesticides, plant-immune stimulators and elicitors of increased plant growth and yield. In this study, the effect of *T. harzianum* secondary metabolites was investigated *in vitro* on two major grain crops namely wheat and maize. Following the identification of several growth promoting secondary metabolites that included harzianolide, T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone and T39butenolide, wheat and maize seeds were selected to determine possible growth enhancement by metabolite stimulation.

The effect of treatment was observed on maize shoot lengths, root masses and root lengths. Maize shoot length was most affected by enhancing growth significantly when compared to control data. Wheat shoot length, root mass and root length were most affected with a significant increase of 36.8% for shoot length, 45.1% for root mass and 25.3% for root length when compared to untreated plants. These results hold promise for future studies concerning field and glasshouse trials to establish whether increased growth in the first 10 days could correlate to increased yield.

1 Introduction

A number of environmental factors including temperature, nutrients, light, and microorganisms affect plant growth. The region around the root, known as the rhizosphere, is comparatively rich in nutrients and as much as 40% of plant photosynthesis production can be lost from the roots (Bais *et al*., 2006). Thus, the rhizosphere supports extensive microbial populations capable of exerting beneficial, neutral, or harmful effects on plant growth. The close association between *Trichoderma* and other microorganisms with plant roots is well established. These microorganisms have a direct and indirect influence on the development and growth of host plants (Harman *et al.,* 2004).

Lindsey and Baker (1967) reported that significant increases in plant height and weight were observed in dwarf tomato plants when using *Trichoderma viride*. Other reports have since shown similar growth promotion on a wide variety of industrially significant plants (Vinale *et al*., 2008; Contreras-Cornejo *et al*., 2009). These growth enhancements include increased germination rates, accelerated and increased flowering, increased height and weight of plants, with more developed root systems and increased yield of both grain and fruit crops (Chang *et al*., 1986). Over the years, most of the research in this field focused on plant growth promotion due to the direct effect of *Trichoderma* on plant pathogens as a biological control agent, rather than on the direct influence on the plant. The major focus of this study was devoted to the effects that *Trichoderma* elicits directly on the plant in the absence of pathogens.

Large varieties of plants used in the flower, forestry, grain and vegetable industries have shown growth promotion through *Trichoderma.* The majority of work performed in this regard was on glasshouse grown vegetable crops, especially cucumber, lettuce and green pepper (Vinale *et al*., 2008).

The use of soil beneficial microbes (such as *Trichoderma*) and bacteria for symbiotic relationships with plants for improved nutrition or growth is known as biofertilisation (Vinale *et al.*, 2008). The goal of biofertilisers is to improve soil quality. In this sense, some *Trichoderma* species may be considered as biofertilisers as they possess the ability to rehabilitate soil from xenobiotic substances and alter soil pH (Vinale *et al.*, 2012). *Trichoderma* species are also able to colonise roots and thereby improve plant nutrition, growth and abiotic stress relief. There are several conflicting reports in literature as to which article was the first to report on growth promotion, yet all of these ‘first reports’ demonstrate enhanced development in the plants tested. However, it was an article by Baker and co-workers (1984) that concluded that these growth-promoting factors were caused by something other than direct contact between the plant host and *Trichoderma*.

Some *Trichoderma* strains have been shown to have direct effects on their plant hosts. These include increased growth and yield, nutrient uptake, germination percentage, stimulation of plant immunity, defence against biotic and abiotic threats, and increased efficiency of fertilizer uptake and incorporation (Shoresh, Harman, & Mastouri, 2010). It was recently made known that secondary metabolites are key facilitators of many of these positive effects. They act as microbe-associated molecular patterns (MAMPS) and as auxin-like analogues at low concentrations (Vinale *et al.*, 2008).

In vitro experiments on pepper seedlings, using cell-free culture broths from *Trichoderma* species, showed that negative growth influences were detected at high concentrations, whereas using a 1/16 dilution of the same culture exhibited a significant increase in the height and dry weight of the tested plants. These results proved that *Trichoderma* species produce substances in the cell-free culture filtrate responsible for these effects. Thus, they have a phytohormonal effect on pepper seedlings exhibiting the same growth enhancement as natural plant hormones (Brenner, 1981). It was further demonstrated in later studies that the volatile compounds produced by *Trichoderma atroviride* stimulated lateral root formation in *Arabidopsis thaliana* without influencing root length (Vinale *et al.*, 2008).

Secondary metabolites from *Trichoderma* spp. that may act as auxin-like compounds typically have an optimum activity at low concentrations (10^{-5} and 10^{-6} M) while having an inhibitory effect at higher doses (Brenner, 1981; Cleland, 1972).

In maize (*Zea mays*) plants, roots inoculated with living *Trichoderma* spores were affected in their system architecture, yielding increased growth and significantly larger harvests (Bjorkman *et al.*, 1998). Some effects that were reported include enhanced root biomass growth and increased root hair development (Harman *et al.*, 2004). Root systems in plants are responsible for anchorage, water use efficiency, and facilitating the attainment of minerals and nutrients from the soil (Lo´pez-Bucio *et al.*, 2005). Evidence indicated that auxins play a role in the regulation of root system formation and structure. Application of synthetic and natural auxins increases lateral root and root hair development, whereas auxin transport inhibitors decrease root branching (Reed *et al.*, 1998; Casimiro *et al.*, 2001).

The aim of the current chapter was to determine the plant growth promoting benefits of *Trichoderma* species in the absence of any living material, using only the cell-free culture broth containing certain secondary metabolites. Wheat and maize seedlings were tested for increased length and weight after 10 days of growth in metabolite infused water agar.

2 Materials and Methods

2.1 Production of *Trichoderma* Secondary Metabolite Cocktail

A culture broth was obtained and produced as described in Chapter 3 (2.2). Mass spectrometry was used to identify secondary metabolites and the presence of T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone, and T39butenolide, was investigated. This culture broth was used for the experimental section of this chapter.

2.2 Metabolite Infused Agar

In a modified version of Vinale and co-workers (2012), a culture broth of *Trichoderma harzianum* (CGJM 2295), containing secondary metabolites, was filter sterilised and infused in sterilised water agar (5 $g.L^{-1}$) at five different concentrations namely 0, 17, 35, 70, and 140 ml/L, respectively. One treatment served as control and contained no culture filtrate. Glass beakers (500 ml) were filled with 200 ml of infused agar and allowed to set.

2.3 Wheat and Maize Seeds

Wheat seeds from Sensako (CRN 826) and maize seeds from PANNAR (PAN 6Q-345CB) were selected due to their notable drought tolerance and because they are widely cultivated in South Africa.

2.4 Growth Study

Growth parameters regarding the number of repetitions and replications were calculated to ensure statistical reliability. Five treatments of metabolite concentrations were performed in five repetitions with 10 seeds being used per repetition. Metabolite concentrations were estimated using results from Vinale and co-workers (2012).

Estimations based on literature indicated that 35 ml/L of culture filtrate contained metabolite concentrations optimal for plant growth promotion based on natural plant hormone levels. A treatment was then representative of half the optimal concentration (17 ml/L), double the optimal concentration (70 ml/L) and four times the optimal concentration (140 ml/L). Water agar (WA), absent of culture broth containing metabolites, served as control. Seeds were surface sterilised for one minute in 76% EtOH (Merck™) and rinsed with sterile distilled water. Seeds were placed on the agar surface and flasks were covered with plastic bags to ensure minimal moisture loss (Figure 1B).

After 10 days of incubation at room temperature, plants were removed from the agar with all roots and lateral roots intact. Root and shoot lengths were measured and all parts were dried in an oven at 90°C until constant weight was reached. Roots and shoots were weighed and results were statistically analysed.

2.5 Statistical Analyses

NCSS was used to conduct analysis of variance (ANOVA) on all variables measured (Hintze, 2007). Fisher's unprotected test was used to determine the least significant differences (LSDs) at 5% significance level to compare means.

Figure 1
Placement of surface sterilised wheat on metabolite infused WA (A).
Incubation of wheat cultivation in flasks (B).

3 Results

Water agar was infused with a sterile culture broth containing the secondary metabolite cocktail. Maize and wheat seeds were placed on various concentrations of the growth medium and allowed to grow for 10 days. This was done to establish optimal treatment concentrations for increased plant growth. Roots and shoots of each crop were removed from the growth medium and measured prior to dry mass determinations. Analysis of variance was conducted on data collected for shoot masses, shoot lengths, root masses, and root lengths for both maize and wheat.

Analysis of variance (ANOVA) on wheat shoot mass indicated that there was no variation between the treatment means (Table 2A) ($P=0.084^{ns}$). This indicated that no significant increases in shoot mass could be found between treated and untreated wheat seeds.

However, wheat shoot lengths were significantly different between treatments as indicated by ANOVA analysis (Table 2B). The lowest mean shoot length was recorded in the control treatment (mean shoot length = 8.79 cm) and the highest mean shoot length was recorded at T140 (mean shoot length = 13.92 cm). According to Fishers LSD ($P<0.05$) each treatment differed significantly from one another (Figure 3A).

ANOVA analysis indicated that there was significant variation between treatments regarding wheat root masses (Table 2C). The control treatment had the lowest mean root mass (mean root mass = 0.00469 g) and T140 had the highest mean root mass (mean root mass = 0.00852 g). T35 and T70 were grouped together with no significant difference according to Fischer’s LSD ($P<0.05$) (Figure 3B).

Wheat root lengths were significantly different due to the treatments and thus had variation between means as indicated by ANOVA analysis (Table 2D). The control treatment had the lowest mean root length (mean root length = 13.06 cm) and the highest mean root length was recorded at T35 (mean root length = 17.51 cm). However, T18, T35, T70 and T140 clustered together and did not differ significantly from one another according to Fishers LSD ($P<0.05$) (Figure 3C).

Table 1

Analysis of variance (ANOVA) of *Trichoderma harzianum* metabolite treatments for maize indicating shoot mass (g; A), shoot length (cm; B), root mass (g; C) and root length (cm; D).

A		**Shoot Mass**					**C**	**Root Mass**				
Source Term	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**		**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**
Treatment	4	1.62E-04	4.04E-05	1.210	0.344	0.294		1.63E-03	4.07E-04	11.950	**0.000109***	0.999
Replicate	4	6.22E-05	1.56E-05	0.470	0.760	0.132		6.59E-05	1.65E-05	0.480	0.747	0.135
Error	16	5.34E-04	3.34E-05					5.45E-04	3.41E-05			
Total (Adjusted)	24	7.58E-04						2.24E-03				
Total	25											
B		**Shoot Length**					**D**	**Root Length**				
Source Term	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**		**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**
Treatment	4	43.083	10.771	5.15	**0.007***	0.898		346.051	86.513	10.850	**0.000***	0.998
Replicate	4	5.200	1.300	0.62	0.653	0.164		26.9569	6.739	0.850	0.517	0.212
Error	16	33.435	2.090					127.593	7.975			
Total (Adjusted)	24	81.718						500.601				
Total	25											

** Term significant at alpha = 0.05*

ANOVA analysis of maize shoot mass (Table 1A) indicated no significant differences due to the treatments ($P=0.344^{ns}$).

However, maize shoot length (Table 1B) indicated significant variation between the treatments. The lowest mean shoot length was recorded at the T140 treatment (mean shoot length = 13.25 cm) and the highest mean shoot length was recorded at T17 (mean shoot length = 17.16 cm). According to Fisher's LSD ($P<0.05$) T17 and T140 differed significantly from one another. Furthermore, the control treatment and the T35 treatment were grouped together and thus were not significantly different (Figure 2A).

ANOVA analysis indicated that maize root masses between treatments were significantly different (Table 1C). The treatment T140 had the lowest mean root mass (mean root mass = 0.04 g) and T35 had the highest mean root mass (mean root mass = 0.06 g). However, T35, T70 and T17 were grouped together according to Fischer's LSD ($P<0.05$) (Figure 2B). The control treatment and treatment T140 differed significantly from one another and the formerly mentioned grouping.

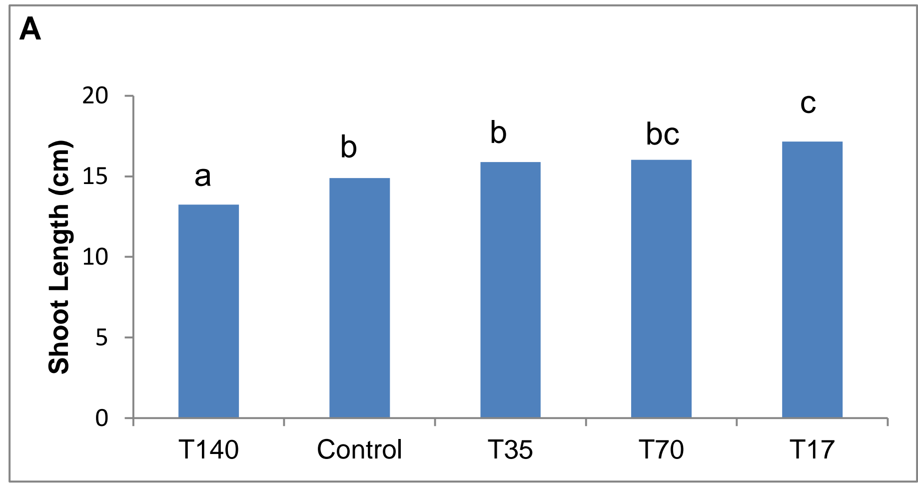
Maize root length means had significant variation due to the treatments as indicated by ANOVA analysis (Table 1D). The T140 had the lowest mean root length (mean root length = 15.69 cm) and the highest mean root length was recorded at T17 (mean root length = 26.78 cm). Treatments T17 and T140 differed significantly from one another, which is similar to the shoot length results. Furthermore, the control treatment and the T35 treatment were grouped together and thus were not significantly different (Figure 2C).

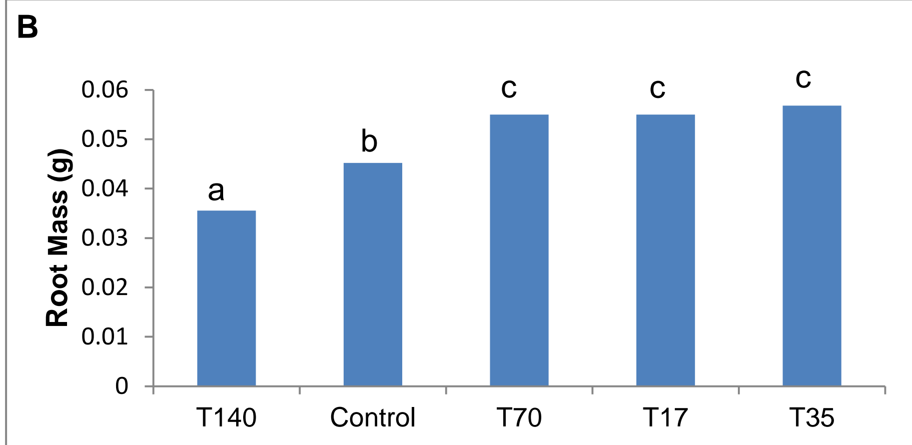
Table 2

Analysis of variance (ANOVA) of *Trichoderma harzianum* metabolite treatment for wheat shoot mass (g; A), shoot length (cm; B), root mass (g; C) and root length (cm; D).

A		**Shoot Mass**					**C**	**Root Mass**				
Source Term	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**		**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**
Treatment	4	1.26E-04	3.15E-05	2.49	0.08	0.57		4.540E-05	1.135E-05	13.99	0.00*	1.00
Replicate	4	5.05E-05	1.26E-05	1.00	0.44	0.25		2.320E-06	5.801E-07	0.72	0.59	0.18
Error	16	2.02E-04	1.26E-05					1.298E-05	8.111E-07			
Total (Adjusted)	24	3.79E-04						6.069E-05				
Total	25											
B		**Shoot Length**					**D**	**Root Length**				
Source Term	**DF**	**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**		**Sum of Squares**	**Mean Square**	**F-Ratio**	**Prob Level**	**Power (Alpha=0.05)**
Treatment	4	71.35	17.84	30.52	0.00*	1.000		68.29	17.07	5.06	0.01*	0.89
Replicate	4	10.10	2.53	4.32	0.01*	0.833		15.02	3.76	1.11	0.38	0.27
Error	16	9.35	0.58					54.01	3.38			
Total (Adjusted)	24	90.80						137.32				
Total	25											

** Term significant at alpha = 0.05*





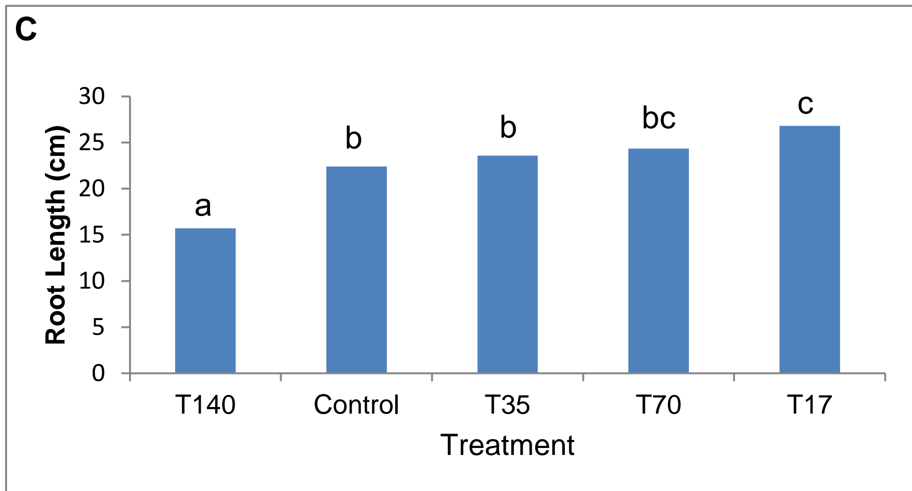
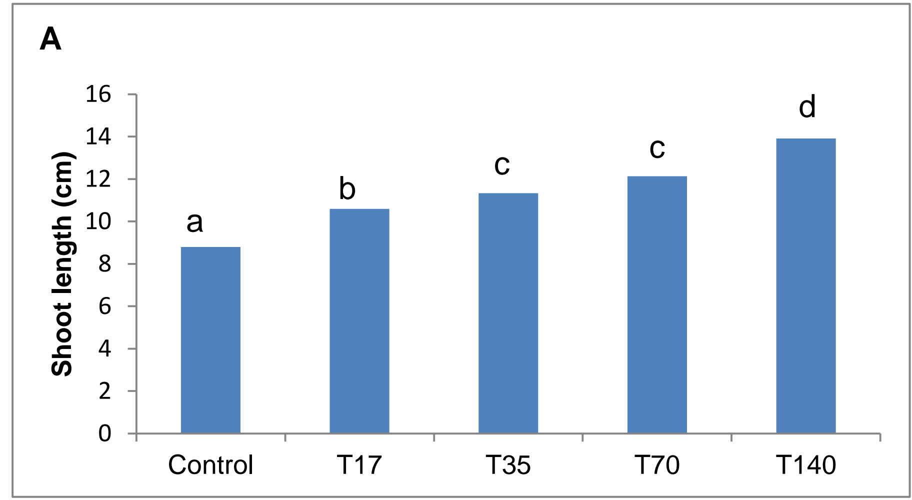
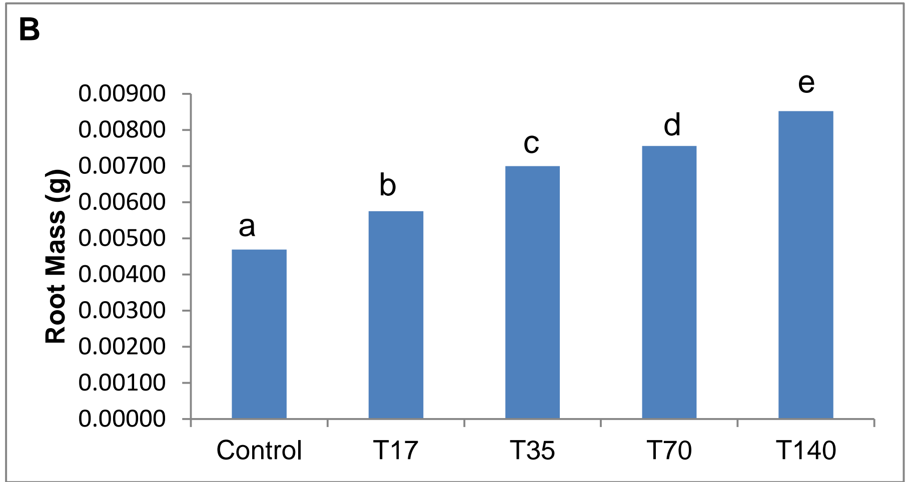


Figure 2

Means of (A) shoot length (cm), (B) root mass (g), and (C) root length (cm) of maize plants grown in four different *Trichoderma* treatments 17 mL.L^{-1}(T17), 35 mL.L^{-1} (T35), 70 mL.L^{-1} (T70) and 140 mL.L^{-1} (T140)*

*Means with the same letter do not differ significantly from one another





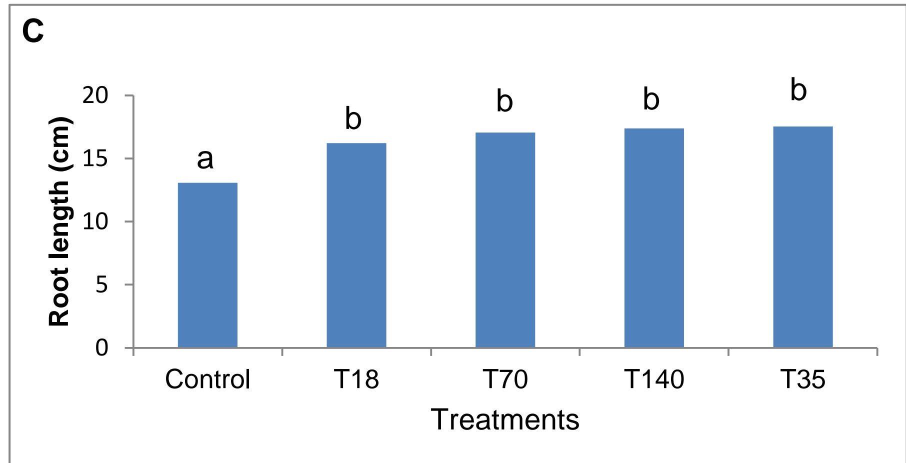


Figure 3

Means of (A) shoot length (cm), (B) root mass (g), and (C) root length (cm) of wheat plants grown in four different *Trichoderma harzianum* metabolite treatments 17 $mL.L^{-1}$(T17), 35 $mL.L^{-1}$ (T35), 70 $mL.L^{-1}$ (T70) and 140 $mL.L^{-1}$ (T140)

[*] Means with the same letter do not differ significantly from one another

4 Discussion

Trichoderma species are common soil fungi that colonise roots and stimulate plant growth. They have been applied to a wide range of plant species for the purpose of growth enhancement, with positive effects on plant weight, crop yields, and disease control. Their agricultural use could be expanded and more readily accepted if the mechanisms of their growth enhancement were known (Contreras-Cornejo *et al*., 2009). Some strains of *Trichoderma* also have the ability to improve photosynthetic efficiency and respiratory activities in plants. All of these capabilities are a result of their capacity to alter and regulate plant gene expression (Shoresh *et al*., 2010).

Literature states that the majority of metabolite formation occurs during the stationary growth phase of most organisms and thus 28 days of cultivation was used to achieve metabolite formation. After 28 days a small amount of culture broth was analysed qualitatively for metabolite formation using mass spectrometry and it was confirmed that T22azaphilone, T39butenolide, harzianolide and 1,8-dihydroxy-3-methyl-anthraquinone were present. Serial filtration was performed using cheesecloth followed by a series of filters in descending pore sizes from 10 µm, 8 µm, 3 µm, 0.45 µm and finally 0.22 µm. This array of filters allowed for minimal pore clogging and assured a sterile filtrate, which was tested in PDB for any growth before proceeding to further tests.

To study the growth promoting benefits of *Trichoderma harzianum* (CGJM 2295), wheat and maize seeds were used as model organisms due to their popularity and agricultural significance in South Africa. During growth, it was visibly noticeable that seeds grown on metabolite-containing medium grew faster with a more developed root system, especially lateral roots and root hair formation. Internal fungi of the seeds were also supressed and did not contaminate the growth medium as readily when compared to the control plants (Figure 4). The results obtained in this study correlates with literature concerning growth promotion with special reference to development of a structurally enhanced root system (Harman *et al*., 2004).

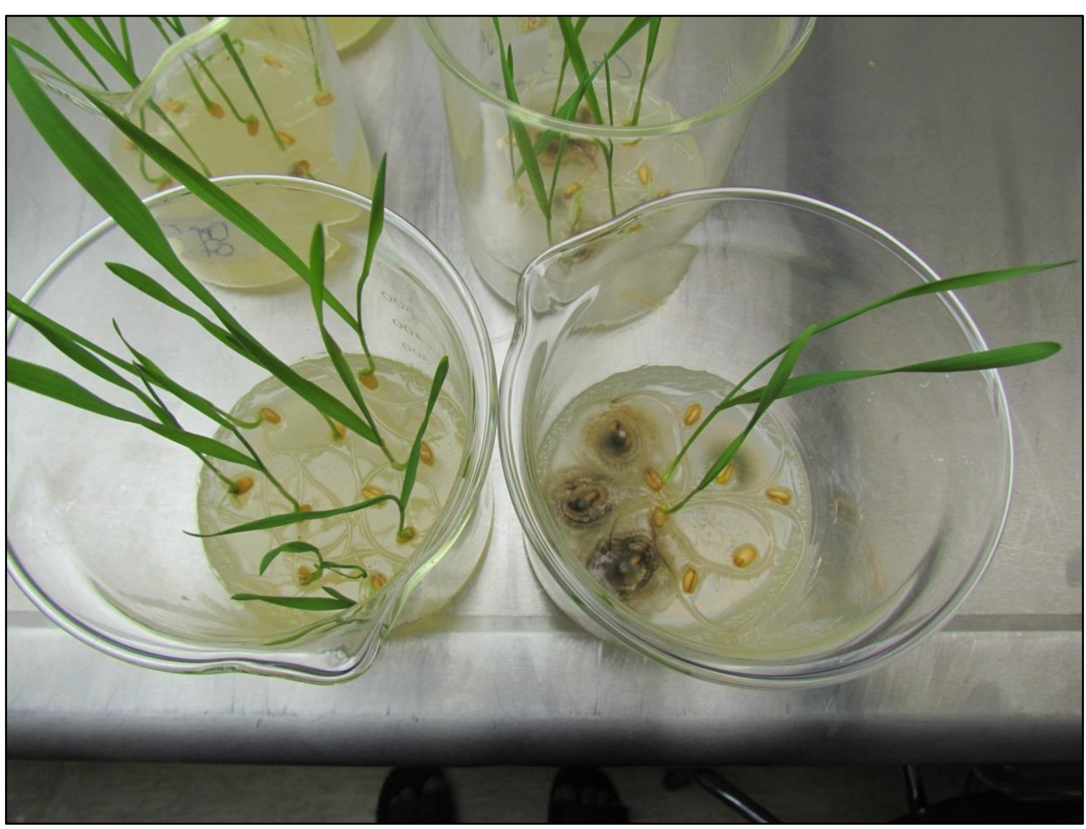


Figure 4
Increased inhibition of internal pathogens in wheat due to metabolite anti-microbial properties. Treatment 140 $mL.L^{-1}$ (Left) and control (right) indicating internal fungal contaminants emerging from the seed.

Secondary metabolites that were identified from *Trichoderma harzianum* (CGJM 2295) have all been found to enhance plant growth, inhibit phytopathogens and stimulate plant defences leading to increased immunity (Vinale *et al.*, 2006; Vinale *et al.*, 2008; Vinale *et al.*, 2009; Haman *et al.*, 2004). The results of this study correlate and corroborate these findings.

Wheat and maize grown on metabolite infused agar showed significant growth enhancement when compared to the control groups in this research. In maize, it has been shown that, in addition to induction of carbohydrate metabolism and photosynthesis-related proteins, the starch content of leaves in *Trichoderma*-inoculated plants was higher with greater chlorophyll concentrations (Shoresh and Harman, 2008).

Similar results were obtained in this study in wheat seedlings at a metabolite treatment of 140 $mL.L^{-1}$ (Fig. 5). This would be beneficial for plants under drought conditions, especially if stress is extended to result in carbon deprivation due to stomatal closure.

Figure 5
Difference in observable chlorophyll intensity for wheat between control (left) and treatment at 140 $mL.L^{-1}$ (right) on wheat seedlings.

Wheat plants were significantly enhanced with regards to shoot length, root length and root dry mass for all metabolite treatments. Root dry mass of wheat, after 10 days of growth, at a treatment concentration of 140 $mL.L^{-1}$ showed an average increase of 45.1% compared to the control, as well as 25.3% increase in root length. The reason for a greater biomass rather than an increase in length is due to the formation of root hair and lateral root formation. This increase in root development and biomass will have a significant impact on crop safety and production with regards to yield in the agricultural sector. Although shoot length was also significantly increased by 36.8%, shoot weight remained unaffected. A possible explanation for this is that, because of the absence of UV stimulation during the growth period, plants increased shoot length in search of UV light without achieving adequate UV stimulation for sustainable photosynthesis; thus leading to lower levels of carbohydrate formation and consequently insignificant influence on biomass production.

In maize plants, at a treatment concentration of 17 $mL.L^{-1}$, there was a significant increase of biomass for root length with an increase of 16.8% when compared to control plants, a root mass increase of 20.4% and a shoot length increase of 13.4%. As described previously, the difference in weight enhancement of root length versus weight was attributed to increased lateral root and root hair formation. Interestingly, the best concentration of culture broth containing the metabolite cocktail for treatment in wheat, was detrimental to growth in maize with root mass, root length and shoot length being significantly less than the control group. A possible explanation for these surprising results is that maize seedlings are more susceptible to the influence of the metabolite concentrations. According to literature, growth promoting metabolites act as analogues to natural plant growth hormones, whereby at the right concentration, growth of the plant can be enhanced, but when concentrations of the analogous metabolite reach unnaturally high levels the host plant growth is inhibited. *Trichoderma* secondary metabolites that may act as auxin-like compounds typically have an optimum activity at low concentrations (10^{-5} and 10^{-6} M) while having an inhibitory effect at higher doses (Brenner, 1981; Cleland, 1972).

Another possible explanation for this occurrence is the fact that wheat is a C3 photosynthesis plant and maize is a C4 photosynthesis plant. Because *Trichoderma* secondary metabolites influence plant photosynthesis, it may possibly be the reason why differences in growth promotion were observed. C3 plants have a disadvantage in hot and dry conditions in that their photosynthetic efficiency is partially diminished because of photorespiration. When the CO_2 concentration in the chloroplast drops below approximately 50 ppm, the catalyst rubisco that helps to fix carbon, begins to fix oxygen instead. This is highly wasteful of the energy that has been collected from the light, and causes the rubisco to operate at perhaps a quarter of its maximal rate. In C4 plants the advantage that comes from its two-stage process is that the active pumping of carbon into the bundle sheath cell, blocks oxygen to produce an environment with 10-120 times as much CO_2 available to the Calvin cycle, and the rubisco tends to be optimally utilized. The high CO_2 concentration and the absence of oxygen implies that the system never experiences the detractive effects of photorespiration (Moore *et al*., 1995)

In this study, it was shown that the use of secondary metabolites from *Trichoderma harzianum* (CGJM 2295) significantly enhance the growth of wheat and maize. It is, however, not known which metabolites specifically were the cause of these effects or whether it was due to a cumulative effect. The use of this treatment in agriculture would be more readily accepted once the exact mechanisms of this growth enhancement are elucidated. However, the potential for novel biofertilisers and plant growth stimulators is not without merit and further studies to determine possible yield increase would provide a greater insight into industrial relevance and application.

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Summary

The world population is growing at an exponential rate, placing extreme pressure on already scarce natural resources. This leads to changes in our environment with consequences such as climate change. It is predicted that South Africa will develop a more semi-arid and dry climate, which will endanger agricultural sustainability and place even more pressure on the already insufficient food supply. The need to increase agricultural productivity and quality has consequently led to an excessive use of chemical fertilisers, generating severe environmental pollution and, with global climate change, places increased pressure on world food production. It has become imperative that alternative means of crop protection and productivity enhancers are investigated. The use of *Trichoderma* and *Trichoderma*-based products as biofertilisers, biopesticides, and growth stimulators are alternatives to sustaining high production with low ecological impact.

Trichoderma (teleomorph *Hypocrea*) is a fungal genus with its members found in ecosystems around the world. *Trichoderma* species can reduce the severity of plant diseases by inhibiting phytopathogens through antagonistic and mycoparasitic capabilities. In recent decades, it has been established that some *Trichoderma* strains can interact directly with roots, increasing plant growth, enhancing resistance to disease and increasing tolerance to abiotic stresses. This makes species of *Trichoderma* potential model organisms for sustained improvement of crop yield and health in agriculture.

Trichoderma species are prolific producers of secondary metabolites. More than 120 structurally distinct *Trichoderma* secondary metabolites have been determined and described analytically, with more being discovered continually. Secondary metabolites are natural products that are biologically active. The vast majority of these compounds, however, have obscure or unknown functions, but they do play an important role in the functioning of the producing fungus, and are crucial to their diversity.

The close association of *Trichoderma* and other microbes with plant roots has been well established. These microbes have direct and indirect influence on the growth of host plants, of which secondary metabolites are key instigators. Growth influences include increased germination rate, more rapid and increased flowering, increased height and weight of plants with more developed root systems, and increased yield of both grain and fruit crops. This positive symbiosis between *Trichoderma* and the host plant is dependent on a plethora of environmental factors. If these factors are not suitable for the fungus the benefits they convey will not be as noticeable and may not be present at all. Metabolites of *Trichoderma* species could be used as new biopesticides and biofertilisers based on their active compounds rather than on the living culture. This could have a significant beneficial impact on increasing crop yields and improving crop health.

In this study, *Trichoderma* species diversity was investigated in Southern Africa in order to identify possible candidates which may provide plant growth enhancing benefits due to secondary metabolite stimulation. ITS gene sequencing was conducted to identify 54 *Trichoderma* species isolates accessible from the CGJM culture collection at the University of the Free State and from a variety of regions and host substrates. A phylogenetic tree was constructed to determine relatedness among species. In total, 54 isolates were identified using molecular techniques representing 11 species and three of the four taxonomic sections of *Trichoderma. Trichoderma atroviride* was the most prominent isolate with 24 strains and *Trichoderma harzianum* constituted 13 of the total isolates with preference to agricultural niches. Other species identified were *T. asperellum*, *T. gamsii*, *T. citrinoviride*, *T. viride*, *T. longibrachiatum*, *T. reesei*, *T. spirale* and *T. hamatum*. This study revealed a great diversity despite the limited number of samples represented in the CGJM culture collection. This indicates that South Africa has a richness of *Trichoderma* species, the full extent of which will only be realised through a comprehensive study.

Trichoderma harzianum (CGJM 2295) and *Trichoderma virens* (CGJM 2307) were selected for metabolite screening as literature indicated higher possibilities for the production of the desired metabolites. *Trichoderma* species were cultivated in broth culture to produce possible plant growth promoting secondary metabolites. The culture broth was analysed for secondary metabolites as indicated in literature and T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone, T39butenolide, and a metabolite similar to gliotoxin were qualitatively detected and identified using liquid mass spectrometry with transition monitoring. All of these metabolites have previously been shown to have growth promoting benefits on plants, indicating that *Trichoderma* species from natural environments in South Africa do possess the ability to produce these metabolites.

Following the identification of secondary metabolites produced by *Trichoderma harzianum* (CGJM 2295), the goal was to determine the plant growth promoting benefits of these metabolites in the absence of the living organism, using only the cell-free culture broth containing the secondary metabolites. Wheat and maize seedlings were tested for increased length and weight after 10 days of growth on metabolite infused water agar. Wheat shoot length, root mass and root length were most affected with a significant increase of 36.8% for shoot length, 45.1% for root mass and 25.3% for root length when compared to untreated plants. In maize plants there was a significant increase of biomass in root length with an increase of 16.8% when compared to untreated plants, root mass increase of 20.4% and a shoot length increase of 13.4%.

The results of this study indicated the possible use of *Trichoderma* secondary metabolites as novel plant growth enhancers. These results also hold promise for future studies with regards to field and glasshouse trials to establish whether increased growth in the first 10 days could ultimately correlate to increased yield. *Trichoderma* biotechnology is a field of study with endless possibilities and applications, and may be a contributing factor to overcoming challenges in the production of sufficient food stores, improving crop protection and enhancing plant yield in agriculture in South Africa.

Opsomming

Die wêreld se bevolking groei teen 'n eksponensiële koers en plaas geweldige druk op die reeds beperkte natuurlike hulpbronne. Dit lei tot veranderinge in die omgewing, met gevolge soos klimaatsverandering. Daar word beraam dat Suid-Afrika 'n meer semi-droë en woestynagtige klimaat gaan ontwikkel, wat landbou volhoubaarheid in gevaar stel en plaas selfs meer druk op die reeds onvoldoende kosvoorraad. Die behoefte aan die verhoging van landbouproduktiwiteit en kwaliteit het tot oormatige gebruik van chemiese bemesting gelei. Dit het ernstige omgewingsbesoedeling en globale klimaatsverandering tot gevolg wat intensiewe druk plaas op die produksie van wêreld voedsel. Hierdie faktore het dit genoodsaak dat alternatiewe middels vir gewasbeskerming en produktiwiteit ondersoek word. Die gebruik van *Trichoderma* en *Trichoderma* gebaseerde produkte vir bio bemesting, bio insektisiedes en groei stimulators kan 'n wesenlike alternatief vir die handhawing van 'n hoë landbou produksie met lae ekologiese impak wees.

Trichoderma (teleomorf *Hypocrea*) is 'n swam genus wat regdeur die wêreld se ekosisteme gevind word. *Trichoderma* spp. kan die omvang van plantsiektes verminder deur inhibisie van plantpatogene deur hul antagonistiese en mikoparasitiese vermoëns. In die afgelope dekades is daar gevind dat sommige *Trichoderma* isolate direk interaksie kan hê met wortels om ‘n toename in plantegroei, verhoging in weerstand teen siektes en toename in verdraagsaamheid teen abiotiese stresfaktore te bewerkstellig. Hierdie kenmerke maak *Trichoderma* 'n potensiële model organisme vir volhoubare verbetering van die oeskwaliteit, kwantiteit, asook gewas beskerming.

Trichoderma spesies is uitstaande produseerders van sekondêre metaboliete. Meer as 120 struktureel differensieërbare sekondêre metaboliete van *Trichoderma* is beskryf en analities bepaal, en meer word voortdurend ontdek. Sekondêre metaboliete is natuurlike produkte wat biologiese aktiwiteit toon. Die oorgrote meerderheid van hierdie verbindings het egter onduidelike of onbekende funksies, maar speel egter 'n belangrike rol in die funksionering van die swam en is 'n belangrike element van hul diversiteit.

Die rol van *Trichoderma* en ander mikrobes se noue assosiasie met plantwortels is reeds bewys en hierdie mikrobes het direkte en indirekte invloede op die gasheer plant se groei, en sekondêre metaboliete is die sleutel drywer van hierdie indirekte invloede. Groei invloede sluit in: verhoogde ontkieming, vinniger en verhoogde blomvorming, vermeerdering in hoogte en gewig van plante met meer ontwikkelde wortelstelsels, en verhoogde opbrengs van beide graan en vrugte gewasse. Hierdie positiewe simbiose tussen *Trichoderma* en die gasheer plant is afhanklik van 'n verskeidenheid van omgewingsfaktore. As hierdie faktore nie geskik is vir die swam nie sal die voordele wat hulle bewerkstellig nie so opvallend wees nie, en mag selfs nie sigbaar wees nie. *Trichoderma* se metaboliete kan gebruik word as nuwe bio insektisiedes en bio bemesting wat gebaseer is op die aktiewe verbindings in plaas van die lewende mikrobe self. Dit kan 'n beduidende positiewe impak maak met verhoogde gewas opbrengste en verbetering in gewas gesondheid.

In hierdie studie is die diversiteit van *Trichoderma* ondersoek in Suider-Afrika met die doel om moontlike kandidate te identifiseer wat plant groei verbetering kan bied weens sekondêre metaboliet stimulasie. Die ITS geen area se DNS basispaaropeenvolgorde is bepaal om 54 *Trichoderma* isolate, beskikbaar in die CGJM kultuurversameling by die Universiteit van die Vrystaat en uit 'n verskeidenheid van streke en gasheer substrate, te identifiseer. ‘n Filogenetiese boom is opgestel om die verwantskappe tussen spesies vas te stel. In totaal is 54 isolate geïdentifiseer met behulp van molekulêre tegnieke, insluitende 11 spesies en drie van die vier taksonomiese seksies van *Trichoderma* is gedifferensieer. *Trichoderma atroviride* was die mees prominente isolaat met 24 stamme en *T. harzianum* wat 13 verteenwoordigers gehad het. Ander spesies wat geïdentifiseer was is *T. asperellum*, *T. gamsii*, *T. citrinoviride*, *T. viride*, *T. longibrachiatum*, *T. reesei*, *T. spirale* en *T. hamatum*. Hierdie studie het groot diversiteit getoon ten spyte van 'n beperkte hoeveelheid isolate in die CGJM kultuur versameling. Hierdie dui daarop dat Suid-Afrika ‘n ryke diversiteit van *Trichoderma* spesies het, en die volle omvang daarvan sal slegs besef word deur ‘n meer omvattende studie.

Trichoderma harzianum (CGJM 2295) en *Trichoderma virens* (CGJM 2307) is gekies vir metaboliet bepaling aangesien literatuur op ‘n hoër waarskynlikheid gedui het vir die produksie van die gewenste metaboliete. *Trichoderma* spesies is gekweek in ‘n vloeibare kultuur medium om moontlike groei bevorderende sekondêre metaboliete te produseer. Die vloeibare kultuur is ontleed vir sekondêre metaboliete met betrekking tot bekende literatuur en T22azaphilone, 1-, 8-dihydroxy-3-methyl-anthraquinone, T39butenolide, harzianolide en n metaboliet, soortgelyk aan gliotoxien, was kwalitatief gevind en geïdentifiseer met behulp van vloeibare massaspektrometrie deur oorgang monitering. Al hierdie metaboliete het voorheen groeibevordering op plante getoon, wat aandui dat die Suider-Afrikaanse *Trichoderma* spesies ook oor die vermoë beskik om hierdie metaboliete te produseer.

Na aanleiding van die identifisering van sekondêre metaboliete wat deur *T. harzianum* (CGJM 2295) geproduseer is, was die doel om die plant groei voordele te bepaal in die afwesigheid van die lewende organisme, en slegs met die selvrye kultuur deur die sekondêre metaboliete. Koring en mielie saailinge is getoets vir lengte en gewig toename na 10 dae van groei op metaboliet bevattende water agar. Koring lootlengte, wortelmassa en wortellengte is die meeste bevorder met 'n beduidende toename van 36,8% in lootlengte, 45,1% in wortel massa en 25,3% in wortel lengte in vergelyking met onbehandelde plante. Vir mielies was daar 'n beduidende toename van biomassa in wortellengte met 'n toename van 16,8% in vergelyking met die onbehandelde plante. Daar was ook ‘n wortelmassa toename van 20,4% en 'n lootlengte verhoging van 13,4%.

Die resultate van hierdie studie dui die moontlikheid aan vir die gebruik van *Trichoderma* sekondêre metaboliete om plantegroei te verbeter. Hierdie resultate hou ook belofte in vir toekomstige studies met betrekking tot veld en glashuis proewe om vas te stel of verhoogde groei in die eerste 10 dae kan korreleer met verhoogde opbrengs. *Trichoderma* biotegnologie is 'n studieveld met eindelose moontlikhede en toepassings, en kan ‘n bydraende faktor wees in die verbetering van gewasproduksie in Suid-Afrika.

Keywords

Trichoderma, Secondary Metabolites, South Africa, Biodiversity, Plant Growth Enhancement, Mass Spectrometry, *Trichoderma* Isolation, Biotechnology.