A new approach for delivery of entomopathogenic fungi for plant protection against insect pests and plant diseases via maize seed coating

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A thesis

submitted in partial fulfilment

of the requirements for the Degree of

Doctor of Philosophy in Plant Protection

at

Lincoln University

by

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Lincoln University

2018

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy

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by

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Entomopathogenic fungi have been recognized mainly for their ability to kill insects which has seen as an advantage for the development of these particular fungi as biocontrol agents in sustainable agriculture programmes. Recently, it was determined that some species of entomopathogenic fungi, including *Metarhizium* spp., also have the capability to associate with roots and aerial parts of the plant. The potential benefits from the plant-fungal association are as broad as nutrient acquisition, plant growth promotion, protection against biotic or abiotic factors and even increase of the induced defence response in the plant through modification of the phytohormone content. However, many of these the positive effects are uncertain and we are far from understanding the complex interaction between the plant, the fungus and a biotic challenger. Additionally, the establishment of the plant-fungal association relies on the specificity of the fungus and plant interaction. The complex taxon *Metarhizium anisopliae* had gone through an intense molecular revision, where more than 30 new species of this genus have been described. *M. anisopliae*, and some other members of the genus such as *M. robertsii* and *M. brunneum*, have been described as strongly associated with plants roots, and at the same time with the ability to infect insect pests. Some strains are associated with the rhizosphere and can even colonise the plant as endophytes. One of the main constrains in the use of this type of fungi for the control of soil dwelling insects or pathogens in the agriculture are the contact to the target pest, and the survival of the biocontrol agent in the environment. The soil offers an appropriate environment for fungi, where conditions of moisture and temperature are suitable. This PhD study contributes to the goal of developing improved biological control agents from selected rhizosphere competent, entomopathogenic fungi, delivered efficiently through seed coating. Using molecular approach, isolates of several genera of entomopathogenic fungi were characterised and species assigned based on phylogenetic comparisons. Among the species described, this is the first report of the presence of recently described new species of *Metarhizium* spp. in New Zealand, such as *Metarhizium novozealandicum*, *M. robertsii*, *M. brunneum*, *M. guizhouense* and *M. frigidum*. Presence of specific genes associated with plant and insect colonisation abilities were also determined, to aid

selection of potential plant associated isolates. In addition, the selection of *Metarhizium* isolates with the potential to survive in the plant rhizosphere was evaluated for first time by spectrophotometric determination of fungal growth in root exudates. Conidia from the fungal isolates where then used to coat maize seed, to determine the effect of each fungal isolate –plant interaction on plant growth and resistance to insect and disease damage. In presence of the grass grub *Costelytra giveni* (Coleoptera: Scarabaeidae) and the maize disease fungus, *Fusarium graminearum* (Ascomycete), some entomopathogenic fungal treatments produced improved growth performance of the maize seedlings. It was also determined the fungal infection of the grass grubs and the decrease in the *Fusarium graminearum* rot root due to the seed coating with entomopathogenic fungi. Particularly, the *M. anisopliae* and *M. robertsii* isolates had significantly higher colonization of the rhizosphere than the other isolates. It was also demonstrated that colonisation of the rhizosphere by *M. anisopliae* produced changes in the levels of salicylic acid and jasmonic acid when compared to control plants. This is the first report where the effect of *M. anisopliae* on the content of the phytohormones was evaluated. The seed coating was further developed by replacing fungal conidia with resistant structures known as microsclerotia (MS). Production of these resistant structures in *M. guizhouense* and *M. novozealandicum* was evaluated for first time. Maize seeds coated with MS grew better than control plants when grown in presence of *F. graminearum*. The association between *Metarhizium* spp and maize roots, after seeds were coated with MS, was possible to determine using fluorescent and laser confocal microscopy. These observations on maize have not been previously published where soil conditions were appropriate for MS germination and hyphae developed along the maize roots which associated with the ecto- and endo-rhizosphere. These studies also confirmed the suitability of using conidia of isolates of *Metarhizium* transformed with the fluorescent proteins mcherry and GFP to coat maize seeds for the observation of plant colonisation. This is the first report of using a blastospore-based transformation system for *M. anisopliae*. Overall the results suggest that seed coating with microsclerotia of *Metarhizium* can be used as delivery system for the control of plant pathogens and insect pests and improve the opportunity for close association with plant roots after conidia germination and hyphal growth.

Keywords: Entomopathogenic fungi, endophyte, rhizosphere-competent fungi, *Zea mays*, *Metarhizium* spp., microsclerotia, seed coating, bioprotection, plant induced response, biological control agent delivery, fungal characterization, plant root-fungal interaction, biotic stress, *Fusarium graminearum*, *Costelytra giveni*

Acknowledgements

Through this acknowledgement, I would like to express my profound appreciation to the many individuals that have supported and encouraged me during the course of my PhD program. First, I am very grateful to my supervisors, Prof. Travis Glare, Prof. John Hampton, Dr Trevor Jackson, Dr Michael Rostas and Dr Per Wessman. It has been their shared skills and vast knowledge, combined with their guidance and enthusiasm that encouraged me to pursue every challenge we came across.

I am also very grateful to Dr Nora Altier and Dr Jose Terra for the encouragement to start this great challenge and support during these years. I look forward to contributing to the expertise required in the next challenges that await for us at INIA - Uruguay. I am would like also to thank INIA and ANII from Uruguay for the financial support towards the realization of this PhD.

I would like to say a big thanks, to Brian Kwan, Fariba Nourozi, Leila Dadian and Norma Merrick for their enduring helpful disposition to sort out problems and obstacles, leaving all aside to look after my requirements and urgent requests. I am really thankful for your considerable help to make a difficult life science easier. I am also very grateful to Dave Saville for his expertise and support in the statistical analysis. I am also grateful to Dr Maria Moran-Diez for her assistance and guidance in the molecular aspects of this thesis, and Nic Cummings for all the assistance with the molecular identification of the fungal isolates. To my favourite consul, Maria Elena Duter, thank you for your assistance in the germination trials and for bringing me a piece of Uruguay during these long years away from home.

Other big thanks to "Las Lolas Locas": Jo Narciso (Mom) and Jenny Brookes. This couple made not only my day at the lab, but they were always kind and helpful help. My special thanks, to “Mom Lab”, for her invaluable assistance in the last months that allow me to run experiments while in the hard stage of writing up the thesis. I am also grateful to Dianne Fyfe who helped with all the process, requirements and formalities for the completion of the PhD during all these years.

I am very grateful to my office's mates, Jessica Dunbar and Jessica Yardley, for their cheering up and for all the happy moments we share combined with professionalism and assistance. Specials thanks to the staff and students in the Bioprotection Research Centre, to make a place to work an enjoyable home to be. Special thanks also to Sandy Wilson, for all her help but also for looking after us with treats and tricks to enrich our souls.

The professional side it would not be completed if we don't have a family and friends that support us in the hard moments that everyone come through. My sincere gratitude to the "Twisted Minds", my true family everywhere on the world. Finally, I would like to say thanks to my very own family at home, that encouraged me to finish my objectives and pursue the challenges.

List of acronyms

AMF	Arbuscular mycorrhizae
BPM	Between paper method
BSM	*Beauveria* selective medium
CC	Complete coating
CFU	Colony forming unit
ConA-AF633	Concanavalin-A conjugated with Alexa Fluor 633
CS	Control seeds
DCM	Methylene chloride
DE	Diatomaceous earth
DPI	Days post inoculation
EF	Entomopathogenic fungi
EF1-α	Elongation Factor 1 alpha
ET	Ethylene
GAs	Gibberellins
GFP	Green fluorescent protein
IR	Induced Resistance
ISR	Induced Systemic Resistance
ITS	Internal transcribed spacer
JA	Jasmonic acid
LBA	Luria-Bertani Agar
LBB	Luria-Bertani Broth

LSD	Least significant differences
MAD1	*Metarhizium* adhesin protein 1
MAD2	*Metarhizium* adhesin protein 2
MC	Methyl cellulose
MEB	Malt extract broth
MGT	*Metarhizium* clade: *M. majus* and *M. guizhouense*
MRT	*Metarhizium* raffinose transporter
MS	Microsclerotia
MSM	*Metarhizium* selective medium
OD	Optical density
PARB	*Metarhizium* clade: *M. pingshaense*, *M. anisopliae*, *M. robertsii* and *M. brunneum*
PRP	Pathogenesis-related proteins
PS	Pure seeds
RE	Roots exudates
SA	Salicylic acid
SAR	Systemic Acquired Resistance
SDB	Sabouraud Dextrose Agar
SGM	Sand germination method
THSM	*Trichoderma harzianum* selective medium
TMS	Trimethylsilyldiazomethane
W_a	Water activity
WGA-AF488	Wheat germ agglutinin conjugated to Alexa Fluor 488

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Chapter 1

Introduction

1.1 Entomopathogenic fungi

Entomopathogenic fungi (EF) are natural enemies of a broad range of insects and are found in different ecosystems around the world (Roberts & St Leger, 2004; Vega *et al*., 2009; Moonjely, Barelli & Bidochka, 2016). Species belonging to this group of fungi are identified based on their ability to cause diseases in insects and to produce fungal growth on insect cadavers (Vega *et al*., 2009; Moonjely, Barelli & Bidochka, 2016). EF are distributed in approximately 90 genera and are known to infect over 1000 insect species in terrestrial and aquatic habitats (Roberts & Huber 1981; Moonjely, Barelli & Bidochka, 2016). The most common genera of EF include *Aspergillus*, *Beauveria*, *Culicinomyces*, *Hirsutella*, *Lecanicillium*, *Isaria*, *Metarhizium*, *Entomophthora* and *Tolypocladium* (Inglis *et al*., 2001). Most EF consist of collections of isolates that are specific to the insect taxon on which they were found or to closely related species. However, there is a wide range of insect specificity in EF, with some species being obligate pathogens of a single or very few taxonomically related insect species (i.e. *Entomophthora*), through to some species which can infect a wide range of hosts such as *Metarhizium* spp. and *Beauveria* spp. (Moonjely, Barelli & Bidochka, 2016).

The process of fungal insect colonization has been documented particularly well for *Metarhizium* spp. and *Beauveria bassiana* (Ortiz-Urquiza & Keyhani, 2013; St. Leger, Wang & Fang, 2011). The first stage is the adhesion of hydrophobic fungal spores to the insect cuticle. Subsequently, a series of process involving spore germination, extension of the germination tube into hypha and the formation, in some cases, of specific structures that assist cuticle penetration known as appressoria. These structures, in conjunction with hydrolytic enzymes which help to degrade the insect cuticle, assist the penetration into insect cavities (Moonjely, Barelli & Bidochka, 2016). Once inside the body, the fungus shifts from hyphae growth to the formation of hyphal bodies, protoplasts and blastospores that disperses with the haemolymph and invade the haemocoel of the insect body. During this process, the fungus has several mechanisms to avoid the natural insect immune system and sometimes produce mycotoxins that weaken the insect (Ortiz-Urquiza & Keyhani, 2013; Moonjely, Barelli & Bidochka, 2016). Finally, the fungus produces hyphae invading the insect´s cadaver and protrudes to the external environment through soft tissues of the insect articulations. Once the fungus has broken through to the insect surface, it produces infective conidia again (Ortiz-Urquiza & Keyhani, 2013; Moonjely, Barelli & Bidochka, 2016). Spore germination on the host (during infection), and hyphal growth along with sporulation on the cadaver, occur mostly at night when the relative humidity exceeds 90% and

temperatures are moderate (e.g. 18-20°C). Solar radiation is lethal to these processes (Butt, Jackson & Magan, 2001).

The capacity of EF to parasite insects led to work which first began in the 1800s to exploit these fungi as biological control agents for insect pests of crops (Vega *et al*., 2009). In particular, the anamorphic EF *Beauveria bassiana* (Balsamo) Vuillemin and *Metarhizium anisopliae* (Metchnikoff) Sorokin from the order Hypocreales (Ascomycota), have been intensively studied for development as biopesticides since these species have the ability to infect a range of insect pests (Bruck, 2005; Meyling & Eilenberg, 2007; Ownley *et al*., 2010).

1.2 Entomopathogenic fungi as endophytes

EF have traditionally been associated only with insects, and little attention has been paid to other possible ecological roles (Bruck, 2010; Wyrebek *et al*., 2011). Nowadays, it is known that many EF play additional roles in nature as plant endophytes, antagonists of plant pathogens, beneficial rhizosphere-associates and possibly even plant growth promoters (Goettel *et al*., 2008; Hu & St. Leger, 2002; Meyling & Eilenberg, 2007; Ownley *et al*., 2010; Sasan & Bidochka, 2012; Vega *et al*., 2009). In particular, the endophytic ability of some strains of *Metarhizium* and *Beauveria* show potential for use in conservation biological control (Meyling & Eilenberg, 2007), where microbial pathogens are used without an inundative approach.

Endophytic fungi are biotrophs which live asymptomatically within host-plant tissues without harming the host (Raman *et al*., 2012). Fossil records indicate that this intimate association was occurring more than 400 million years ago (Gao *et al*., 2011). Fungal endophytes gain by inhabiting the vascular plant tissues which offer not only nutrition, but also protection from predation and probably less competition for resources with other organisms (Raman *et al*., 2012). In certain situations, the presence of the endophyte can modify the physiological properties of a host plant, improving its competitiveness against stressful abiotic conditions and also promoting plant growth (Faeth, 2002). In addition, endophytic fungi are unique among plant microbial symbionts because they are producers of a diversity of secondary compounds that may benefit the plant through direct defences against phytophagous insects and diseases (Faeth, 2002; Raman *et al*., 2012).

In the last 30 years, most exploitation efforts related to fungal endophytes have been concentrated on *Epichlöe* spp. of grass endophytes. Recent evidence suggests that there is a close phylogenetic relationship between *Epichlöe* spp. and some EF. Hypocreales contains the largest number of fungal entomopathogens including two of the most widely studied, *B. bassiana* and *M. anisopliae* (Bruck, 2010). The closest relatives of grass symbionts, *Claviceps* and *Epichlöe* are in the family Clavicipitaceae

(Ascomycota, Hypocreales), which also includes the genus *Hypocrella*, pathogens of scale insects and white flies, and *Metarhizium* (Spatafora *et al*., 2007; Wyrebek *et al*., 2011).

EF have been recovered as endophytes either from natural habitats or from inoculated plants (Benhamou & Brodeur, 2000; Vega *et al*., 2008). For example, *B. bassiana* grows endophytically within the tissues of *Zea mays* L. (maize) and is believed to enter through the plant cuticle after germination of conidia and hyphal growth across the leaf surface (Bing & Lewis, 1991; Wagner & Lewis, 2000; Bruck, 2010). Also, it was demonstrated that endophytic isolates of *B. bassiana* effectively controlled European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambiadea) (Lewis *et al*., 2002) while being non-pathogenic to *Z. mays* (Bing & Lewis, 1991; Vidal, 2015). Natural occurrence of *B. bassiana* as an endophyte has been recorded from cacao (*Theobroma cacao*), poppy (*Papaver somniferum*), coffee (*Coffea* spp.), tomato (*Lycopersicon esculentum*), *Echinacea purpurea*, cotton (*Gossypium* spp.), snap bean (*Phaseolus vulgaris* L.), soy (*Glycines max* L.) switchgrass (*Panicum virgatum* L.) and pines (*Pinus* spp.) (Meyling & Eilenberg, 2007; Ownley *et al*., 2010; Reay *et al*., 2010).

The colonization of cotton leaves also occurred with *Lecanicillium lecanii* (Zimmermann) Zare and Gams (Hypocreales: Clavicipitaceae) where the presence of the fungus reduced reproduction of the aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae). These effects were considered to be mediated by fungal metabolic products (Raman *et al*., 2012) since *L. lecanii* is capable of producing several secondary metabolites with antibacterial, antifungal, cytotoxic and insecticidal properties (Ownley *et al*., 2010). Furthermore, *B. bassiana* can provide plants with protection from soil dwelling soil phytopathogens like *Rhizoctonia solani* and *Pythium myriotylum* (Griffin, 2007; Ownley *et al*., 2008; Vega *et al*., 2009).

Studies on plants endophytically colonised by *B. bassiana* and *Lecanicillium* spp. revealed the induction of genes associated with plant defence, stress response, energetic metabolism and photosynthesis (Ownley *et al*., 2010; Raman *et al*., 2012). Endophytic colonization by these EF is capable of enhancing plant growth (Tefera & Vidal, 2009). Coincidentally, these mechanisms of growth promotion and induction of defence associated genes against pathogens are similar to those observed in plants colonised with growth promoting rhizobacteria and mycorrhizae (Harman *et al*., 2004; Vinale *et al*., 2008). However, these proposed mechanisms of effect have not yet been demonstrated.

Contrary to *Beauveria* and *Lecanicillium* which can be endophytic mainly in the aerial parts of the plants, *Metarhizium* spp. are found in plant roots. Members of this genus were found to be pathogenic to >600 insect species, and the use of *Metarhizium* as a biocontrol agent has mostly focused on its ability to kill insects directly, with little consideration of plant associations (Veen, 1968; Zimmermann, 2007; Behle *et al*., 2013; Wyrebek *et al*., 2011). Recent studies suggest another ecological role of these

fungi, showing that it is not only endophytic but also intimately associated below ground with the rhizosphere (Hu & St. Leger, 2002; Meyling & Eilenberg, 2007; St Leger, 2008; Wyrebek *et al*., 2011).

The rhizosphere is the region of soil in which the release of root exudates influences the soil microbiota (Bruck, 2010). This region of the roots has been identified as a potential reservoir for *Metarhizium*, with propagules persisting in the inner rhizosphere while decreasing over time in bulk soil (Hu & St. Leger, 2002; Meyling & Eilenberg, 2007). Moreover, it has been demonstrated that both endophytic capability and insect pathogenicity of *Metarhizium* are coupled to provide an active method of nitrogen transfer from infected insects to plant hosts via fungal mycelia, probably in exchange for carbon (Behie *et al*., 2012). Additionally, *M. robertsii* was suggested as a plant-growth-promoting fungus since not only does it initiate root hair development earlier, but also roots were significantly longer in the presence of the fungus. Stimulation of lateral root development is considered an early phase of interaction in non-phytopathogenic root-colonizing fungi (Sasan & Bidochka, 2012). Accordingly, rhizosphere competent microorganisms are those that cause enhanced plant growth in response to developing roots (Bruck, 2010).

The finding that *Metarhizium* is both an insect parasite and a plant endophyte makes sense when considering the recurrent evolutionary shifts from animal to plant hosts in the Clavicipitaceae family (Spatafora *et al*., 2007). While some clavicipitaceous species strictly associate with animals or plants, others are "lost in translation" and colonize hosts from both kingdoms, retaining both evolved niches (Selosse *et al*., 2018). This bifunctional lifestyle of *Metarhizium*, as insect pathogen or colonizer of plant rhizosphere, is further exemplified in the differential expressions of genes (Behie *et al*., 2012; Sasan & Bidochka, 2012; Wang *et al*., 2005). *M. anisopliae* produces two different proteins, MAD1 and MAD2, used for adhesion to insect and plant surfaces, respectively (Wang & St Leger, 2007; Wyrebek & Bidochka, 2013; Liao *et al*., 2014). These proteins are differentially produced in response to insect hemolymph or in the presence of plant root exudates (Bruck, 2010). In addition, a plant carbon transporter, *Metarhizium* raffinose transporter (MRT), has been reported for *Metarhizium* and is required for successful root colonization (Behie *et al*., 2012; Fang & St Leger, 2010).

1.3 Endophyte fungal transmission

Endophytes are possibly found in all plants and form internal localized infections in foliage, roots, stems, fruits and bark. Most of them are horizontally transmitted via spores, while a much smaller portion form systemics infections in above-ground tissues and are vertically transmitted via hyphae growing into seeds (Faeth, 2002). However, vertical transmission is not always effective since hyphae fail to grow into some seed heads or tillers, or hyphae can lose viability in seeds and plants (Faeth, 2002). In order to keep symbionts at high frequencies among the plant population the endophyte should be horizontally transmitted, either by conidia or hyphal transmission, to neighbouring plants

(Faeth, 2002). For this reason, it is assumed that possibly all plants harbour horizontally transmitted endophytic species with endophyte-plant interactions varying from antagonistic to mutualistic (Saikkonen *et al*., 2010).

Endophytic fungi are often regarded as plant-defending mutualists and the presence of *B. bassiana* in internal plant tissue has been discussed as an adaptive protection against herbivorous insects. In ecological terms the endophytic association benefits both fungus and plant and this supports the bodyguard hypothesis (Elliot *et al*., 2000; Ownley *et al*., 2010; Posada & Vega, 2005; White *et al*., 2002). However, in the field of plant pathology, the "disease triangle" is a central concept based on the principle that disease is the result of an interaction between a host, a pathogen, and the environment (Bruck, 2010). In the case of beneficial endophytic fungi, the "disease triangle" would involve also an antagonist microorganism. These antagonistic effects in the intimate association of *Metarhizium* with roots could be the same as those observed in mycorrhizal fungi against plant pathogenic fungi. According to Faeth (2002) it can be consequence of: i) reduced sugar exudation caused by mycorrhizal colonisation and consequent non-detection by pathogens, ii) competition and physical exclusion of the pathogen from root surface, iii) release of secondary metabolites with antimicrobial effect, iv) induction of the plant systemic response or v) an improvement of plant performance in general and, additionally in the case of *Metarhizium*, iv) potential parasitism of insects.

The transmission mechanisms of EF into plants is far from clear. However, taking into consideration the above mentioned it is possibly a combination of both vertical and horizontal transmission.

1.4 The plant microbiome

Plants harbour a wide diversity of microorganisms both inside and outside their tissues, in the edosphere and ectosphere, respectively (Vandenkoornhuyse *et al*., 2015). These microorganisms, which mostly belong to Bacteria and Fungi, are involved in major functions such as plant nutrition and plant resistance to biotic and abiotic stresses. Hence, the microbiota impact plant growth and survival, two key components of fitness. Plant fitness is therefore a consequence of the plant *per se* and its microbiota, which collectively form a holobiont (Partida-Martínez & Heil, 2011; Vandenkoornhuyse *et al*., 2015).

Microbiome complexity and the rules of microbiotic community assemblage are not yet fully understood. It has been suggested that the plant can modulate its microbiota to dynamically adjust to its environment (Vandenkoornhuyse *et al*., 2015). In this case, it has been hypothesized that plants have the ability to select their symbionts based on nutrient allocation (Vandenkoornhuyse *et al*., 2015). For instance, in a rhizobium-legume symbiosis, plants have been shown to detect and penalize *Rhizobia* defective in N_2 fixation by reducing resource allocation, while in arbuscular mycorrhizae (AM)

symbiosis is stabilized by both the control of carbon embargo against a deficient AM and by the transfer of phosphorous to plants conditioned by a plant carbon allocation (Kiers *et al*., 2003; Kiers *et al*., 2011; Vandenkoornhuyse *et al*., 2015). The foundations for the establishment of entomopathogenic fungus – plant associations might be ruled by the same postulates mentioned above, but it is still unclear where the demands from plants to EF are set. Because of the dual lifestyle of the fungal entomopathogens, as plant endophytes or pathogens of insects, the demands from plants for a successful symbiotic establishment could rely exclusively on nutrient exchange or only on protection against pest or diseases, or a combination of both. Additionally, the systemic induction of pathogen resistance in leaves can impair the capacity of plant roots to establish their interaction with rhizobia or AMF, while leaf infestation with whitefly (*Bemisia tabaci*) affected the composition of the bacterial rhizosphere with likely negative effects on PGPB. In summary, the net outcome of most, if not all, plant-endophyte interactions are highly conditional and depends on the detailed biotic and abiotic environment as much as on the specific genotypes of both endophyte and plant host (Partida-Martínez & Heil, 2011; Fernandez-Conradi *et al*., 2018).

In order to explain the variable effects observed in plant - fungal interactions two non-exclusive frameworks have been proposed to understand the conditional outcomes for the infection of plants with fungal endophytes: the virulence-resistance balance (Schulz & Boyle, 2005) and cost-benefit relations (Faeth, 2002). The first explains that at the molecular level, all endophytes require at least some virulence to infect their host while the host requires some resistance traits to control their density. The partial suppression of plant defences represents a crucial step not only during the infection of plants by fungal endophytes but also in the nodulation or mycorrhization of plants roots. Maintaining the balance between host resistance and endophyte virulence represents a key factor that decides whether the endophyte can infect the plant at all without causing visible disease symptoms (Schulz & Boyle, 2005; Partida-Martínez & Heil, 2011). The second framework implies that endophytes compete with the primary metabolism of plants for certain nutrients, and their presence can incur a considerable cost in terms of reduced plant growth rates under limiting soil nutrient condition. However, this cost can be counterbalanced. For example, AM provides the plant with otherwise inaccessible nutrients and endophytes can enhance the resistance of their host against herbivores (Faeth, 2002; Partida-Martínez & Heil, 2011). In conclusion, the costs for the plant to harbour fungal endophytes is high in both cases: rich nutrients soils and in the absence of biotic or abiotic stressor factors. Additionally, these costs will be proportional to the extent of endophytic density in the plant tissues (Partida-Martínez & Heil, 2011).

Plant-associated fungi comprise a wide range of lifestyles, such as biotrophy, necrotrophy and hemibiotrophy. Biotrophic fungi require actively metabolizing plant tissues and avoid extensive

damage while keeping their host alive. They include pathogenic as well as mutualistic forms. Necrotrophic fungi, which kill host cells in advance of their own growth and obtain nutrients from the dead cells, comprise only pathogenic forms. An intermediate category is represented by hemibiotrophic fungi (Zuccaro *et al*., 2011). These require living host cells during part of their life cycles but switch to necrotrophy at later colonization stages with detrimental effects on host survival and fitness and therefore have been classified as pathogens. For fungal entomopathogens a likely scenario appears to be that the fungus uses the plant as a vector to enhance its transmission rate among different insect hosts (Vega *et al*., 2009; Ownley *et al*., 2010; Partida-Martínez & Heil, 2011). Fungi as endophytes need nutrients to survive and keep a basal metabolic activity functioning, which has a cost for both the plant and the fungus. At first sight, the interaction might appear to be symptomless. However, the additive ecological functions supported by the plant microbiome are acknowledged to be a major trait that extend the plant's ability to adapt to many kinds of environmental conditions and changes, which is of primary significance in view of the sessile lifestyle of plants (Bulgarelli *et al*., 2013; Vandenkoornhuyse *et al*., 2015). In this context, it is hard to decipher what the overall result of the presence of the EF is in combination with the other endophytes that could be also present in the plant.

1.5 Are plant endophytes an extension of the innate vegetal defence?

In plants, *Induced Resistance* (IR) is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant´s innate defences are potentiated against subsequent biotic challenges (Vallad & Goodman, 2004). The two most clearly defined forms of induced resistance are *Systemic Acquired Resistance* (SAR) and *Induced Systemic Resistance* (ISR), both are characterized by the nature of the elicitor and the regulatory pathways involved. SAR has been studied extensively in the case of dicotyledonous plants where it was determined that micro-lesions induced by necrotizing pathogens trigger a local accumulation of salicylic acid (SA), with mitogen-activated protein kinases, H_2O_2 and other signals being involved. Local defence responses are accompanied by an enhanced expression of defence-related genes: pathogenesis-related genes (PR genes) which respond rapidly to challenges by pathogens and are under the control of the protein non-expressor of pathogenesis-related genes 1 (NPR1) (Waller *et al*., 2007). ISR, which is triggered by non-pathogenic rhizobacteria, depends on both, NPR1 and the jasmonic acid/ethylene pathway, but not on SA (Waller *et al*., 2007).

In interactions between non-pathogenic rhizosphere microbes and plants, the phytohormones SA, jasmonic acid (JA) and ethylene (ET) regulate symbiosis and mediate IR elicited by several groups of non-pathogenic rhizosphere microbes (Zamioudis & Pieterse, 2012; Pieterse *et al*., 2014). The role of these plant hormones is to function as signal molecules regulating plant growth, development and responses to biotic and abiotic stimuli.

The classic form of SAR can be triggered by exposing plants to biotrophic pathogens, non-pathogenic microbes, and insect herbivores with a piercing-sucking feeding mode (like aphids and whiteflies). It can also be elicited artificially with chemicals (Vallad & Goodman, 2004; Pangesti *et al*., 2013). Depending on the plant and the elicitor, a period of time is required for the establishment of SAR which corresponds to the time required for the coordinated accumulation of pathogenesis-related proteins (PRP) and SA throughout the plant (Vallad & Goodman, 2004). To activate a defence response, SA signalling is transduced via the regulatory protein NPR1, which functions as transcriptional co-activator of SA-responsive genes such as PRP and it also functions as an important node modulating SA- and JA-signalling crosstalk (Pangesti *et al*., 2013). Simultaneously, SA-dependent signalling is crucial in interactions of plant roots with non-pathogenic microbes. It has been suggested that in the initial stage of symbiosis, non-pathogenic microbes are sensitive to SA-regulated defence responses (Zamioudis & Pieterse, 2012). SA-signalling has been reported to negatively affect rhizobial, mycorrhizal, and rhizobacterial colonization (van Spronsen *et al*., 2003; Doornbos *et al*., 2011; Pangesti *et al*., 2013).

On the other hand, ISR is potentiated by plant growth-promoting rhizobacteria (PGPR) of which the best characterized are strains within several species of *Pseudomonas* that cause no visible damage to the plant´s root system. Unlike SAR, ISR does not involve the accumulation of PRP or SA, but instead relies on pathways regulated by JA and ET (Vallad & Goodman, 2004). The phytohormone JA is a lipid-derived compound playing a prominent role in various aspects of plant development like seed germination, root growth, and flower development and in plant defence against various aggressors (Wasternack, 2007; Pieterse *et al*., 2012). Induction of JA-signalling mainly occurs after attack by necrotrophic pathogens, tissue-chewing insects such as caterpillars, and cell-content feeding insects such as thrips (Pangesti *et al*., 2013). Interestingly, JA-signalling has also been described as the main pathway in ISR against aboveground herbivores and is stimulated by root-associated microbes (Van Oosten *et al*., 2008; Pineda *et al*., 2010; Jung *et al*., 2012).

During the past few years, evidence has accumulated that plants have a sophisticated mechanism to actively recruit non-pathogenic root associated microbes following attack by pathogens or insects. For instance, foliar infection by the pathogen *Pseudomonas syringae* triggers the secretion of malic acid by *A. thaliana* roots that attract the beneficial rhizobacterium *B. subtilis* (Rudrappa *et al*., 2008; Lakshmanan *et al*., 2012). Therefore, by regulating its root secretion in the form of carbon-rich exudates, plants are able to shift rhizosphere microbiota composition affecting microbial diversity, density and activity (Pangesti *et al*., 2013). The main conclusion emerging from this is that, in addition to the plant´s innate immunity systems, the plant's microbiota can be seen as an external component of plant defence (Vandenkoornhuyse *et al*., 2015).

Alternatively, like some plant pathogens, certain root endophytes have been successful in evading or manipulating the plant hormone signalling in different ways. For instance, by synthesizing auxins and auxin analogs with gibberellins (GAs) which probably attenuate SA signalling, or using effectors which modify the hormonal signalling pathways as is the case for mycorrhizal fungi. Some others endophytes use transient accumulation of JA at an early stage of mycorrhiza formation; and root-nodule formation supposedly to 'bypass' the SA-triggered response (Pieterse *et al*., 2012; Vandenkoornhuyse *et al*., 2015). In the case of fungal endophytic entomopathogens there are still no clear clues about the interaction of these fungi with the natural plant immune defence. The chances are high that the interaction between EF and plants should follow some mechanism similar to other endophytic fungal interactions like with mycorrhizae or biotrophic fungi like *Piriformospora indica*.

1.6 Seed coating

Biocontrol of insect pests with EF has been promoted as a viable alternative to chemical pesticides. To date, more than 170 entomopathogenic fungal strains have been commercialized as biocontrol agents and many of them are based on *Beauveria* spp. and *Metarhizium* spp. (de Faria & Wraight, 2007; Krell *et al*., 2017). Apart from their pathogenic lifestyle, in which they inhabit the soil as insect pathogens, the recent reports that fungal entomopathogens are capable to grow endophytically within plant tissues has encouraged researchers to consider their possible use as endophytes in biological control programs (Ownley *et al*., 2010; Vega *et al*., 2008; Vidal & Jaber, 2015; Krell *et al*., 2017). Furthermore, a key advantage of microbial control agents over chemical pesticides is their potential to replicate and persist in the environment providing a long-term control (St. Leger, 2008). On the other hand, biocontrol agent survival under environmental challenging conditions, such as high temperatures, UV-radiation and low humidity, have been one of the main factors constraining their use and effectiveness (Inglis *et al*., 2001). A new avenue could therefore be to exploit not only the capacity of EF to infect insects but also their ability to live endophytically.

If an endophyte-associated defence against natural enemies of the plant-host is to be useful, colonisation needs to occur at highly susceptibility stages of the plant such as during seed germination and in seedlings. In these stages, predation, pathogen attack and herbivory put pressure on the plant (Faeth, 2002). For this reason, seed treatments could be an effective delivery method for EF in order to optimize the control of soil dwelling phytopathogens or insects (St Leger, 2008). Seeds have already proved to be an important delivery vehicle for a variety of beneficial microbes for plant growth promotion or antagonist of plant pathogens (Barea *et al*., 2005; St. Leger, 2008). Additionally, since seeds are sown, the soil environment will protect the biological control agent from the most adverse abiotic conditions to which it would be exposed when applied as foliar formulation. For instance, maize

seed treated with *M. anisopliae* conidia resulted in significant increases in stand density and stock and foliage area, while at the same time wireworms infected with *M. anisopliae* were recovered from treated plots, suggesting that the increase in yield may have been due to wireworm control (Kabaluk & Ericsson, 2007). Moreover, successful endophytic colonisation of cucumber roots was obtained with blastospore root-application of *Lecanicillium muscarium* also resulted in powdery mildew resistance response in leaves (Hirano *et al*., 2008). Additionally, a stable formulation that allows microorganisms to be coated to seeds by the supplier and sold to farmer in a "ready to drill" state, would likely enhance uptake of seed-coated biologicals (Swaminathan *et al*., 2015).

On the other hand, plant-rhizosphere specificity must be taken into account in order to optimize delivery against a target insect and ensure sustainability in the plant rhizosphere (St Leger, 2008; Wyrebek *et al*., 2011). In a review article, Bruck (2010) suggested that rhizosphere competence in insect-pathogenic fungi was dependent upon the host plant. The genus *Metarhizium* was found to be strongly associated with habitat type, with isolates from agricultural/open field habitats belonging predominantly to *M. robertsii*, while *M. brunneum* and *M. guizhouense* isolates were found in forested soils. Since no differences were found between these groups regarding virulence or host specificity, there may be some selective ecological ability to survive or inhabit a certain plant rhizosphere for these species. For instance, *in vitro* conidial germination experiments demonstrated that switchgrass root exudates was a favourable medium for germination of *M. robertsii* conidia compared with *M. brunneum* or *M. guizhouense* (Wyrebek *et al*., 2011). In other studies, the use of a rhizosphere-competent isolate of *Metarhizium* provided nearly 80% control against a target insect within two weeks of exposure to inoculated roots (Bruck, 2005).

The use of *Metarhizium* strains with confirmed rhizosphere competence in a seed coating should provide a way to assure the establishment of the biocontrol agent in the roots, allowing an improved opportunity for protection against pathogens.

1.7 Potential formulation additive to improve seed coating and biocontrol properties: microsclerotia

The development of new formulations that contribute to the viability and quality of microorganisms has been identified as one of the limiting factors in the adoption of biocontrol agents as an alternative to the use of conventional pesticides or plant growth promotors (Glare *et al*., 2012; Swaminathan *et al*., 2015).

In general, *Metarhizium*-based bioinsecticides have been formulated as aerial conidia for foliar application of a broad range of crops. After application, *Metarhizium* has also been found as endophytes in oil-seed rape (Batta, 2013), potato (Ríos-Moreno *et al*., 2016), broad bean (Jaber &

Enkerli, 2017), alfalfa, tomato, and melon (Resquín-Romero *et al*., 2016; Krell *et al*., 2017). Aerial conidia are usually mass-produced under expensive and labour-intensive conditions by solid-state fermentations. In contrast, submerged culture techniques are less expensive and easily up-scalable, resulting in high cell densities of fungal propagules (Krell *et al*., 2017).

In liquid fermentation, *Metarhizium* spp. are capable of growing in a variety of different morphological forms, such as blastospores and mycelial pellets, submerged conidia, microsclerotia and finely dispersed mycelium (Jackson & Jaronski, 2009). Blastospores are usually less resistant to environmental stresses than aerial conidia (Jenkins & Goettel, 1997; Leland *et al*., 2005) and are often produced in low quantities (Fargues *et al*., 2002; Jackson & Jaronski, 2009) making production processes and applications as biocontrol agents inefficient. In contrast, submerged conidia are less sensitive during drying (Leland *et al*., 2005), but only a few *Metarhizium* strains have been reported to produce these conidia (Jenkins & Prior, 1993; Jenkins & Goettel, 1997; Kassa *et al*., 2004). Similarly, formation of microsclerotia resistant to desiccation has been described for a few *Metarhizium* spp. (Jackson & Jaronski, 2009a; Mascarin *et al*., 2014).

Recently, Jackson and Jaronski (2009a) reported the capacity of *M. brunneum* to produce melanised compact hyphal aggregates of 200 – 600 µm in diameter known as microsclerotia (MS). These structures are resistant to desiccation and capable of producing conidia under favourable conditions. Also, others EF like *L. lecanii* and *B. bassiana* are able to produce MS (Wang *et al*., 2013). Additional benefits of MS are that in just 4 days up to 20.5 g/L biomass containing 3.4 x 10^7 microsclerotia/L can be obtained (Behle *et al*., 2013). These resistant structures once formulated as granules and applied to moist potting mix produce infective conidia within 2 weeks (1.24 x 10^{10} conidia/g microsclerotia granules) and remain viable for up to 8 weeks after application (1.84 x10^{10} CFU/g microsclerotia). Another advantage of MS for industrial production as biopesticides is that they can be mass produced using stirred-tank bioreactors (100 L) without reducing product quality or stability (Jackson & Jaronski, 2012).

Microsclerotia from EF formulated in diatomaceous earth are particularly suited for soil applications since propagules are protected from biotic and abiotic stress factors, antagonist microorganisms or desiccation and have been shown to proliferate in the soil, producing large quantities of conidia (Vega *et al*., 2009; Jackson & Jaronski, 2012). Currently, fungal biological pesticides generally contain conidia, which were subjected to a series of steps that include harvest, drying, formulation, package, shipping and storage, which can adversely affect the biological activity of the conidia (Behle *et al*., 2013). For the above mentioned, the incorporation of MS in the seed coating would avoid some of these processes which, once they are in the appropriate environment, will provide infective conidia with optimal fitness for pest control (Jackson & Jaronksi, 2009; Jackson & Jaronksi, 2012; Behle *et al*., 2013).

In addition, the nature of microsclerotia as resting structures will assist the viability of fungal entomopathogens during long storage after coating onto the seed surface.

1.8 Thesis aim

This study contributes to the goal of developing improved biological controls from selected insect pathogenic, rhizosphere competent, EF delivered efficiently through seed coating. This PhD study uses molecular and phenotypic characterisation, microbial formulation studies, laboratory and glasshouse experiments, and molecular transformations to establish a base of knowledge for development of improved biological controls. This work was divided into four objectives;

Objective 1. Characterization of EF, based on molecular and phenotypic studies, for the selection of fungal isolates with rhizosphere competence and biocontrol activity against *Costelytra giveni*. This objective was addressed in chapter 2 through the following activities: First, isolates were identified to species level using molecular techniques, which helped to determine the natural ability of certain isolates to behave exclusively as entomopathogens and/or to associate with plants. Secondly, assessing the presence of genes related with the fungal ability to adhere to insect or plant surfaces. Thirdly, determining fungal rhizosphere-compatibility by growing EF in roots exudates. Finally, ascertaining the natural entomopathogenicity of the isolates against larvae of *C. giveni*. The genetic and phenotypic factors assessed were analysed to identify potential indicators for selection of biocontrol fungi.

Objective 2. Determination of the effect of selected EF coated onto maize seeds on plant development in the presence of *C. giveni* and *Fusarium graminearum*. The activities for this objective are described in chapter 3. First, the effects on germination and plant growth of entomopathogenic fungal conidia coated onto maize seeds, were determined. Second, maize plants coated with selected entomopathogenic fungal isolates were grown in the presence of *C. zealandica* and *F. graminearum* and plant performance was evaluated. Third, the ability of the fungal isolates coated onto maize seeds to form an association with roots or to become endophytic was determined. Finally, changes in concentrations of salicylic acid and jasmonic acid in maize plants growing from fungal coated seeds were determined.

Objective 3. Production of fungal microsclerotia and their use in delivery of fungal biocontrol. The activities related with this objective are reported in chapter 4. First, the ability of the selected entomopathogenic fungal isolates to produce microsclerotia was determined. Second, microsclerotia viability was evaluated and coated to maize seeds. Third, plant growth performance after microsclerotia seed coating was evaluated in the presence of *F. graminearum*. Fourth, the ability of

the entomopathogenic fungal isolates, coated to seeds as microsclerotia, to associate with roots was determined through fluorescent and laser confocal microscopy.

Objective 4. Determination of entomopathogenic fungal isolates coated to maize seeds to become endophytic. Activities for this objective are reported in chapter 5. First the transformation of entomopathogenic fungal isolates to express fluorescent proteins, GFP and mcherry. Second, maize seed coating with conidia from the transformants and observation with laser confocal microscopy.

The final outcome of these studies is improved knowledge in the selection of EF with rhizosphere compatibility and biocontrol activity against *C. giveni* and *F. graminearum*. Additionally, a goal was to improve the delivery of EF in seeds coating using microsclerotia to increase the survival of the biocontrol fungal agent during the stages of storage, delivery, commercialization and finally, application. Finally, the study determined the level of association with the plant, as root colonizers or endophytes, their effect on plant growth and the possible role in the induced response in maize plants.

Chapter 2

Characterization of the entomopathogenic fungi

2.1 Introduction

The control of insect pests with entomopathogenic fungi requires a significant understanding of the interactions between the target insect, the fungal entomopathogen, and the environment (Jackson, Dunlap & Jaronski, 2009). Traditionally, the selection of fungal entomopathogens for development as microbial agents has involved isolation of the fungus from soil or infected insects, followed by bioassays on the target pest and then studies of mass production. An additional step is the characterization of the ecological constraints of the candidate isolates relative to the environment in which insect pests are targeted (Bruck, 2010).

It has recently been discovered that many entomopathogenic fungi (from the genera *Metarhizium*, *Beauveria*, *Lecanicillium*) also have close associations with plant hosts (Vega *et al*., 2008; Ownley *et al*., 2010) but there has been little research to determine the strength of symbiosis between specific fungal species and plant populations. Improved understanding of the traits of interaction between a fungus and plant host will assist the selection of new fungal biocontrol agents with improved performance as biopesticides. Rhizospheric competence or endophytic ability is a desirable attribute for a biopesticidal microbe as it will enable the agent to persist in the environment long after application which would help to maintain crop protection throughout the year and minimize application costs (Bruck, 2010; Moonjely, Barelli & Bidochka; 2016).

This study is focussed on examining these factors for entomopathogenic fungi with pathogenic activity against *Costelytra giveni* and determining their rhizosphere competence and endophytic ability with a view to improving their impact as biocontrol agents in soil. In this study molecular and phenotypic approaches for identification and characterisation of the fungal isolates have been used and elucidation of their interactions with the host insects and plants undertaken.

2.1.1 Identification of entomopathogenic fungi

Traditional identification of fungi has been based on morphological and phenotyping characteristics of fungal cultures (Humber, 2012). The classification of *Metarhizium* based on morphological characters was reviewed by Tulloch (1976) who only accepted *Metarhizium flavoviride* and *Metarhizium anisopliae* as species. Originally, morphological characteristics were based on the shape of the conidia, conidiogenous cells, presence or absence of a subhymenial zone, and whether conidia adhere laterally to form prismatic columns (Rombach *et al*., 1987). Later, the limitations of these morphological

characters in distinguishing between species of *Metarhizium* spp. were realized (Glare *et al*., 1996; Driver *et al*., 2000).

Early molecular studies on *Metarhizium* used sequences of the internal transcribed spacers (ITS) of rDNA to discriminate between *M. anisopliae*, *M. flavoviride* and *M. album* (Bidochka *et al*., 1993; Driver *et al*., 2000). From these works it was concluded that *M. anisopliae* was composed of four varieties and, most importantly, that the ITS sequences alone were clearly deficient to resolve lineages of *M. anisopliae* isolated from different localities and insect hosts (Driver *et al*., 2000).

Bischoff *et al*. (2006, 2009) conducted the first multi-locus phylogenetic analyses of *Metarhizium* with sequences of the Elongation Factor 1-α (EF1-α), RNA polymerase II largest subunit (RPB1), RNA polymerase II second largest subunit (RPB2) and β-tubulin. They considered that the genus *Metarhizium* was composed of at least 12 distinct species. It was soon noted that the intron sequences of the 5´portion of the EF1-α was most effective delimitating species boundaries in *Metarhizium* and so this gene displaced the ITS sequences as the principal tool for molecular identification (Wyrebek *et al*., 2011; Hernández-Dominguéz & Franco, 2017; Rehner & Kepler, 2017). This can be seen when the phylogeny of Driver *et al*. (2000) derived from ITS data is compared against the 5'-EF tree of Bischoff *et al*. (2009). Although the phylogenetic resolution of ITS sequences is still superior to other legacy genes, when compared to 5'-EF, its information ranks among the lowest, second only to 3'-EF (Bischoff *et al*., 2009; Kepler & Rehner, 2013).

The discrepancy between the 5' and 3' prime portions of EF are due to the presence of three introns in the 5' prime portion. Data from orthologous protein coding genes, are typically highly conserved due to stabilizing selection that preserves their essential functions (Kepler & Rehner, 2013). Such genes are generally less variable and informative at lower taxonomic levels. By contrast, non-coding regions, such as introns of protein coding genes and the ITSs of nrDNA, have shown utility at discrimination between closely related species (Kepler & Rehner, 2013). The variability in these introns, and the reliability of amplification, led Bischoff *et al*. (2009) to recommend its use as a marker for diagnosis of species in *the M. anisopliae* clade.

Recently, the genus *Metarhizium* has gone through a deep reclassification where new species have been described and species have been reallocated from other genera. For example, some species from *Metacordyceps*, *Nomuraea*, *Chamaeleomyces* and *Paecilomyces* have been transferred to *Metarhizium* (Kepler *et al*., 2014). Currently, the genus is comprised of 36 species, with the addition of the recently described *M. blattodea* (Montalva *et al*., 2016, Glare *et al*., 2018).

The most frequently isolated species of *Metarhizium* from soils and insects are among the so-called "PARB" clade which include: *Metarhizium pingshaense*, *M. anisopliae*, *Metarhizium robertsii* and *M.*

brunneum (Bischoff *et al*., 2009; Rehner & Kepler, 2017). Additionally, these species possess unique biogeographic patterns of dispersal or ecological adaptations. The *M. pingshaense* isolates are mainly from Australasia, *M. robertsii* predominating in North and South America and, *M. brunneum* in Europe, however both the latter species are found in Australasia. Finally, *M. anisopliae* has a worldwide distribution been found in all the continents (Rehner & Kepler, 2017). In addition to the PARB clade was defined the MGT clade to include species of *Metarhizium majus* and *M. guizhouense*. However, currently, none of these new species of *Metarhizium* have been described in New Zealand. Furthermore, most of the isolates provided for the present work were classified as *M. anisopliae* var. *anisopliae* (which is a defunct and confused variety) or still need to be identified to a species level (Table 1).

An essential first step to elucidating the use of different *Metarhizium* spp. isolates as entomopathogens, endophytes or soil-adapted fungi is to accurately define them at a species level (Rehner & Kepler, 2017). This identification in *Metarhizium* spp. is significant since some species are known to be plant species-specific or adapted to different agroecosystems (Meyling & Eilenberg, 2007; Vega *et al*., 2009; Bruck, 2010). Previous studies have shown that *M. robertsii* was associated with grass roots, *M. brunneum* was associated with shrubs and trees, and *Metarhizium guizhouense* was found only with tree roots (Wyrebek *et al*., 2011). Thus, different species and isolates of *Metarhizium* have different capabilities and maybe, ecological roles, which make the selection of isolates for a particular biocontrol purpose extremely important (Jackson, Dunlap & Jaronski, 2009). For the above-mentioned reasons, molecular identification using the EF1-α gene is necessary to determine the proper identification to a species level and characterize the isolates.

2.1.2 Entomopathogenic fungi molecular characterization

Variation in activity among *Metarhizium* species could be explained by differences in functional genomics approaches which have confirmed that this genus can up-regulate different genes in the presence of plants or insects, demonstrating that it has specific sets of genes for a bifunctional lifestyle. For instance, the genome of *M. robertsii* is larger than *M. acridum* and encodes more toxins and extracellular enzymes, which could explain the versatility of *M. robertsii* as an insect pathogen or a plant endophyte (St. Leger, Wang & Fang, 2011). In other studie on the ecological significance of *Metarhizium* in agronomic soils, it was found that *M. flavoviride* had lower virulence toward *Tenebrio molitor* larvae than *M. brunneum* or *M. robertsii* isolates, but was still pathogenic to above ground weevils. Additionally, *M. flavoviride* was not found associated to plants roots (Keyser *et al*., 2015). These results suggested that certain species of *Metarhizium* play a role in the regulation of at least some insect populations.

Metarhizium differently express two adhesin genes, *mad1* and *mad2*, used for the adhesion to insect cuticles or plant cell walls, respectively (Wang & St Leger, 2007; Sasan & Bidochka., 2012). The genes *mad2* and *mrt* seem to play an important role in the ability of *Metarhizium* to survive in the rhizosphere of maize plants. Mutants defective in the *mad2* or *mrt* genes were unable to promote plant growth and colonize roots or rhizospheric populations were reduced, respectively (Liao *et al*., 2014). On the other hand, there was no differences in rhizospheric compatibility between wild-type isolates and mutants defectives in the *mad1* gene. For these reasons, a molecular characterization of the isolates of the present work will include the determination of the presence in the isolates of the *mad*1, *mad*2 and *mrt* genes. The *mad*1 and *mad*2 genes would help to determine the ability of the isolates to adhere to surface of insects and plants, respectively. The *mrt* would support from a molecular perspective the potential of rhizosphere competence of the isolates since this gene encodes for a transmembrane protein associated with the assimilation of oligosaccharides from the roots to the fungus. However, root exudates have a complex composition and also a more practical approach will be needed determining isolates´ rhizosphere competence.

2.1.3 Rhizosphere competence determination

The rhizosphere can be distinguished in three different interacting regions: the outer rhizosphere, the rhizoplane and the roots. The outer rhizosphere contains the soil that loosely adhered to the roots and is the region where the root exudates influence the soil microbiota. The rhizoplane is the portion of the rhizosphere directly in contact with the root surface resulting in the soil tightly adhering to the roots. The roots themselves are also an important component of the rhizosphere, particularly for endophytic microorganisms (Bruck, 2010). The rhizosphere is a region extending a few millimetres into the soil surrounding the roots where the release of root exudates influences the soil microbiota and may provide a favourable environment for fungal entomopathogens. It is in the rhizosphere that complex interactions between roots, root exudates, beneficial and pathogenic microorganisms and invertebrates take place (Bruck, 2010). Root exudates fall into two main classes of compounds: low molecular weight compounds like amino acids, organic acids, sugars, phenolics and other secondary metabolites, and high molecular weight compounds such as polysaccharides and proteins (Dakora & Phillips, 2002; Bruck, 2010; Carvalhais *et al*., 2011).

Fungal entomopathogens can occupy any one of the three regions of the rhizosphere depending on the grade of rhizosphere competence and their endophytic capabilities. For example, the persistence of *M. anisopliae* (ARSEF 1080) was demonstrated after several months at 10^5 propagules/g soil in the inner rhizosphere while in bulk soil decreased from 10^5 to 10^3 in the same period of time (Hu & St. Leger, 2002). While the notion of fungal populations increasing in the rhizosphere is not new, and rhizosphere competence is an attribute intrinsic to some microbes, the rate of colonization also

depends on the host plant (Bruck, 2005). The knowledge that even isolates of the same species have different attributes and abilities neither is new. For these reasons fungal rhizosphere competence should be considered in the selection of biocontrol agents, especially interactions with plant roots and their exudates, in order to select those fungal isolates that are highly competitive and able to persist in the environment where they were applied.

The use of spectrophotometric methods to determine fungal growth in liquid media containing roots exudates might help to assess fungal rhizosphere competence in entomopathogenic fungi. Previous research on fungal liquid cultures found that absorbance measures increase linearly with mycelium density (Granade, Hehmann & Artis, 1985). Characteristic growth curves for different fungal species were obtained using spectrophotometric determination in a microbroth kinetic system when fungal development was monitored over time allowing different growth stages during the fungal growth to be distinguished (Llop *et al*., 2000; Meletiadis, te Dorsthorst & Verweij, 2003). The authors of these works proposed the microbroth kinetic system could be used as a reproducible methodology to describe fungal growth curves of different species in the presence of antifungal drugs at different concentrations and in agreement with the National Committee on Clinical Laboratory Standards (NCCLS) standard M-38A (NCCLS, 2002). Therefore, this strategy was tested to determine growth of entomopathogenic fungi in the presence of root exudates as an indicator of rhizosphere competence.

2.1.4 Objective of this chapter

The aim of this part of the study was to characterize entomopathogenic fungi, including pathogenic activity against *C. giveni* and rhizosphere competence, for its use as biocontrol agents in soil. The strategy for this selection was based in a molecular and phenotypic approach.

First, molecular identification of the isolates to species level was conducted, since most of the isolates had only been identified to genus. Second, the presence of certain genes that had been related to the ability of fungi to adhere to insects or to plants were determined. Third, a new method for evaluating growth of different isolates of entomopathogenic fungi in root exudates to determine potential rhizospheric competence was tested. Finally, the natural entomopathogenicity of the isolates against larvae of *C. giveni* was determined by bioassay. Together, molecular characterisation, growth in roots exudates and pathogenicity, might improve the selection of entomopathogenic fungal biocontrol agents with pathogenicity against a specific pest and at the same time, with improved ability to survive on roots and so, to persist in soil.

2.2 Materials and methods

2.2.1 Fungal isolates and culturing

Live culture stocks were obtained from the Bio-Protection Research Centre (BPRC), AgResearch (AgR) and from ARSEF (United Stated Department of Agriculture, Agricultural Research Service Collection of Entomopathogenic Fungal Cultures, Ithaca, New York). The initial identification, description and origin of the isolates used in this study are described in Table 2.1. Fungi were grown on potato dextrose agar plates (PDA) at 22°C in light dark conditions (12:12 hrs) and kept at 4°C in slant tubes containing PDA for short term storage or as conidia suspension in glycerol (30%) at -80°C for long storage.

2.2.2 Fungal DNA extraction

Three protocols for DNA extraction were used for the study. For phylogenetic studies for fungal identification the genomic DNA was extracted directly from mycelia grew on PDA plates of 10 days old using the PowerPlant® Pro DNA Isolation Kit (Mo BIO Laboratories, Inc.) according to the manufacturer's instructions and recommendations for the extraction of fungal DNA. Extracted DNA was diluted 1:10 in sterile distilled water prior to PCR amplification and stored at -20°C. For the molecular characterization by PCR of the isolates (determination of the presence of adhesin genes and transmembrane sugar transporter) the genomic DNA was extracted directly from mycelia grew on PDA plates of 10 days old using the Extract-N-Amp™ Plant PCR Kit (SIGMA-ALDRICH®) according to the manufacturer's instructions and recommendations for the extraction of fungal DNA. For molecular characterization by dot-blot studies, the genomic DNA was extracted from frozen mycelia ground with liquid N_2 and using the Gentra Puregene Tissue Kit (Qiagen) according to the manufacturer's instructions and recommendations for the extraction of fungal DNA.

2.2.3 PCR amplification of the *Elongation Factor 1-alpha* gene

The sequence of the entire elongation factor 1-alpha (EF1-α) was obtained using a set of three pair of primers (Bischoff, Rehner, & Richard, 2006; Kepler & Rehner, 2013; Rehner & Buckley, 2005). The 5' EF1-α intron was amplified with the pair EF1T and EF2T. The EF1-α exon was amplified with the set of pairs 983F, and 1567R and the pairs 1577F and 2218R (Table 2.2). Each of the corresponding amplicons with the three pair of primers obtained from the EF1-α were identified as region A, B and C, respectively. PCR products obtained were visualized by gel electrophoresis to confirm amplicon size and then the PCR product was purified using the HighPrep™ PCR purification kit before sequencing.

Table 2.1 Voucher information for the specimens used in this study

Code N°	Species	Origin	Source	Location	Collection [a]
A1080	*Metarhizium anisopliae*	Insect larvae	*Trichoplusia ni* (Lep: Noctuidae)	Florida, USA	ARSEF
Bb18	*Beauveria bassiana*	Endophyte from leaf husk	*Zea mays* (Poa: Poaceae)	Ashburton, Canterbury	BPRC
Bb21	*Beauveria bassiana*	Endophyte from leaf husk	*Zea mays* (Poa: Poaceae)	Ashburton, Canterbury	BPRC
F11	*Metarhizium anisopliae var. anisopliae*	Insect cadaver from farm soil	*Costelytra giveni* (Col: Scarabaeidae)	Rakaia, Canterbury	AgR
F120	*Metarhizium anisopliae var. anisopliae*	Insect cadaver	*Listronotus bonariensis* (Col: Curculionidae)	North Island, New Zealand	AgR
F133	*Metarhizium anisopliae var. anisopliae*	Soil forest	Soil isolation using *Galleria mellonella* (Lep: Galleriidae)	Matangi, North Island.	AgR
F137	*Metarhizium anisopliae var. anisopliae*	Soil maize	Soil isolation using *Galleria mellonella* (Lep: Galleriidae)	Te Kawa, North Island	AgR
F138	*Metarhizium anisopliae var. anisopliae*	Soil maize	Soil isolation using *Galleria mellonella* (Lep: Galleriidae)	Monovale, North Island	AgR
F142	*Metarhizium anisopliae var. anisopliae*	Soil forest	Soil isolation using *Galleria mellonella* (Lep: Galleriidae)	Gordonton, North Island	AgR
F144	*Metarhizium anisopliae var. anisopliae*	Soil pasture	Soil isolation using *Galleria mellonella* (Lep: Galleriidae)	Pirongia, North Island	AgR
F148	*Metarhizium anisopliae var. anisopliae*	Insect cadaver	*Costelytra giveni* (Col: Scarabaeidae)	Southland, New Zealand	AgR
F16	*Metarhizium anisopliae*	Insect cadaver	*Costelytra giveni* (Col: Scarabaeidae)	Nelson, Nelson-Marlborough Region	AgR
F178	*Metarhizium anisopliae var. anisopliae*	Insect cadaver from farm soil	*Wiseana* spp. (Lep: Hepialidae)	West Coast, New Zealand	AgR
F264	*Metarhizium* sp.	Insect cadaver from farm soil	*Costelytra giveni* (Col: Scarabaeidae)	Methven, Canterbury	AgR
F30	*Metarhizium* sp.	Insect cadaver	*Costelytra giveni* (Col: Scarabaeidae)	Mid-Canterbury, New Zealand	BPRC
F31	*Metarhizium anisopliae*	Insect cadaver	*Costelytra giveni* (Col: Scarabaeidae)	Mid-Canterbury, New Zealand	BPRC
F327	*Trichoderma harzianum*	Endophyte from roots	*Mentha* spp. (Lam: Nepetoideae)	Waitakaruru, Waikato	BPRC
F387	*Metarhizium* sp.	Insect cadaver	*Aphodius tasmaniae* (Col: Scarabaeidae)	Hawkes Bay, North Island	AgR
F401	*Metarhizium* sp.	Insect cadaver from farm soil	*Costelytra giveni* (Col: Scarabaeidae)	Te Anau, New Zealand	AgR
F447	*Metarhizium* sp.	Endophyte from roots	*Actinidia deliciosa* (Eri: Actinidiaceae)	Avondale, Auckland	BPRC
F628	*Metarhizium* sp.	Insect cadaver	*Pyronota festiva* (Col: Scarabaeidae)	Buller, New Zealand	AgR
F672	*Metarhizium* sp.	Endophyte from root	*Pinus radiata* (Pin: Pinaceae)	Taupo, New Zealand	AgR
F98	*Metarhizium anisopliae var. anisopliae*	Insect cadaver from farm soil	*Costelytra giveni* (Col: Scarabaeidae)	Methven, Canterbury	AgR
F99	*Metarhizium anisopliae var. anisopliae*	Insect cadaver from farm soil	*Costelytra giveni* (Col: Scarabaeidae)	Methven, Canterbury	AgR
Bk41	*Metarhizium* sp.	Endophyte from leaves	*Actinidia deliciosa* (Eri: Actinidiaceae)	Nelson, Nelson-Marlborough Region	BPRC
MFI	*Metarhizium* sp.	Insect cadaver Pine plantation	*Arhopalus tristis* (Col: Cerambycidae)	Auckland, New Zealand	BPRC
WH#2	*Metarhizium* sp.	Insect cadaver	*Hemideina broughi* (Ort: Anostostomatidae)	West coast, New Zealand	BPRC
WH#8	*Metarhizium* sp.	Insect cadaver	*Hemideina broughi* (Ort: Anostostomatidae)	West coast, New Zealand	BPRC

[a] Abbreviations for collections: ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; BPRC, Bioprotection Research Centre Fungal Collection, Lincoln, Canterbury; AgR, AgResearch Fungal Collection, Lincoln Canterbury. All isolates were isolated from New Zealand but for the strain reference A1080.

PCR amplifications were performed in a total volume of 25 µl, which included 2.5 µl of 10X PCR buffer (10mM Tris/HCl pH 8.0, 50 mM KCl, 1.5 – 2.0 mM $MgCl_2$), 0.75 µl of dNTP mix (10 mM each dATP, dCTP, dGTP, and dTTP), 1 µl each of the opposing amplification primers (10 µM), 0.25 µl *Taq* polymerase (FastStart *Taq* DNA Polymerase, Roche) and 2 - 20 ng of the genomic DNA (Rehner & Buckley, 2005). PCR for all primer combinations both loci was performed using a touchdown PCR procedure (Rehner & Buckley, 2005).

Table 2.2. Primers used for the amplification by PCR of the *Elongation factor 1- alpha* gene and ITS region.

Primers for:	Region	Sequence 5'-3'	Amplicon (bp)
Elongation Factor			
EF1T	**A**	ATGGGTAAGGARGACAAGAC	770
EF2T		GGAAGTACCAGTGATCATGTT	
EF1-983F	**B**	GCYCCYGGHCAYCGTGAYTTYAT	500
EF1-1567R		ACHGTRCCRATACCACCRATCTT	
EF1-1577F	**C**	CARGAYGTBTACAAGATYGGTGG	500
EF1-2218R		ATGACACCRACRGCRACRGTYTG	
ITS			
ITS4	**D**	TCCTCCGCTTATTGATATGC	600
ITS5		GGAAGTAAAAGTCGTAACAAGG	

Touchdown PCR amplifications were initiated with a 4 min initial denaturation at 95°C, followed by 20 amplification cycles each consisting of 30 s denaturation at 95°C, a 30 s annealing step (touch down) and a final extension at 72°C for 90 s. The annealing temperature in the first amplification cycle was 66°C and successively reducing the Tm by 0.5°C over the next cycles to reach a final Tm of 56°C. An additional 40 amplification cycles were then performed, each consisting of 30 s denaturation at 95°C, a 30 s annealing step at 56°C and a final extension at 72°C for 90 s. Finally, an additional final extension step for 90 s at 72°C was performed.

2.2.4 PCR product purification and sequencing

Amplicons were purified using HighPrep™ PCR purification kit based on paramagnetic beads technology. This PCR clean up system is designed for an efficient purification of PCR amplicons. The purification consists of removal of salts, primers, primer-dimers, dNTPs, whereas DNA fragments are selectively bound to the magnetic beads particles. Highly purified DNA is eluted from the magnetic beads particles with low salt elution buffer or water and can be used directly for downstream applications. Sequencing of the amplicon DNA sequences were determined using an ABI Prism 3130xl

Genetic Analyser with a 16 capillary 50 cm array installed and using Performance Optimized Polymer 7. In the sequencing reaction were used 0.5 µl BDT (ABI PRISM® BigDye® Terminator v3.1 Cycle Sequencing Kit), 2.0 µl buffer (BigDye® Terminator v3.1 5x sequencing buffer), 1.0 µl of each primer (5µM), DNA template volume variable and H_2O up to a final total volume of 10 µl. The post sequencing reaction clean-up used the HighPrep™ DTR Dye Terminator Removal System by MAGBIO.

2.2.5 Phylogenetic analysis

For a particular isolate, there were in total three pair of sequences conforming to the three regions in which the EF1-α was split for sequencing purposes. For each region, both sequences forward and reverse were quality determined with the ChromasPro 2.6 Program (Technelysium Ltd). A consensus sequence was obtained from both strands for each corresponding region was completed in Geneious 9.1.4. Sequence alignments were performed in ChromasPro using default settings, and manual adjustments and matrix concatenation were performed in Geneious 9.1.4. Trimmed alignment length for the EF1-α was 1700 bp. Finally, using the program MEGA 6.0 (Tamura *et al*. 2013) the three consensus region sequences were aligned to obtain the whole spam of the gene EF1-α of each isolate. The final data set consisted of 25 complete EF1-α sequences from isolates of *Metarhizium* spp. and 18 EF1-α reference sequences of different *Metarhizium* species. The Neighbour-Joining method was used to obtain an optimal tree with the sum of branch length of 0.45 with a 1000 bootstrap test using the program MEGA 6.0.

2.2.6 Determination of the presence of adhesin genes (*mad1* and *mad2*) and the *Metarhizium* raffinose transporter (*mrt*)

2.2.6..1 Primer design for mad1*,* mad2 *and m*rt *genes from* Metarhizium *spp.*

Primers for the corresponding genes *mad1*, *mad2* and *mrt* for *Metarhizium* spp. were designed using the primer blast tool available in NCBI/Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) which ensure a full primer-targ*et al*ignment. The *mad1* and *mad2* genes were amplified using the following pair of primers respectively: MAD1F (5´-TGCTGTCTTCGTCGTCTTACA) and MAD1R (5´-CGCAGTGCCACTTGATCTTG); MAD2F (5´-ACCAGCACAGAAAGCAGACT) and MAD2R (5´-TGATTGGCAGGCTTGTTCCA). Finally, for the *Metarhizium* rafinose transporter gene amplification was performed using two pairs of primers: MRT1F (5'-CAGCCTCGCAGAATCTCCAT) and MRT1R (5´-GCAGACGATTTCTGTTCGGC); MRT2F (5´-GATGGAGAAGGAGCTCACGG) and MRT2R (5´-TCACCTTGCGCATCGAATCT). PCR amplification were performed in a total volume of 25 µl, which included 2.5 µl of 10X PCR buffer (10mM Tris/HCl pH 8.0, 50 mM KCl, 1.5 – 2.0 mM MgCl2), 0.75 µl of dNTP mix (10 mM each dATP, dCTP, dGTP, and dTTP), 1 µl each of the opposing amplification primers (10 µM), 0.25 µl *Taq* polymerase (FastStart *Taq* DNA Polymerase, Roche) and 5-20 ng of the genomic DNA (Rehner & Buckley, 2005). PCR for all primer combinations was performed using a touchdown

PCR procedure (Rehner & Buckley, 2005). Touchdown PCR amplifications were initiated with a 4 min initial denaturation at 95°C, followed by 20 cycles of amplification cycles which each consisted of 30 s denaturation at 95°C, a 30 s annealing step (touchdown) and a final extension at 72°C for 90 s. The annealing temperature in the first amplification cycle was 66 °C and successively reducing the Tm by 0.5°C over the next cycles to reach a final Tm of 56°C. An additional 40 amplification cycles were then performed, each consisting of 30 s denaturation at 95°C, a 30 s annealing step at 56°C and a final extension at 72°C for 90 s. Finally, an additional final extension step for 90 s at 72°C was performed. The amplification of the sequences was visualized by gel electrophoresis in agarose (2%) in buffer TAE (Tris-base, acetic acid and EDTA).

2.2.7 Dot-blot

The DNA for hybridization probes for each of the genes, *mad1*, *mad2* and *mrt,* were obtained by PCR with the same programme used the amplification of these genes and PCR reactions but with nucleotides labelled with digoxigenin (DIG) using the PCR DIG Labeling Mix^{plus}. The dot blot was done following the protocol and recommendations described in Brown (2003), which follows a sequence of steps: DNA fixation, pre-hybridization, hybridization, stringency washes, detection and development. Briefly, the nitrocellulose membrane was marked where DNA should be loaded and then denaturalized DNA was applied in successive rounds of 1 µl until the recommended concentration was reached. The DNA was fixed to the nitrocellulose membrane by baking for 2 h at 80°C. For prehybridization the nitrocellulose membrane is put into a hybridization tube with 20 ml of prehybridization solution warmed up to 65°C and Salmon Testes DNA (10 mg/ml). The membrane and prehybridization solution were incubate at 65°C overnight in the hybridization oven in continuous rotation. For hybridization the prehybridization solution was removed and replaced by hybridization solutions warmed up to 65°C. The hybridization solution contained Salmon Testes DNA and the DIG-labeled probe DNA. The membrane was left to hybridize at 65°C in the hybridization oven overnight. After hybridization, the membrane was subjected to a series of high and low stringency washes at 65°C in rotation. The detection stage was done by removing the low stringency washing solution from the hybridization tube and washing twice the dot-blot with washing buffer warmed at 65°C. After washes the dot-blot was incubated with blocking solution for 30-50 min at room temperature. Then blocking solution was discarded and the dot-blot incubated for 30 min with the antibody solution at room temperature. The dot-blot was washed twice with washing buffer and incubated in rotation at 23°C and then equilibrated with detection buffer at room temperature in rotation. The development was done placing the blot with DNA side facing up into a Ziploc plastic bag and immediately applying 0.5 ml of the CDP-Star solution distributing uniformly onto the membrane. Finally, after incubation the dot blot was exposed to an X-ray film for 20 min in the dark and developed.

2.2.8 Measure of radial growth rate

The fungal radial growth rate (RGR) was determined according to the method of Trabelsi *et al*. (2017). Conidial suspensions were obtained by spreading conidia of the fungal isolate onto the surface of a PDA plates incubating at 22°C in light:dark conditions (12:12 h). After 14 days conidia were harvested from the PDA plates into 3 - 5 mL of 0.01% Triton X-100 using a sterile hockey stick to help dislodge the conidia from the fungal colony. The suspension was transferred from the Petri plate to a Falcon tube of 15 mL containing 5 glass beads of 0.5 mm diameter. Conidia concentration was determined using an improved Neubauer chamber and conidial suspension was adjusted to 1 x 10^4 conidia/mL with a solution of 0.01% Triton X-100. For the determination of the radial growth rate, 10 µl of the conidial suspension of each fungal isolate was placed at the centre of a PDA plate per triplicate and incubated at 22 ± 2°C. The diameter (D) of the fungal colony was determined as two perpendicular diameters, and value averaged. Radial growth rate was calculated using the following formula where: radial growth rate (mm/day) = RGR max (D max/2)/number of days. Growth experiments were repeated at least three times.

2.2.9 Spectrophotometric determination of fungal growth in root exudates and in PDB

2.2.9..1 Maize seed sterilization

Maize seeds were surface sterilized using the procedure of Martinez & Wang (2009) with some modifications. Approximately 200 g of seeds were placed in a 500 ml beaker along with a stir bar. Then ethanol (80%) was added and the beaker covered with an aluminium foil and placed on a stir plate and stirred at medium speed. After 3 min the ethanol was decanted, and seeds were washed with sterile water. After the first wash, $HClO_4$ (50%) was added to the beaker containing the seeds and covered with the paper foil and stirred at medium speed. After 15 min the seeds were washed for four times with sterile water. The procedure with $HClO_4$ (50%) and subsequent rinses with sterile water was repeated again. Finally, seeds were dried using sterile paper towels and filtered air in the laminar flow cabinet. All maize seed surface sterilization procedures were carried out in a laminar flow.

2.2.9..2 Root exudate collection

Root exudates were obtained following the methodology described by Wyrebek, Huber, Sasan, & Bidochka (2011) with minor modifications. Approximately 40 surface-sterilized maize seeds were transferred to an Erlenmeyer flask (250 ml) containing 100 ml of sterile water. Flask were closed with sterile cotton plugs to avoid contamination while allowing gas transfer. The flasks containing the seeds were maintained on an orbital shaker at 140 rpm at room temperature. Once 90% of the seeds were physiologically germinated (protrusion of the radicle), samples were kept on the shaker at 25°C for an additional four days (in total approximately eight days). Root exudates were collected by vacuum

filtration through a series of filters to reduce clogging. First exudates were filtered through a Whatman cellulose filter (grade 2, 8 µm), then through 0.8 µm Millipore filter and finally 0.2 µm Millipore filter (Figure 2.1). Composition of roots exudates in sugars, organic acids and amino acids were determined by HPLC (Shimadzu).

Figure 2.1 Root exudates collection. Sterilized maize seed was incubated in distilled water at room temperature in an orbital shaker platform at 140 rpm for 8 days

2.2.9..3 Spectrophotometric determination of fungal growth in RE and in PDB

The fungal growth was determined using the spectrophotometric methodology described by Meletiadis, Meis, Mouton, & Verweij (2001) with some modifications. For all the isolates tested the concentration of the conidial suspension was obtained in two stages. First, the suspension was adjusted to 1 x 10^5 conidia/ml in 0.01% Triton X-100, and then to 1 x 10^4 conidia/ml using the medium where fungal growth would be tested, PDB or roots exudates. From this conidial suspension (1 x 10^4 conidia/mL) in 50% RE or PDB, 220 µl were taken and transferred to a well of a 96-well plate. Each isolate was tested in duplicate per plate. The spectrophotometer was set at 25°C, 200 RPM and at 405 nm. The plate was subjected to 10 s of orbital agitation before the optical density (OD) was measured every 15 min.

2.2.9..4 Kinetic parameters determination

In order to compare the growth curves for each isolate in both nutrient media, a number of kinetic parameters were calculated based on the changes of the OD over time with the Skanlt Sofware 3.2.0.36 RE for Multiskan GO (Multiskan GO 1.00.40) and based on the obtained growth curve of each isolate in each medium. The kinetic parameters determined were: the highest OD (Maximum), the average growth rate (Rate, s^{-1}), the integral (slope), the OD change as OD_{max}– OD_{min} (Change), the Maximum rate (Max. rate min^{-1}) and the time at which the maximum growth rate was reached (T MaxR, h.). Additionally, from each curve the exponential growth was determined and equations that describe

kinetic microbial growth (Widdel, 2010) were calculated: the specific growth rate (μ min^{-1}); the duplication time or generation time (Td, h^{-1}) and the time when the lag phase ends (Tlagf, h^{-1}).

2.2.10 *Costelytra giveni* bioassays with entomopathogenic fungal isolates

Bioassays were conducted using the method of Glare (1994) with some modifications. Second and 3rd instar larvae of *C. giveni*, were field collected and kept individually in sections of a 24 well-plate with a small piece of carrot at 18 ± 2°C in the dark. After 72 hrs, larvae were checked for feeding and natural mortality. Only healthy, feeding larvae were selected for bioassays. Conidia were harvested with 3 – 5 mL of a solution of 0.01% Triton X-100 added to 15 days old fungal colonies grown in half strength PDA at 20 ± 2 °C in light:dark conditions (12:12 h). Conidial suspensions were quantified with an improved Neubauer chamber and adjusted to 10^5, 10^7 and 10^9 conidia/mL. Soil was semi-sterilized at 90°C for 48 hrs and then 10 grams of soil was added to Falcon tubes of 50 mL. To adjust the final soil moisture, each Falcon tube received 1 mL of 0.01% Triton X-100 and 1 mL of the corresponding conidial suspension (20% w/w) or 2 mL of 0.01% Triton X-100 and 1 mL of the conidial suspension (30% w/w). After soil and conidial suspension were mixed, one larva of *C. giveni* was added and the tube was loosely closed to allow gas exchange (Figure 2.2).

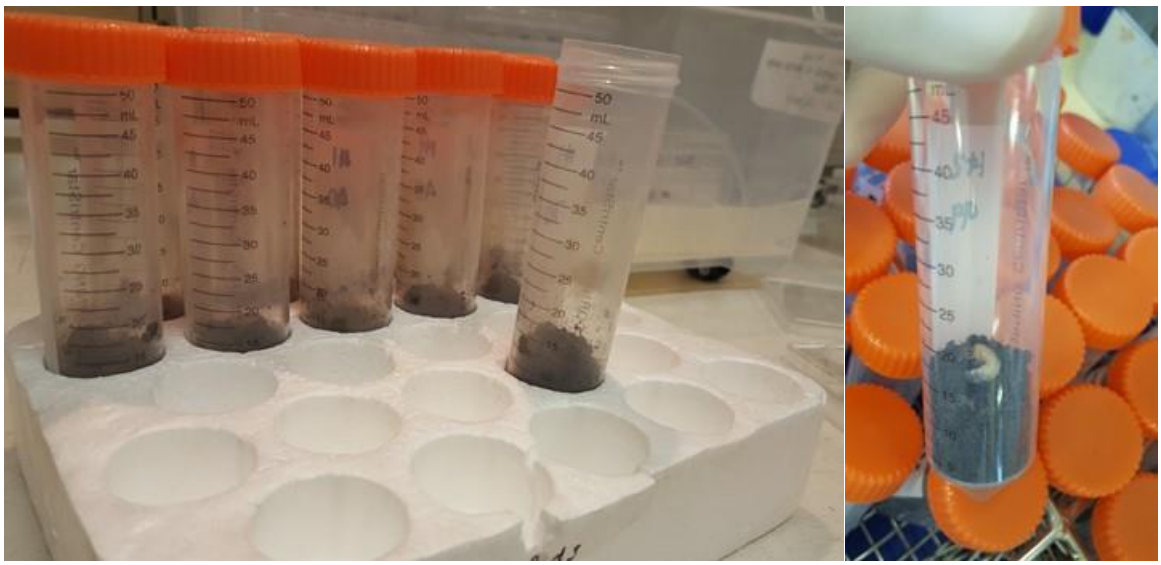

Figure 2.2 *Costelytra giveni* bioassays with entomopathogenic fungi. Soil was semi-sterilized, and 10 grams of soil were added to Falcon tubes of 50 mL. Each tube received 1 mL of the corresponding conidial suspension and moisture was adjusted to 20% w/w or 30% w/w. One larva of *C. giveni* was added, tube was closed loose to allow gas exchange and incubated at 20 ± 2 °C. Every 5 days larvae were checked for survival, mortality or fungal infection.

Larvae were kept for at least 40 days at 20 ± 2 °C and were checked every five days for mortality and fungal infection (mycosis). Dead larvae were kept in the Falcon tubes with the lid closed tight to keep the humidity in the system and checked also every five days for signs of developing mycelia and

sporulation. For each bioassay, tubes were arranged in a randomized block design with four blocks. In each block, there were seven isolates at each conidial concentration (10^5, 10^7 and 10^9) plus an untreated control (CS). The controls *M. anisopliae* A1080 and CS were replicated three times in each block. The bioassay was carried out three times. In order to combine data over the three bioassays, treatment means were input into an analysis of variance which treated bioassays as "blocks", with treatments having a seven (isolates) by three (conidial concentrations) factorial structure. Untreated controls were omitted from this statistical modelling. The three conidial concentrations (10^5, 10^7 and 10^9) were also further modelled using linear and quadratic polynomial contrasts (assuming equal spacing on a log scale). Comparison of isolates employed an unprotected least significant difference (LSD) procedure (Saville, 2015).

2.3 Results

2.3.1 Molecular characterization of fungal isolates – Optimization of techniques

The molecular characterization was divided into two parts: molecular identification of the strains and detection of genes associated with the fungal capacity to attach to surfaces of either insects (*mad1*) or plants (*mad2*). Additionally, a third gene known as *Metarhizium* raffinose transporter (*mrt*) was also investigated. The identification of the strains at a molecular level targeted the elongation factor 1 alpha (EF1- α).

2.3.2 Molecular identification of fungal cultures – Phylogenetic tree

The *Metarhizium* spp. isolates were identified by PCR amplification of the genomic sequence of the elongation EF1-α. The whole EF1-α gene consists of 2477 bp with the 5´region being the most informative region for the correct identification of different fungal isolates to species level. A total of 27 consensus sequences with a total span of 1700 bp were obtained for the EF1-α gene of each isolate.

The Neighbour-Joining method for the phylogenetic study of the data set allowed the identification of the *Metarhizium* isolates to species level (Figure 2.3). A section of the obtained phylogenetic tree strongly corresponded to the backbone topology of the PARB species complex, with *M. brunneum* forming the basal-most clade and *M. robertsii* parallel to a clade containing *M. pingshaense* and *M. anisopliae*. Interestingly, only five of the study isolates belonged to this common and world-wide distributed clade and none of the isolates were aligned to the *M. pingshaense* clade. Most of the remaining isolates, 57%, belonged to the *M. novozealandicum* clade. Another section of the obtained phylogenetic tree also coincides to the split of *M. lepidiotae* and the MGT clade which includes *M. majus* and *M. guizhouense*. Only three isolates fell in this clade. Finally, two isolates belonged to clade of cold active species: *Metarhizium frigidum*.

From all the isolates previously identified as *M. anisopliae*, the majority fell in the *M. novozealandicum* clade and others in *M. robertsii*, *M. brunneum* and *M. guizhouense*, except for F672 which belongs to *M. anisopliae*. The *Metarhizium* isolates originally obtained from plant material as endophytes belonged *to M. robertsii* (F447), *M. anisopliae* (F672) and *M. guizhouense* (Bk41). The isolate F144 previously identified as *M. anisopliae* var. *anisopliae*, in this study its sequence corresponded to that of *Isaria fumosorosea* and was the only isolate outside of the genus *Metarhizium*. This isolate was not included in the phylogenetic tree.

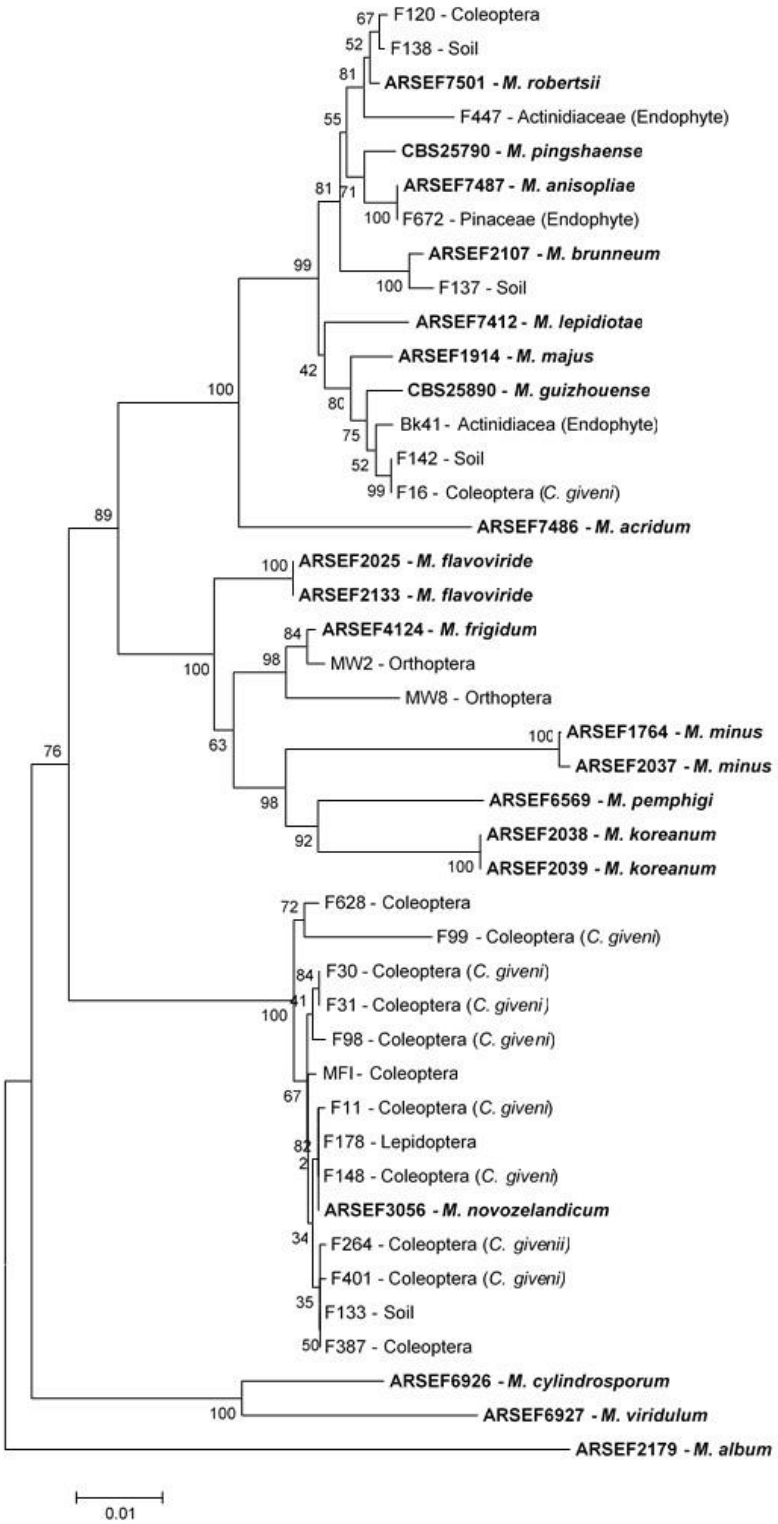

Figure 2.3 Molecular identification to the species level of *Metarhizium* spp. isolates. Neighbour-Joining phylogeny inferred from the analysis of the *elongation factor 1-alpha* gene (EF1-α). Support values were obtained from 1000 bootstrap replicates. Percentage of replicate trees in which the associated taxa clustered together are shown next to the branches. The analysis involved 43 nucleotide sequences, including reference strains obtained from GenBank (in bold). All positions containing gaps and missing data were eliminated. There was a total of 1353 positions in the final dataset.

2.3.3 Determination of the presence of adhesin genes (*mad1* and *mad2*) and the *Metarhizium* raffinose transporter (*mrt*)

Approximately 2 ng of genomic DNA from each isolate was used for PCR amplification of the targeted genes sequences, *mad*1, *mad*2 or *mrt*, using the pair of primers selected from the NCBI described in the methodology. However, using these set of primers did not result in gene amplification in most of the entomopathogenic isolates (Figure 2.4).

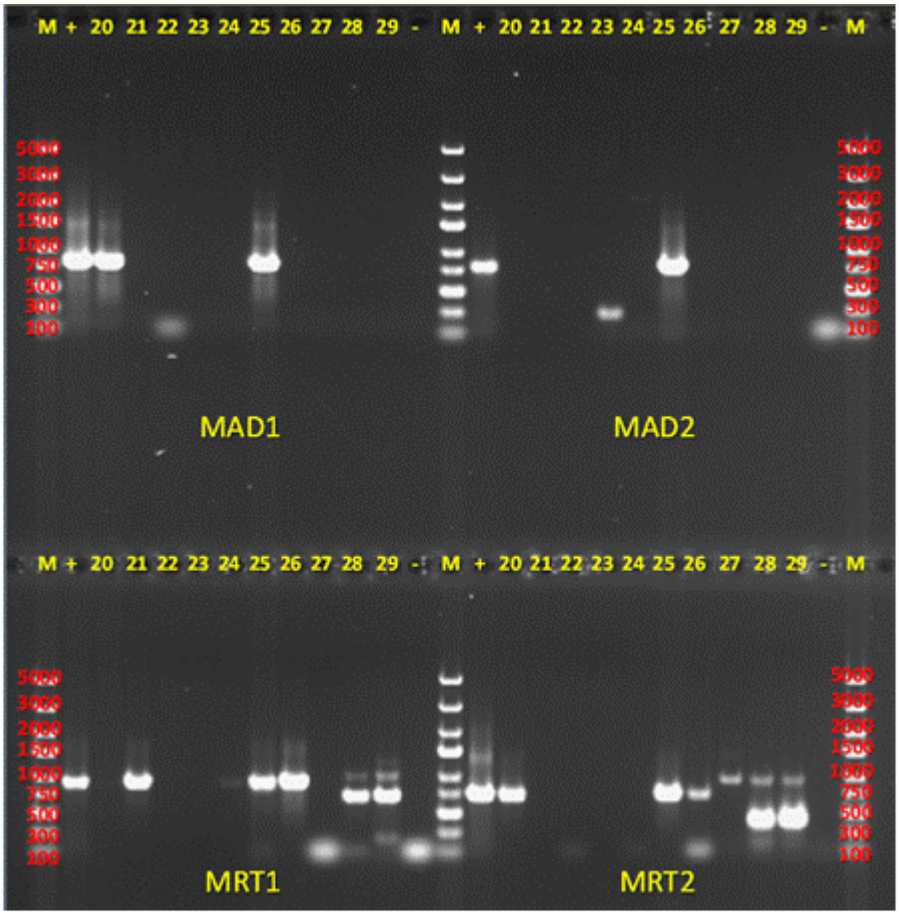

Figure 2.4 Molecular characterization of entomopathogenic fungi. Amplification of genes related with the fungal capacity to attach to the surfaces of either, insects (*mad1*) or plants (*mad2*), and their capacity to associate with roots (*mrt*). M, Molecular marker; +, Positive control; 20= F447; 21= C14; 22= MW#2; 23= MW#8; 24= B14; 25= Bk41; 26= MFI; 27= F327; 28= Bb18; 29= Bb21; -, Negative control. *Metarhizium*: 20 – 26; *Trichoderma*: 27; *Beauveria*: 28-29.

A new set of degenerated primers were designed which contemplated the differences that might exist between different species. The new primers used and the size of the corresponding expected amplicon are given in Table 2.3.

Table 2.3. Primers used for the amplification of target genes related with the adhesion of *Metarhizium* to insect or plant surfaces, *mad1* and *mad2*, respectively, and the *Metarhizium raffinose transporter* gene (*mrt*).

Primers for:	Code	Sequence 5'-3'	Amplicon (pb)
MAD1F*	I	TGCTGTCTTCRTCGTCYTACA	793
MAD1R*		CGCAGTGCCACTTGATCTTG	
MAD2F*	J	ACCAGCACAGAAAGCAGWST	799
MAD2R*		TGATTGGCAGGCTTGKTCCA	
MRT1F	H	CAGCCTCGCAGAATCTCCAT	738
MRT1R		GCAGACGATTTCTGTTCGGC	
MRT2F*	K	GATGGAGAAGGAGCTCACRG	738
MRT2R*		TCMCSTTGCGCRTCGAATCT	

**All pair of primers were degenerated but for the pair H.*

In Table 2.4 are shown the results obtained in this molecular characterization with the genes *mad1*, *mad2* and *mrt* using the entomopathogenic fungal isolates of this study. The size of the obtained amplicons corresponded to the expected size using either non-degenerated and degenerated (*) primers: *mad1** (793 bp); *mad2** (798 bp); *mrt1* (918 bp) and *mrt2** (738 bp).

Using this new set of degenerated primers in a touchdown PCR, gene amplification was obtained in a higher number of isolates than previously, but results seemed to be genus specific given the fact that no amplification was obtained in the *Beauveria* isolates (Figure 2.5).

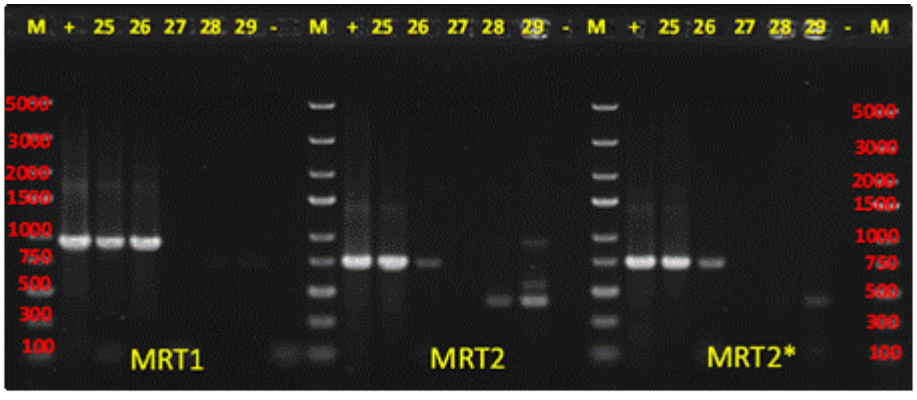

Figure 2.5 Molecular characterization of entomopathogenic fungi. Amplification of genes related with the fungal capacity to associate with roots (*mrt*) using non-degenerated and degenerated primers (*). M, Molecular marker; +, Positive control; 25= BK41; 26= MFI; 27= FCC327; 28= Bb18; 29= Bb21; -, Negative control. *Metarhizium*: 25 – 26; *Trichoderma*: 27; *Beauveria*: 28-29.

The *mad1* gene, which is related to the ability of the fungi to attach to insects, was found in only 33.3% of the isolates and its presence was homogenously distributed among the different species (Table 2.4). As expected, both *B. bassiana* isolates, *T. harzianum* F327 and *I. fumosorosea*, seemed not to have a homologous sequence and no amplification was determined.

Table 2.4. Determination of the presence of the genes *mad1*, *mad2* and *mrt* by molecular techniques. Using non-degenerated and degenerated (*) primers, the presence of the target genes was determined by amplification with PCR. The results, absence (0), presence (1), were confirmed in three different PCR amplifications. To corroborate the results obtained by PCR, a second molecular technique, a Dot-blot was applied using hybridization probes also obtained by amplification with non- and degenerated (*) primers. Scoring for the Dot-blot is absence (-), presence (+).

		PCR				Dot-blot			
Collection N°	**Species**	**MAD1***	**MAD2***	**MRT1**	**MRT2***	**MAD1***	**MAD2***	**MRT1**	**MRT2***
A1080	*Metarhizium anisopliae*	1	1	1	1	+	+	+	+
F30	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
Bb18	*Beauveria bassiana*	0	0	0	0	-	+	+	-
Bb21	*Beauveria bassiana*	0	0	0	0	-	-	+	-
BK41	*Metarhizium guizhouense*	1	1	1	1	+	+	+	+
F31	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F11	*Metarhizium novozealandicum*	0	0	1	1	-	+	-	-
F120	*Metarhizium robertsii*	1	1	1	1	+	+	+	+
F133	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F137	*Metarhizium brunneum*	1	1	1	0	+	+	+	+
F138	*Metarhizium robertsii*	1	1	1	0	+	+	+	+
F142	*Metarhizium guizhouense*	1	1	1	1	+	+	+	+
F144	*Isaria fumosorosea*	0	0	0	0	+	+	+	+
F148	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F16	*Metarhizium guizhouense*	1	1	1	1	+	+	+	+
F178	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F264	*Metarhizium novozealandicum*	0	0	1	1	+	-	+	+
F327	*Trichoderma harzianum*	0	0	0	0	-	-	+	-
F387	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F401	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F447	*Metarhizium robertsii*	1	1	1	1	+	+	+	+
F628	*Metarhizium novozealandicum*	0	0	1	1	+	-	+	+
F672	*Metarhizium anisopliae*	1	1	1	0	+	+	+	+
F98	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
F99	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
MFI	*Metarhizium novozealandicum*	0	0	1	1	+	+	+	+
MW#2	*Metarhizium frigidum*	0	0	0	1	-	-	+	+
MW#8	*Metarhizium frigidum*	0	0	0	1	-	+	+	+

The *mad2* gene, related with the ability of the fungi to attach to plant surfaces occurred in 31% of the isolates, and only in those that previously had had amplification of the *mad1* gene. On the other hand, the gene which putatively confers the ability of fungi to assimilate sugars from roots exudates, *mrt*, was widely distributed among the different species and detected in 80% of the isolates. The gene seemed to be present in all *Metarhizium* isolates except for the isolates *M. frigidum* MW#2 and MW#8. In the case of the other genera, both *B. bassiana* isolates, *T. harzianum* F327 and *I. fumosorosea* F144, the gene *mrt* was not amplified with the PCR conditions used. The use of degenerated primers for the *Metarhizium raffinose transporter*, *mrt*2*, allowed amplification in all the *Metarhizium* isolates, but not for the other genera (Table 2.4).

The *M. novozealandicum* clade was the only where none of the isolates had amplification of *mad1* and *mad2*, although all of them were isolated from insects, except for the F133 isolate obtained from soil. On the other hand, isolates from this clade, did have the *mrt1* and *mrt2* genes. Another two isolates obtained from insects that had the *mrt* gene but not *mad1* and *mad2* were in *M. frigidum*.

Comparison between PCR results and Dot-blot showed several results where one was negative and other positive. In general dot-blot is less discriminating as hybridisation can occur with less than exact matches, whereas PCR requires exact or nearly exact primer matches to the gene of interest.

The probes for each gene *mad1*, *mad2* and *mrt* were obtained through a touchdown PCR. The incorporation of the digoxigenin-11-dUTP increased the molecular weight in the amplifying sequence and in a gel electrophoresis the probe runs slower when compared to same unlabelled DNA sequence (Figure 2.6).

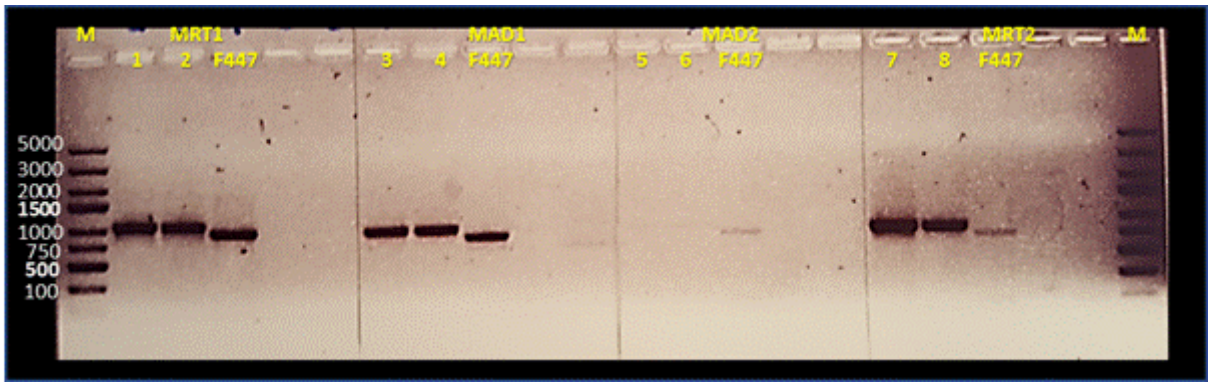

Figure 2.6 Hybridization probes for the Dot-blot technique. The probes of the corresponding genes *mrt*1, *mad*1, *mad*2 and *mrt*2 were obtained through amplification by PCR with marked nucleotides. The product of the PCR was run in a gel of agarose (2%) together with the amplicon of the same sequence but using normal nucleotides. Hybridization probes: 1, 2, 3, 4, 5, 6, 7, and 8; F447, amplicon using normal nucleotides.

The Dot-blot also requires a high concentration of genomic DNA to be hybridized to the nitrocellulose membrane. In previous studies it was determined that around 300 ng/mL of pure genomic DNA would be necessary. The concentration of DNA (ng/µL) obtained after extraction and the volume necessary to apply onto the nitrocellulose membrane to reach the appropriated final DNA concentration are in the Table 2.5.

Table 2.5. DNA concentration obtained after extraction.

Collection N°	Species	DNAo (ng/µl)	Vol. (µl)	DNAf (ng/µl)
A1080	*Metarhizium anisopliae*	124	2.5	310
F30	*Metarhizium novozealandicum*	324	1.5	486
Bb18	*Beauveria bassiana*	370	1.5	555
Bb21	*Beauveria bassiana*	422	1.5	633
Bk41	*Metarhizium guizhouense*	234	1.5	351
F31	*Metarhizium* novozealandicum	394	1.5	591
F11	*Metarhizium novozealandicum*	79.2	4.0	317
F120	*Metarhizium robertsii*	166	2.0	332
F133	*Metarhizium novozealandicum*	418	1.5	627
F137	*Metarhizium brunneum*	238	1.5	357
F138	*Metarhizium robertsii*	256	1.5	384
F142	*Metarhizium guizhouense*	117	2.0	234
F144	*Isaria fumosorosea*	360	1.5	540
F148	*Metarhizium novozealandicum*	542	1.5	813
F16	*Metarhizium guizhouense*	234	2.0	468
F178	*Metarhizium novozealandicum*	302	1.5	453
F264	*Metarhizium novozealandicum*	428	1.5	642
F387	*Metarhizium novozealandicum*	462	1.5	693
F401	*Metarhizium novozealandicum*	416	1.5	624
F628	*Metarhizium novozealandicum*	372	1.5	558
F672	*Metarhizium anisopliae*	114	3.0	342
F98	*Metarhizium novozealandicum*	336	1.5	504
F99	*Metarhizium novozealandicum*	310	1.5	465
F327	*Trichoderma harzianum*	332	1.5	498
F447	*Metarhizium robertsii*	388	2	776
MFI	*Metarhizium novozealandicum*	994	1.5	1491
MW#2	*Metarhizium frigidum*	318	1.5	477
MW#8	*Metarhizium frigidum*	264	1.5	396

DNAo, DNA concentration after extraction; Vol, volume applied of DNA to the nitrocellulose membrane; DNAf, final concentration of DNA in the dot blot.

According to the results obtained with the Dot-blot technique, the *mad1* gene for adherence to the insect cuticle was found in 79% of the samples (Table 2.4). The exceptions were *B. bassiana*, *M. novozealandicum* F11, *T. harzianum* F327 and both *M. frigidum* isolates. The *mad*2 gene, related to adherence to the plant surface, was also widely distributed among the different genera found in 82% of the isolates. The *mrt* gene was present in all the isolates but not in *M. novozealandicum* F11. When the same gene was targeted but using the probe obtained with degenerated primers it was not detected in *M. novozealandicum* F11, in *B. bassiana* (Bb18 and Bb21) or in *T. harzianum* F327 (Table 2.4).

Considering the results obtained from the Dot-blot, the *mad1* gene of insect adherence seems to be widely distributed among the *Metarhizium* spp. except for *M. frigidum*. Also, the gene related with attachment of *Metarhizium* spp. to plant surfaces, or the gene related with the transport of sugars from the plant to the fungal hyphae seemed also to be present in almost all the *Metarhizium* isolates.

Despite the fact that both techniques used for determination of the presence of the genes in the isolates, amplification by PCR and hybridization by Dot-blot, had differences in the number of positives obtained for both the *mad1* and *mad2* genes, both techniques agree with the high frequency that the *mrt* gene is distributed in the different *Metarhizium* species. Additionally, the genes *mad1*, *mad2* and the *mrt* seem to have uniformly distributed among the isolates in the PARB and MGT clades. The *M. frigidum* seemed to lack only the *mad2* gene, while some isolates of the *M. novozealandicum* clade might not have the *mad2* gene.

2.3.4 Fungal radial growth in PDA

There were differences in the growth diameter and the radial growth rate of the different isolates ($p<0.01$). The fastest growth was determined for *T. harzianum* F327 which reached a maximum diameter after 4 days of inoculation. The remaining isolates were growing for an additional 15 days before reaching their maximum diameter of close to 9 cm (Figure 2.7). The fastest growth among the entomopathogenic fungi were for *M. guizhouense* Bk41, *I. fumosorosea* F144*, B. bassiana* Bb21 and *M. anisopliae* F672 isolates.

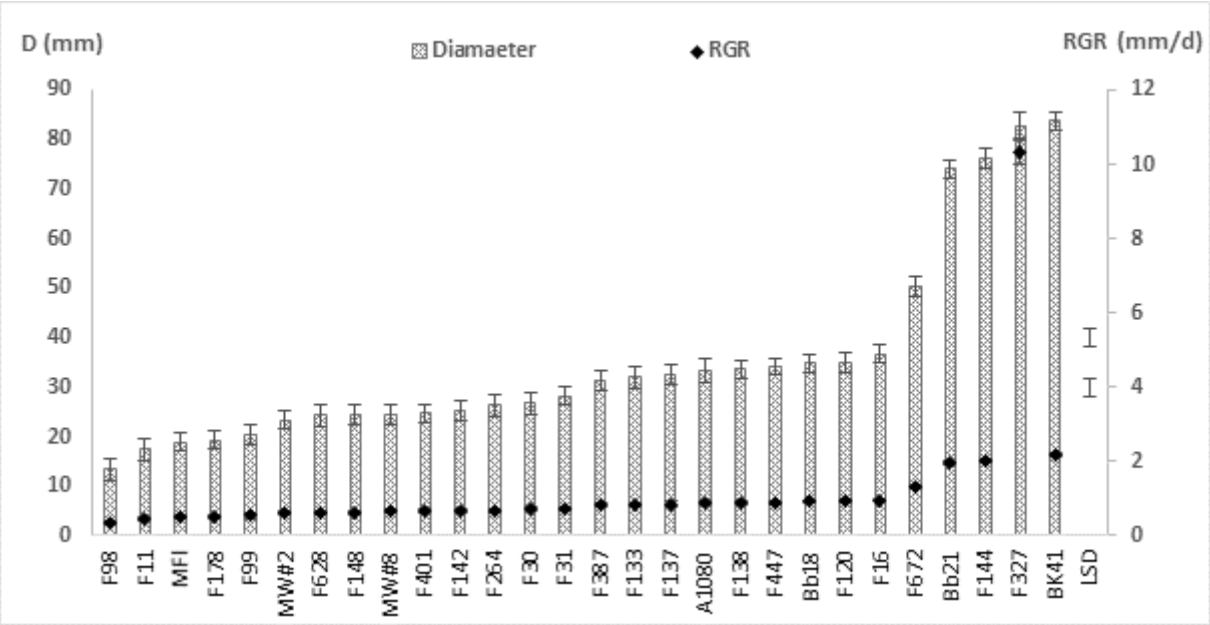

Figure 2.7. Fungal growth in PDA. Fungi were grown on PDA plates at 22°C and diameter was measured at 4 days after inoculation for *T. harzianum* (F327) and at 19 days after inoculation for the entomopathogenic fungi. RGR, radial growth rate. Error bars = standard deviation.

2.3.5 Spectrophotometric determination of fungal growth in root exudates and in potato dextrose broth

Root exudates (RE) include secretions actively released from the root due to osmotic differences between soil solution and the cell. The organic compounds released through these processes can be divided into high and low molecular weight compounds. The former are complex molecules that cannot easily be used by microorganisms (e.g. mucilage, cellulose) while lower molecular weight compounds (LMW) can be categorized into organic acids, amino acids, proteins, sugar, phenolics and other secondary metabolites which in general are easily used by microorganisms.

The composition and amount of the released compounds is influenced by many factors including plant type, climactic conditions, insect herbivory, nutrient deficiency or toxicity, and the chemical, physical and biological properties of the surrounding soil.

The RE in this study were analysed and the principal LMW compounds found are summarized in Table 2.6. Since these were obtained in distilled water, the composition would differ to RE composition in soil.

Table 2.6. Composition of the roots exudates. Low molecular weight compounds like organic acids, amino acids and sugars released by seeds of *Zea mays*. This mixture of different compounds was used for the determination of fungal growth by spectrophotometry.

Organic acids	ppm	Amino acids	ppm
Citric acid	36.2 ± 3.7	Alanine	7.5 ± 2.3
L-Malic acid	24.2 ±3.6	Arginine	3.5 ± 4.9
Shikimic acid	0.4 ± 0.1	Asparagine	5.9 ± 1.3
DL-Lactic acid	21.2 ± 11.0	Aspartate	8.7 ± 1.4
Fumaric acid	0.7 ± 0.2	Cysteine	1.3 ± 0.3
		Glutamate	6.7 ± 1.3
		Glutamine	10.5 ± 2.6
		Glycine	0.8 ± 0.1
		Histidine	2.9 ± 0.2
Sugars	**ppm**	Isoleucine	1.9 ± 0.9
D-Fructose	121.9 ± 18.9	Leucine	7.8 ± 2.5
D-Sorbitol	16.1 ± 1.7	Lysine	1.3 ± 0.5
D-Glucose	1087.3 ± 175.8	Methionine	0.2 ± 0.1
Arabinose	0	Phenylalanine	3.9 ± 1.3
Mannitol	0	Proline	8.6 ± 2.4
Trehalose	0	Serine	2.9 ± 0.7
Raffinose	0	Threonine	2.7 ± 0.7
Ribose	ND	Tyrosine	4.2 ± 1.1
Xylose	ND	Tryptophan	1.0 ± 0.1
Rhamnose	ND	Valine	4.5 ± 1.2

ND, not detected

2.3.6 Characterization of fungal growth

A total of 162 growth curves based on 124,416-time points were obtained. Replicated growth curves were similar for an individual isolate, and data were averaged to depict the characteristic growth curve for each of the 28 isolates. In general, fungal growth curves showed the typical lag phase (where no changes in OD were determined), followed by a rapid increase in OD (first transition period) which preceded the log phase or exponential growth. Then, a second transition period where the OD tended to reach a plateau and finally, the last stage; the stationary phase where no appreciable change in OD occurred (Figure 2.8). This is the case, for example, for *T. harzianum* F327 and *M. guizhouense* F16 while in contrast in the same conditions *M. anisopliae* A1080 and *M. guizhouense* F142 grew continuously in an apparent exponential growth. On the other hand, when the fungi were grown in PDB they showed the lag phase, the first transition period and then grew exponentially till the end of the study 4 days after inoculation (Figure 2.9).

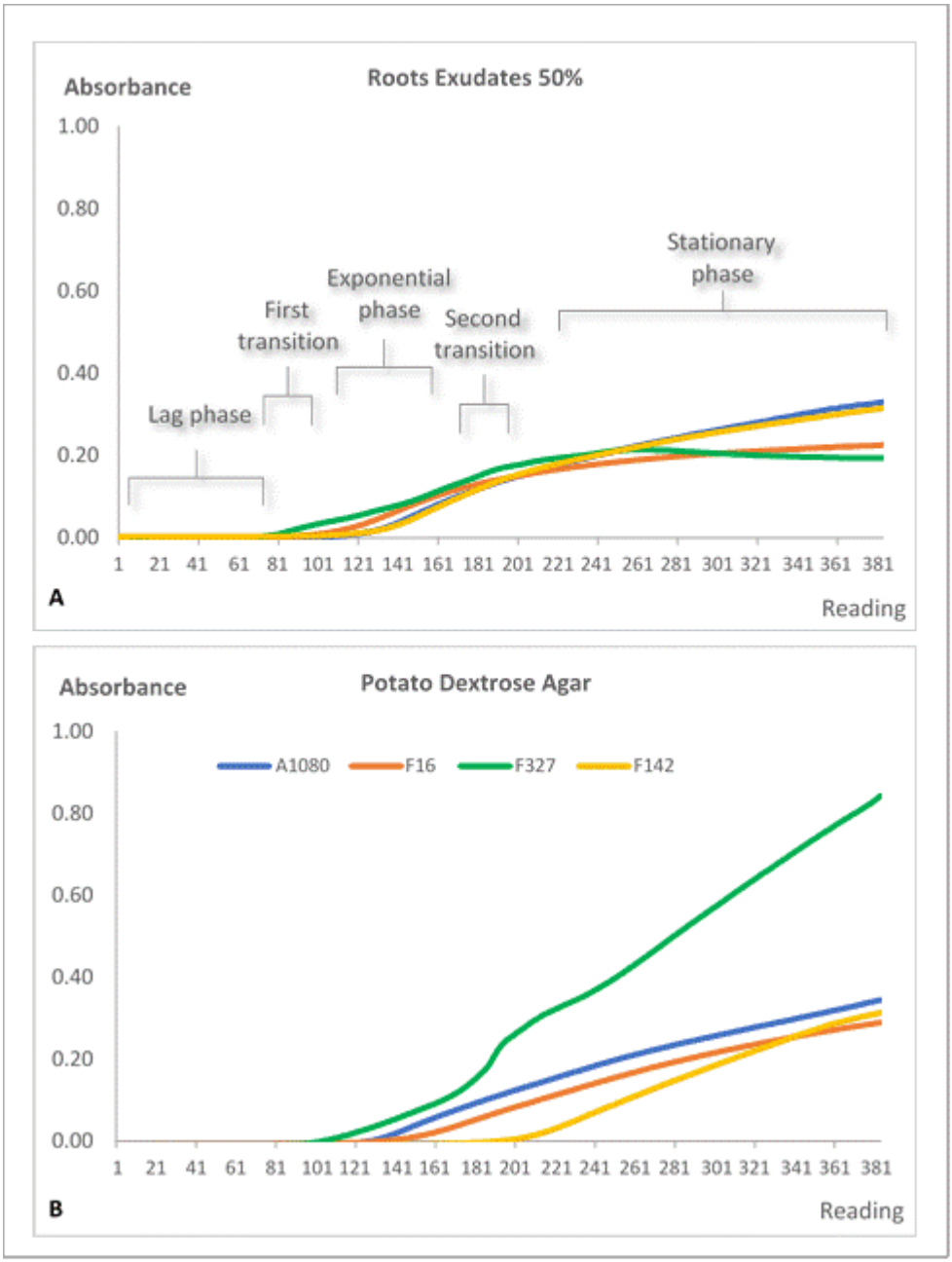

Figure 2.8. Optical density determined by spectrophotometry for fungal growth in roots exudates (A) or potato dextrose agar (B).

During the first 15 hours in RE or 18 hours in PDB, no changes in OD were determined for all the fungi. This period corresponded to the germination of conidia and further elongation of the hyphae which was not detectable until it reached a length of 60 µm. This blind period corresponded to the first 15 hours (60 readings) in RE and to the first 18 hours (73 readings) in PDB (Figure 2.8, A and B respectively). Additionally, only for those fungi in RE which had a stationary phase for the last 40 readings (10 hours) there were no significant differences since no changes in OD were determined.

The values determined for kinetic parameters for fungal growth in each media are depicted in Tables 2.7 and 2.8. These kinetic growth values allowed the characterisation for each isolate of fungal growth on the basis of quantity (change, slope or maximum value), speed (average rate) and time-based. Values have been highlighted with a colour code from green (highest value, percentile 75%), orange – yellow (percentile 50%) to red (lowest, percentile 25%).

Table 2.7. Kinetic parameters determined in the exponential phase growth for fungi grew in half strength roots exudates (RE50%).

Isolate	Change	Max. Rate (min^{-1})	Slope	T MaxR (hr.)	Maximum	Rate (s^{-1})	Value	μ (m^{-1})	$Tlag_f$ (hr.)	ν (m^{-1})	Td (hr.)
A1080	0.242	1.3 E-04	29487	37	0.241	1.1 E-06	0.116	0.0054	19.7	0.0077	2.4
Bb18	0.247	1.6 E-04	30259	51	0.248	1.1 E-06	0.119	0.0041	18.8	0.0059	3.0
Bb21	0.230	1.2 E-04	30875	35	0.229	1.1 E-06	0.121	0.0057	19.9	0.0082	2.3
BK41	0.293	2.0 E-04	38414	46	0.293	1.3 E-06	0.151	0.0050	17.3	0.0072	2.4
F11	0.113	6.6 E-05	8152	65	0.114	4.5 E-07	0.032	0.017	34.1	0.0024	8.4
F120	0.255	1.3 E-04	26678	44	0.253	1.2 E-06	0.105	0.0045	26.6	0.0065	3.3
F133	0.213	1.1 E-04	15792	65	0.214	8.8 E-07	0.062	0.0024	30.0	0.0034	5.0
F137	0.230	1.3 E-04	25825	53	0.228	1.1 E-06	0.101	0.0060	22.3	0.0086	2.0
F138	0.215	1.1 E-04	22379	43	0.214	1.0 E-06	0.088	0.0046	25.3	0.0066	3.5
F142	0.227	1.4 E-04	27196	42	0.227	1.1 E-06	0.107	0.0040	23.8	0.0058	3.2
F144	0.288	1.8 E-04	52675	28	0.386	1.7 E-06	0.207	0.0051	15.1	0.0074	4.2
F148	0.132	7.5 E-05	9356	68	0.134	5.1 E-07	0.037	0.013	35.8	0.018	11.3
F16	0.129	9.3 E-05	20456	32	0.129	5.9 E-07	0.080	0.0060	16.6	0.0087	2.2
F178	0.106	5.9 E-05	7723	68	0.105	4.3 E-07	0.030	0.0022	32.7	0.0032	5.4
F264	0.074	5.2 E-05	4549	74	0.075	2.6 E-07	0.018	0.0021	35.2	0.0031	8.4
F30	0.091	6.0 E-05	6483	62	0.091	3.7 E-07	0.026	0.0022	33.3	0.0032	5.4
F31	0.111	6.6 E-05	8316	61	0.111	4.6 E-07	0.033	0.0022	29.3	0.0031	6.0
F327	0.254	1.1 E-04	36365	29	0.257	1.0 E-06	0.143	0.0081	15.0	0.0117	2.0
F387	0.140	9.9 E-05	8313	73	0.140	5.1 E-07	0.033	0.0029	40.3	0.0042	9.6
F401	0.104	7.8 E-05	5291	79	0.103	3.4 E-07	0.021	0.0026	42.8	0.0038	4.6
F447	0.220	1.3 E-04	22426	47	0.219	1.0 E-06	0.088	0.0057	28.8	0.0083	2.1
F628	0.149	7.5 E-05	11025	66	0.148	6.1 E-07	0.043	0.0045	32.9	0.0065	4.2
F672	0.268	1.5 E-04	36489	34	0.269	1.2 E-06	0.143	0.0060	17.3	0.0086	2.4
F98	0.017	2.4 E-05	1109	84	0.018	4.0 E-08	0.004	0.012	59.1	0.017	10.8
F99	0.083	7.6 E-05	3506	83	0.083	2.5 E-07	0.014	0.0023	49.6	0.0033	7.3
MFI	0.152	1.0 E-04	9448	83	0.150	5.7 E-07	0.037	0.0026	30.8	0.0038	4.4
MW#2	0.060	2.4 E-05	5417	81	0.061	2.5 E-07	0.021	0.018	26.5	0.0027	7.3
MW#8	0.054	2.2 E-05	4346	58	0.053	2.3 E-07	0.017	0.0033	27.1	0.0047	3.7

Kinetic parameters: Change ($OD_{max} - OD_{min}$), Maximum rate (Max. rate min^{-1}); the integral (slope), the time to reach maximum growth rate (T MaxR, hr.), the highest OD (Maximum), the average growth rate (Rate, s^{-1}), the average OD value (value). Additionally, from exponential growth were determined: the specific growth rate (μ min^{-1}); the duplication time or generation time (Td, $hr.^{-1}$) and the time when the lag phase ends (Tlagf, $hr.^{-1}$).

The colour code applied is also consistent with the fact that some high values might not indicate an advantage, i.e. time to max rate, the higher figures are in red since it took longer for the fungi to grow while the highest figures in rate are in green since this is a desirable characteristic. Therefore, parameters values on green for a certain isolate is indicative of a better performance than those values in yellow to red in terms of potential biocontrol attributes. Based on this code performance of fungi can be easily discriminated in each medium (Tables 2.7 and 2.8).

Table 2.8. Kinetic parameters determined in the exponential phase growth for fungi grew in potato dextrose broth (PDB).

Isolate	Change	Max. Rate (min^{-1})	Slope	T MaxR (hr)	Maximun	Rate (s^{-1})	Value	μ (m^{-1})	$Tlag_f$ (hr)	v (m^{-1})	Td (hr)
A1080	0.369	1.3 E-04	98907	37	0.546	1.5 E-06	0.355	0.010	20.7	0.014	12.0
Bb18	0.292	9.6 E-05	81910	63	0.472	1.2 E-06	0.294	0.0066	30.3	0.0095	1.8
Bb21	0.267	1.1 E-04	92975	35	0.450	1.1 E-06	0.333	0.0065	17.9	0.0093	1.8
BK41	0.439	1.3 E-04	107939	54	0.620	1.7 E-06	0.387	0.0089	20.1	0.0129	1.3
F11	0.280	1.2 E-04	82343	68	0.462	1.2 E-06	0.295	0.0039	23.8	0.0056	3.0
F120	0.314	1.2 E-04	91625	39	0.498	1.3 E-06	0.328	0.0025	25.3	0.0037	4.6
F133	0.264	1.1 E-04	78875	57	0.448	1.1 E-06	0.283	0.0200	32.0	0.0289	0.6
F137	0.266	1.1 E-04	88333	38	0.448	1.1 E-06	0.317	0.0112	22.9	0.0162	1.0
F138	0.304	1.1 E-04	88726	39	0.485	1.2 E-06	0.318	0.0070	23.0	0.0102	1.6
F142	0.325	1.3 E-04	83451	53	0.506	1.4 E-06	0.299	0.0069	35.7	0.0099	1.7
F144	0.316	2.0 E-04	105369	40	0.497	1.3 E-06	0.378	0.0094	19.6	0.0135	1.2
F148	0.269	1.0 E-04	77740	71	0.449	1.1 E-06	0.279	0.0185	27.8	0.0267	0.6
F16	0.290	1.1 E-04	88455	43	0.473	1.2 E-06	0.317	0.0081	26.0	0.0117	1.4
F178	0.259	1.0 E-04	80008	58	0.441	1.1 E-06	0.287	0.0165	20.0	0.0237	0.7
F264	0.357	1.7 E-04	88126	78	0.536	1.4 E-06	0.316	0.0156	21.3	0.0225	0.7
F30	0.253	1.2 E-04	76070	69	0.435	1.0 E-06	0.273	0.0148	26.7	0.0213	0.8
F31	0.213	8.1 E-05	72898	60	0.396	8.8 E-07	0.261	0.0044	30.9	0.0064	2.6
F327	0.876	4.9 E-04	153565	47	1.058	3.4 E-06	0.551	0.0631	18.3	0.0910	0.2
F387	0.313	1.6 E-04	77506	63	0.492	1.2 E-06	0.278	0.0197	33.3	0.0284	0.6
F401	0.238	1.5 E-04	74940	85	0.417	9.6 E-07	0.269	0.0312	25.1	0.0450	0.4
F447	0.314	1.0 E-04	88575	40	0.495	1.3 E-06	0.318	0.0118	24.3	0.0170	1.0
F628	0.303	1.1 E-04	83835	64	0.487	1.2 E-06	0.301	0.0164	23.2	0.0237	0.7
F672	0.424	1.6 E-04	107818	36	0.600	1.7 E-06	0.386	0.0089	18.3	0.0128	1.3
F98	0.189	1.1 E-04	62364	91	0.370	6.2 E-07	0.224	0.0206	39.4	0.0297	0.6
F99	0.273	1.3 E-04	75681	71	0.452	1.1 E-06	0.271	0.0167	33.3	0.0240	0.7
MFI	0.152	7.4 E-05	62628	74	0.336	5.4 E-07	0.225	0.012	42.1	0.017	9.9
MW#2	0.119	5.6 E-05	66244	73	0.316	4.8 E-07	0.237	0.0004	36.2	0.0006	26.6
MW#8	0.120	6.2 E-05	59588	73	0.297	4.7 E-07	0.214	0.0320	36.8	0.0462	0.4

Kinetic parameters: Change (OD_{max} – OD_{min}), Maximum rate (Max. rate min^{-1}); the integral (slope), the time to reach maximum growth rate (T MaxR, hr.), the highest OD (Maximum), the average growth rate (Rate, s^{-1}), the average OD value (value). Additionally, from exponential growth were determined: the specific growth rate (μ min^{-1}); the duplication time or generation time (Td, $hr.^{-1}$) and the time when the lag phase ends (Tlagf, $hr.^{-1}$).

In RE50% the best performance among the 28 isolates was *I. fumosorosea* F144, *M. guizhouense* BK41, *M. anisopliae* F672 and *T. harzianum* F327 (Table 2.7, Figure 2.9-A). Concomitantly, these isolates also had the highest maximum growth. This group, together with *M. guizhouense* F16, also had lowest values for both completion of the lag phase and time to reach the maximum rate (Table 2.7). During the period of study, *I. fumosorosea* F144 kept the highest average growth rate, followed close by *M. guizhouense* Bk41 and *M. anisopliae* F672 (Table 2.7). The maximum rate was determined in the *M. guizhouense* Bk41, *I. fumosorosea* F144*, B. bassiana* Bb18 and *M. anisopliae* F672 isolates. Using the kinetic parameters, it was possible to discriminate the different isolates into groups of faster or slower growth ($p<0.01$).

Less differences among the isolates were noted during the exponential growth phase. In this case, the highest velocity was determined in the plant promotor *T. harzianum* F327, which also had the highest generation rate and one of the lowest doubling times. Other isolates, like *M. robertsii* F447, *M. anisopliae* A1080 and F672, and *M. guizhouense* Bk41 and F16 had doubling times below 2.5 hours (Table 2.7).

When fungi were grown in PDB, *T. harzianum* F327 had the best overall growth performance, and higher growth, than the entomopathogenic fungi (Table 2.8). This highlighted the ability of *T. harzianum* to metabolize easily energy sources like dextrose faster than the entomopathogenic fungal species. However, entomopathogenic fungi were close to *T. harzianum* in the time to reach the maximum rate and the time of completion of the lag phase (Table 2.8). In PDB *T. harzianum* had the highest growth rate, followed by *M. anisopliae* A1080 and F672, and *M. guizhouense* Bk41. The maximum rate was determined in *T. harzianum* and *I. fumosorosea* F144.

The parameters specifically related to the exponential growth in PDB medium allowed better discrimination among the isolates ($p<0.01$). The highest specific velocity was determined in the plant promotor *T. harzianum* F327, *M. frigidum* MW#8, and *M. novozealandicum* F401, while the plant growth promotor *M. anisopliae* A1080 had the lowest value. The highest generation rate and lowest doubling times were determined in these isolates also (Table 2.8).

The growth curves for all the isolates in both media, RE50% and PDB, are shown in Figure 2.9. Although there were differences in growth among the isolates, the slow growth in RE50% was probably mainly caused by its complex composition with different sugars, amino acids, and carbonic acids which require the fungal metabolism to adapt to these different components (Table 2.6). The highest growth in RE50% was determined in *I. fumosorosea* F144 although *T. harzianum* seemed to be first to start the exponential growth but was later overtaken by *Isaria*. The entomopathogenic fungi, *M. guizhouense*

Bk41 and *M. anisopliae* F672, were among the entomopathogenic fungi to have the highest growth (Figure 2.9-A). Clearly, growth curves in RE50% allows the discrimination of the fungi better adapted to grow in presence of roots exudates, and this may be extrapolated to growth on the rhizosphere.

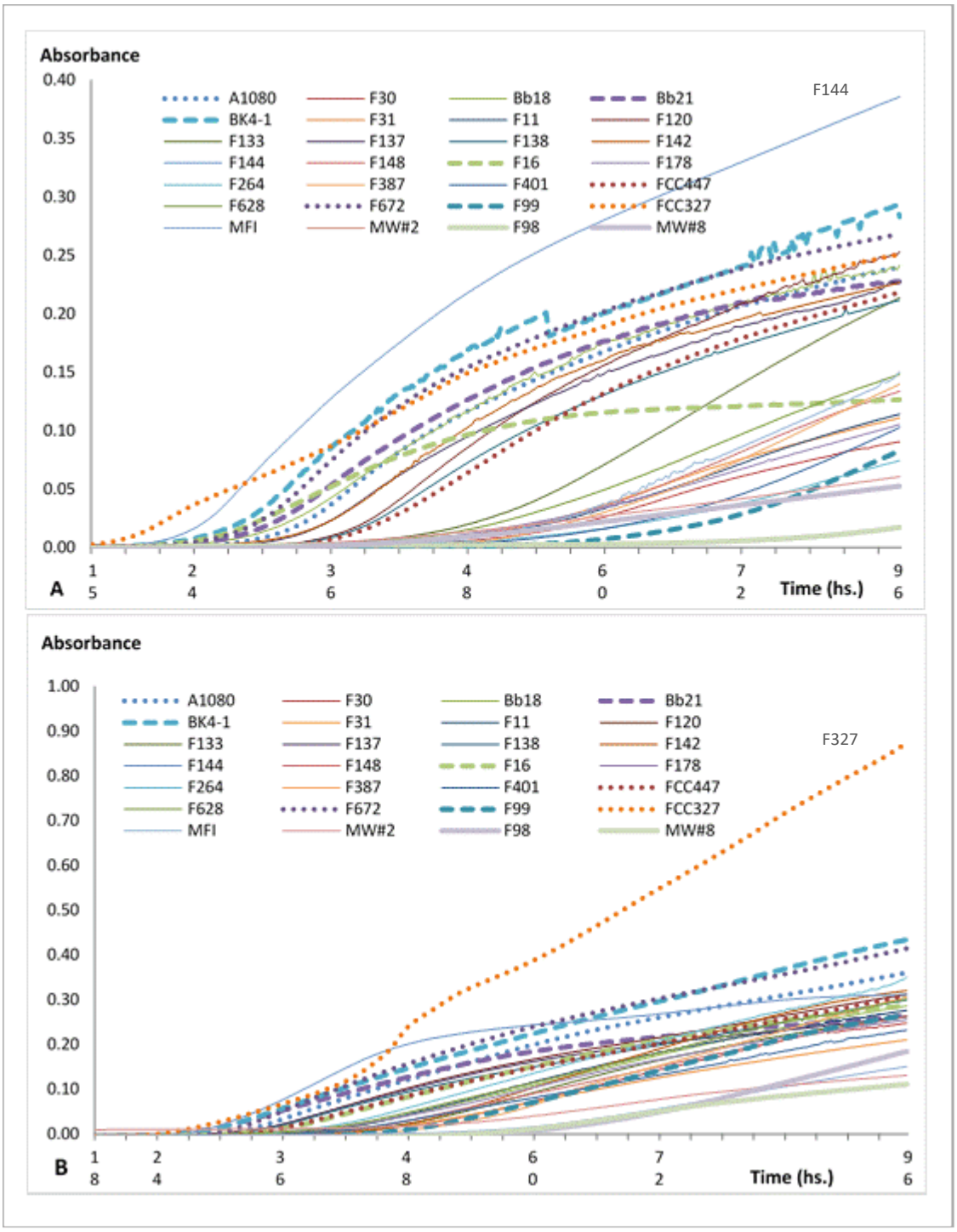

Figure 2.9 Fungal growth of the 28 isolates determined spectrophotometrically in half strength roots exudates (RE50%) (A) and in potato dextrose broth (PDB) (B).

On the other hand, the high growth in PDB of *T. harzianum* clearly highlights the ability of this fungus to metabolize dextrose. This was followed in ability by *M. guizhouense* Bk41, *M. anisopliae* A1080 and F672, *I. fumosorosea* F144 and *B. bassiana* Bb21 which also had the highest growth (Table 2.7 and Figure 2.9-B). In this medium, the fastest ones to enter in exponential growth were *B. bassiana* Bb21, *T. harzianum* F327 and *M. anisopliae* F672 (Figure 2.9-B).

Comparatively, growth in PDB was significantly higher than in RE for all the isolates ($p<0.01$; Figure 2.9). The completion of the lag phase also occurred faster in PDB ($p<0.01$) for most isolates except *B. bassiana* Bb18, *M. guizhouense* F142 and F16, and *I. fumosorosea* F144 (Tables 2.7 and 2.8). Although growth rate was faster in PDB after fungi were in active development ($p<0.01$), the time to reach the maximum rate was similar in both media ($p<0.546$). The maximum rate was, in general, higher in PDB than in RE ($p<0.01$), but while some isolates did not have differences between both media, only two had higher values in RE: *B. bassiana* Bb18 and *M guizhouense* Bk41 (LSD = 4.6×10^{-5}). The kinetic parameters related with growth during the exponential phase (specific velocity, generation rate and doubling generation time) were all higher in PDB than in RE50% ($p<0.01$; Tables 2.7 and 2.8).

Table 2.9. Ranking of isolates by kinetic parameter value in the top ten positions and the last five.

Rank	Change	Max. Rate (min^{-1})	Slope	T Max. (hs.)	Maximum	Rate (s^{-1})	Value	μ (m^{-1})	$Tlag_f$ (hs.)	ν (m^{-1})	Td (hs.)
1	Bk41	F144	F144	F144	F144	Bk41	F144	F327	F327	F327	F327
2	F144	Bk41	Bk41	Bk41	Bk41	F144	F327	F16	F144	F16	F137
3	F672	F672	F672	F672	F672	Bb18	F16	F137	F16	F137	F447
4	F120	F120	F327	F327	F327	F672	F672	F672	Bk41	F672	F16
5	F327	Bb18	Bb21	Bb21	F120	F142	Bb21	F447	F672	F447	Bb21
6	Bb18	A1080	Bb18	Bb18	Bb18	A1080	A1080	Bb21	Bb18	Bb21	F672
7	A1080	Bb21	A1080	A1080	A1080	F137	F142	A1080	A1080	A1080	Bk41
8	F137	F142	F142	F142	Bb21	F120	F138	F144	Bb21	F144	A1080
9	Bb21	F137	F120	F120	F137	F447	F120	BK41	F137	Bk41	Bb18
10	F142	F447	F137	F137	F142	Bb21	Bk41	F138	F142	F138	F142
24	F99	F264	F401	F401	F99	F178	F401	F264	F148	F264	F264
25	F264	MW#2	F264	F264	F264	F264	MW#2	MW#2	F387	MW#2	F11
26	MW#2	F99	MW#8	MW#8	MW#2	F98	F99	F11	F401	F11	F387
27	MW#8	MW#8	F99	F99	MW#8	MW#2	MFI	F148	F99	F148	F98
28	F98	F98	F98	F98	F98	MW#8	F98	F98	F98	F98	F148

There were differences between the kinetic parameters of the isolates in each media tested, RE50% or PDB ($p<0.01$), with the only exception time to max rate ($p = 0.546$). Comparing both media, the change in growth in PDB was on average 56% greater than in RE50% ($p<0.01$; Table 2.7). This fact is true for all the isolates except for *B. bassiana* (Bb18 and Bb21); *M. novozealandicum* (F133 and F137),

M. robertsii F120, and *I. fumosorosea* F144. These isolates had similar growth in either PDB or RE, while the remaining isolates had significantly higher growth in PDB than in RE (LSD = 0.0631). Since some of these fungal isolates are among those with the highest growth, this might indicate that they had the same ability to growth in both conditions, and in particular, indicating a higher adaptability to use a complex source of nutrients than the remaining isolates.

The maximum growth rate (maximum rate) was in general higher in PDB than in RE (Tables 2.7 and 2.8). This was the case, for example, for *M. novozealandicum* F11 and *T. harzianum* F327. Other isolates like *B. bassiana* Bb21 and *M. anisopliae* A1080 and F672 had the same maximum growth rate in both media. Only *B. bassiana* Bb18 and *M. guizhouense* Bk41 had a higher maximum rate in RE than in PDB (LSD = $4.6x10^{-5}$) that could indicate a better adaption of these isolates to the complex nutrient availability present in the RE.

The average rate was also higher in PDB than in RE for most fungal isolates (Table 2.7 and 2.8, $p<0.01$). Only in *B. bassiana* (Bb18 and Bb21), the three *M. robertsii* isolates (F120, F138 and F447) and *M. brunneum* F137 (LSD = 2.3×10^{-7}) the average rate was similar in both media and also values were closer to the average rate determined in PDB.

The average value for specific velocity (μ) and generation rate (ν) represented both in RE only a 27% of the corresponding average values determined in PDB (Table 2.7 and 2.8). Therefore, most of the isolates had a higher μ or ν in PDB than in RE, except for some isolates that did not have significant differences between media. *T. harzianum* had the highest value for both parameters in PDB, but *M. anisopliae* A1080 was the unique isolate with higher μ and ν in RE than in PDB (Tables 2.7 and 2.8). The average doubling generation time (Td) was approximately three hours in PDB and five hours in RE on average ($p<0.01$). In this case, the plant growth promotor *T. harzianum* F327 had the lowest Td in both media while again the entomopathogenic plant growth promotor *M. anisopliae* A1080 had a lower time in RE than in PDB (Tables 2.7 and 2.8).

The completion of the lag phase was approximately two hours earlier in PDB than in RE ($p<0.01$). This is another critical parameter since after this stage, the fungus will start to accelerate the growing process. As expected, the plant growth promotor *T. harzianum* F327 had the shortest lag phase while *M. novozealandicum* the longest (Tables 2.7). Isolates with completion times of the lag phase closer to the value determined in *T. harzianum* F327 were: *B. bassiana* Bb21, *M. guizhouense* F16 and Bk41 and *M. anisopliae* A1080 and F672.

Ranking of the fungal isolates on the basis of specific growth characteristics in RE, the first ten with the best performance were: *M. anisopliae* A1080 and F672; *M. guizhouense* BK41, F16 and F142; *B. bassiana* Bb18 and Bb21; *Metarhizium robertsii* F120 and F447; *Metarhizium brunneum* F137; the plant promotor *T. harzianum* F327 and *I. fumosorosea* F144 (Table 2.9). Among these top ten isolates were found those entomopathogenic fungi isolated originally from plant organs as endophytes: *M. robertsii* F447, *M. anisopliae* F672 and *M. guizhouense* Bk41. In this group were also those which were probe-positive for plant promotion related genes, like *M. anisopliae* A1080 and *T. harzianum* F327.

2.3.7 *Costelytra giveni* bioassays

In general, a higher number of dead and mycotic larvae were found with the higher water content in soil, as expected, demonstrating the importance of moisture for conidia germination and fungal infection ($p<0.01$; Figure 2.10). Mycosis also increased with the concentration of the conidial suspension, with lower percentage of sporulating supporting cadavers at 10^5 conidia/mL than at 10^7 and 10^9 ($p<0.01$), although differences in mycosis at these two higher concentrations were not always significant.

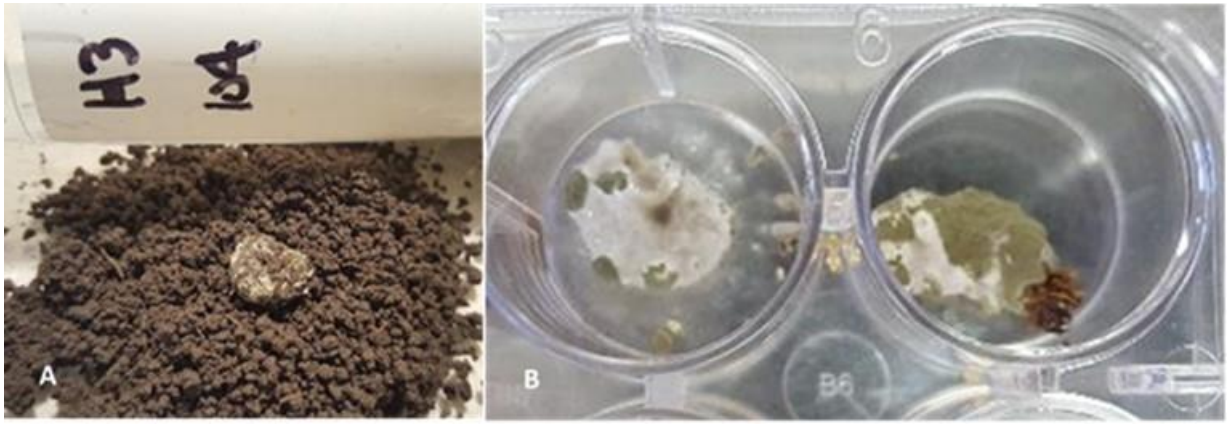

Figure 2.10 Bioassays of *Costelytra giveni* with entomopathogenic fungi. A. Larve with mycelial growth and sporulation. B. Dead larvae with or without signs of fungal infection were transferred to individual wells and kept at 22°C for at least 40 days to determine mycosis.

2.3.7..1 Mortality and mycosis caused by Metarhizium *spp. and* Beauveria bassiana *in 20% (w/v) soil moisture*

At the lowest conidia concentration (1 x 10^5 conidia/mL), inoculation of soil with the *M. guizhouense* BK41 was the first to reach 33% mortality of grass grub larvae, after 15 days post inoculation (DPI) (Table 2.10). By day 20, at this conidia concentration, most isolates presented larval mortalities between 15 and 40% (Figure 2.11). The isolate *M. guizhouense* F16 was the first to cause a mycosis of

25% after 20 DPI (LSD = 11.9; Figure 2.11) while *M. robertsii* F447 was the only isolate to have a value close to 30% by the end of the study ($p<0.01$; Figure 2.12).

Table 2.10. *Costelytra giveni* mortality, in 2nd and 3rd instar larvae, at different conidia concentrations in 20% (w/v) soil moisture over 40 days. All isolates belonged to different species of *Metarhizium* except for *Beauveria bassiana* Bb21.

1 x 10^5 (cond/mL)	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**
A1080	5.5	5.5	11.1	19.5	30.6	63.9	80.5	88.9
Bb21	8.3	16.7	25	25	33.3	83.3	83.3	83.3
Bk41	8.3	25	33.3	41.7	50	50	50	75
F16	8.3	16.7	16.7	25	50	75	75	83.3
F447	0	8.3	8.3	8.3	33.3	50	75	100
F672	0	0	0	0	25	58.3	66.7	75
F99	0	0	0	16.7	41.7	58.3	58.3	83.3
LSD	14.5	20.0	18.9	18.8	24.4	23.3	20.3	16.0
$1x10^7$ (cond/mL)	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**
A1080	0	0	5.5	8.3	41.7	83.3	91.7	94.5
Bb21	0	0	0	8.3	25	58.3	58.3	75
Bk41	0	8.3	8.3	16.7	25	75	91.7	100
F16	8.3	16.7	16.7	41.7	50	83.3	91.7	100
F447	0	0	16.7	16.7	33.3	83.3	100	-
F672	0	16.7	16.7	16.7	41.7	91.7	100	-
F99	25	41.7	58.3	58.3	75	91.7	100	-
LSD	14.5	20.0	18.9	18.8	24.4	23.3	20.3	16.0
$1x10^9$ (cond/mL)	**5**	**10**	**15**	**20**	**25**	**30**	**35**	**40**
A1080	2.8	2.8	8.3	8.3	55.5	88.9	94.5	100
Bb21	0	8.3	8.3	8.3	50	83.3	83.3	91.7
Bk41	0	8.3	8.3	16.7	50	75	100	-
F16	8.3	25	25	50	66.7	100	-	-
F447	0	8.3	33.3	33.3	50	83.3	100	-
F672	0	25	25	33.3	66.7	66.7	100	-
F99	0	8.3	8.3	25	58.3	83.3	91.7	100
LSD	14.5	20.0	18.9	18.8	24.4	23.3	20.3	16.0
Controls	3.3	6.7	6.7	8.3	11.7	11.7	11.7	11.7

At a conidia concentration of 10^7 conidia/mL, *M. novozealandicum* F99 had the highest larval mortality of 42% after 10 DPI ($p<0.01$) which increased to 60% after 20 DPI (Table 2.10). Fungal colonisation and sporulation on the dead larvae was detected at significant levels only after 30 days from inoculation ($p<0.01$). After 35 DPI, 25% of cadavers in the *M. guizhouense* Bk41 treatment supported fungal colonization, while 58% and 75% of cadavers were with mycosis for the *M. anisopliae* F672 and *M. robertsii* F447 treatments, respectively (LSD = 17.6; Figure 2.12).

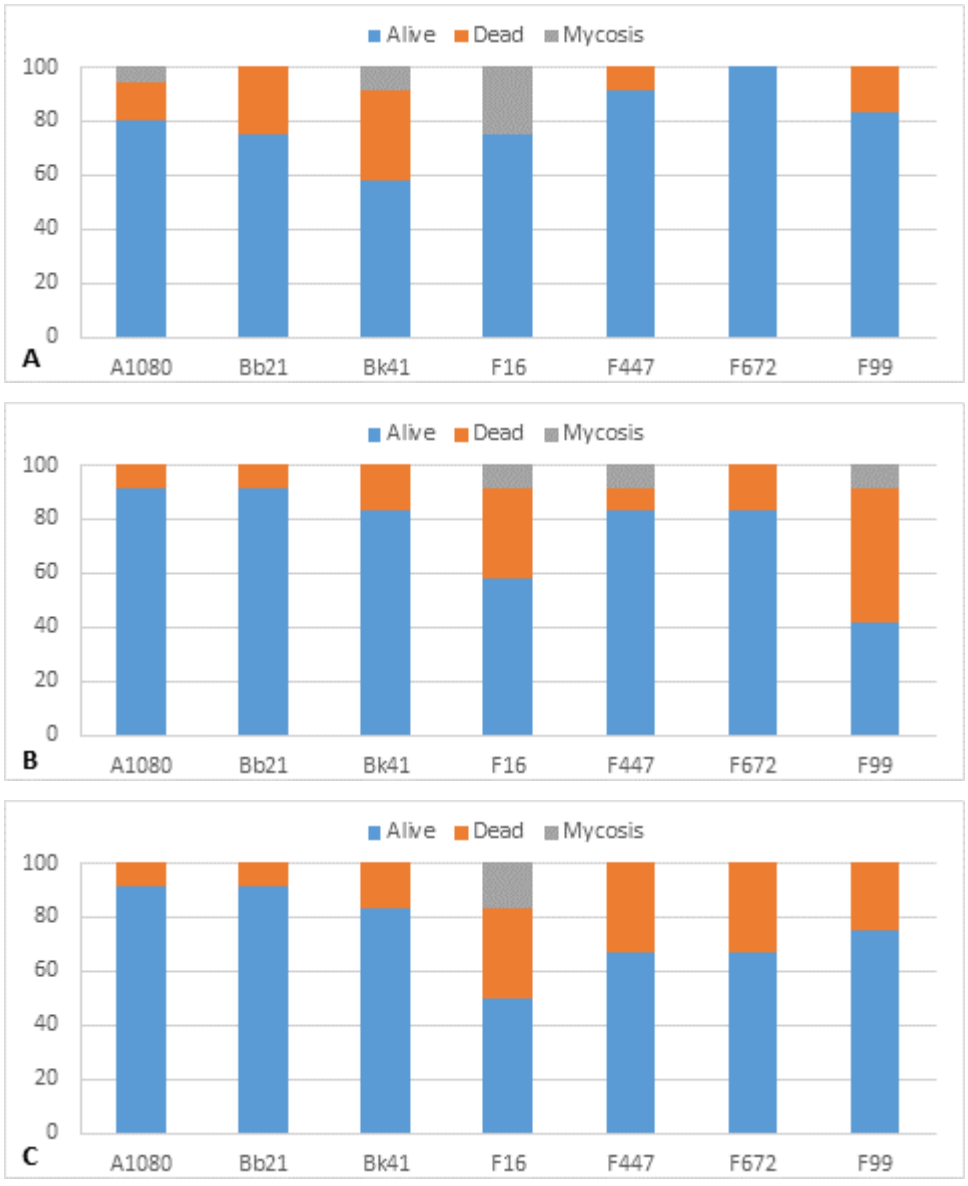

Figure 2.11 ***Costelytra giveni*** **survival, in 2nd and 3rd instar larvae, after 20 days of inoculation with a conidial suspension and at 20% w/w humidity content.** Total larvae population in every treatment was evaluated as percentage of alive, dead without mycosis or dead with mycosis. Therefore, total dead is "dead" + "mycosis". A = 1 x 10^5 conidia/mL; B = 1 x 10^7 conidia/mL; C = 1 x 10^9 conidia/mL. p-values: Alive ($p<0.01$); Dead ($p<0.01$); Mycosis ($p=0.281$). Control dead larva = 8.3%

At 20% w/w moisture content and 20 DPI, larval survival was highest with the *M. anisopliae* F672 treatment at 10^5 conidia/mL, while the lowest survival was in the *M. novozealandicum* F99 at 10^7 conidia/mL ($p<0.01$). Mortality of *C. giveni* larvae with the *M. novozealandicum* F99 treatment at $1x10^7$ conidia/mL was higher than the *M. anisopliae* A1080 treatment at all conidial suspensions, and to *M. robertsii* F477 at conidial suspension 10^5 and 10^7 conidia/mL ($p<0.01$). Mycosis for *B. bassiana* Bb21 was below 1% at 10^5 conidia/mL while no cadavers with signs of fungal infection were observed at the two other higher conidia concentrations.

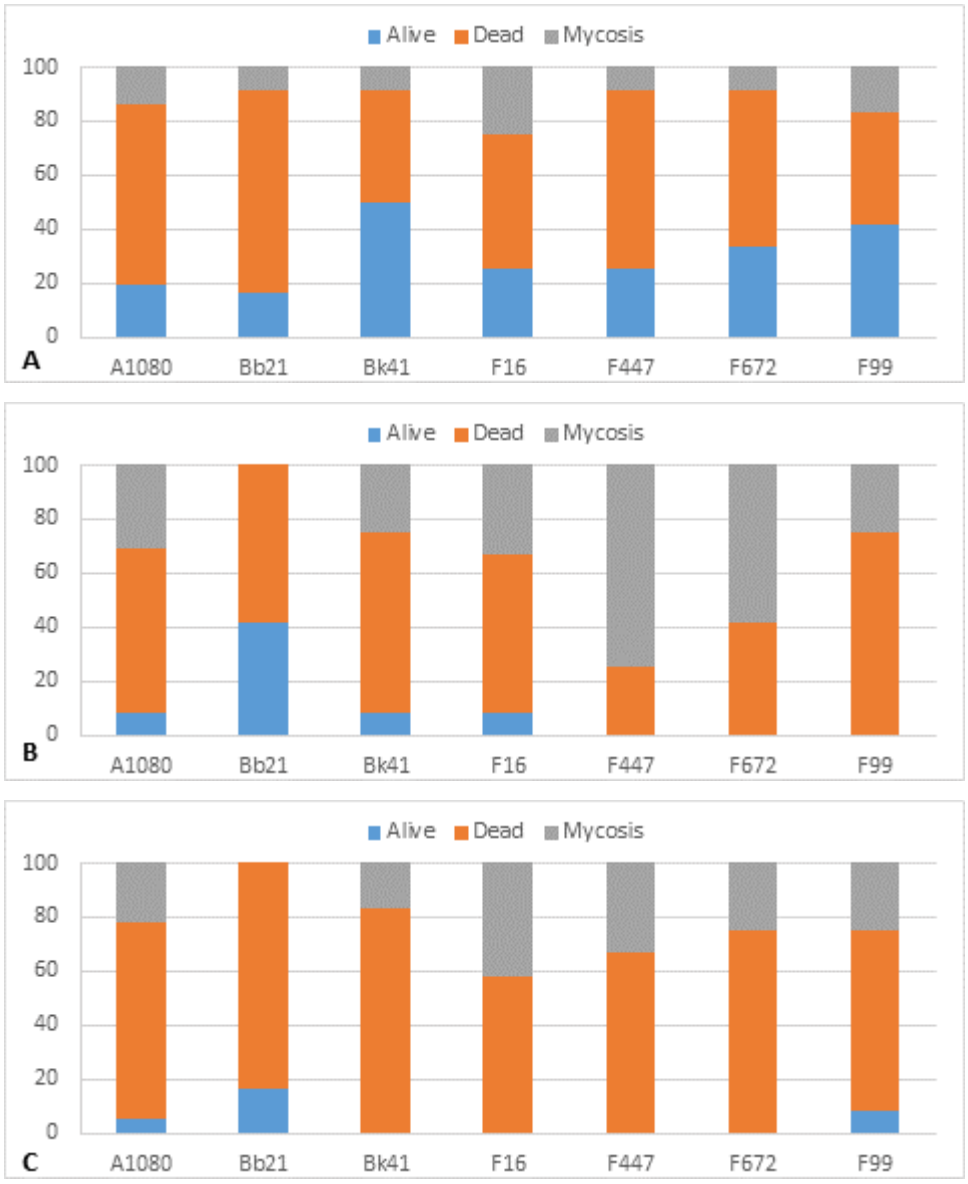

Figure 2.12 ***Costelytra giveni*** **survival, in 2nd and 3rd instar larvae, after 35 days of inoculation with a conidial suspension and at 20% w/w humidity content.** Total larvae population in every treatment was evaluated as percentage of alive, dead without mycosis or dead with mycosis. Therefore, total dead is "dead" + "mycosis". A = 1 x 10^5 conidia/mL; B = 1 x 10^7 conidia/mL; C = 1 x 10^9 conidia/mL. p-values: Alive ($p<0.01$); Dead ($p<0.075$); Mycosis ($p<0.01$). Control dead larva = 11.7%

At the highest conidia concentration (10^9 conidia/mL) *M. guizhouense* F16 and *M. anisopliae* F672 treatment resulted in mortalities of 25% after 15 DPI while in *M. robertsii* F447 had the highest mortality with 33% (Table 2.10). Mycosis signs were first detected in the *M. guizhouense* F16 treatment, representing 17% after 20 DPI (LSD = 16.7) and 42% after 30 DPI (LSD = 17.6; Figure 2.11). At the end of the evaluation, 40 DPI, more fungal infected cadavers were seen in the *M. robertsii* F447 treatment (67%), followed by treatments with *M. guizhouense* Bk41 and F16 with 42% ($p<0.01$).

After 35 DPI, the highest values of *C. giveni* larvae alive were found in the *M. guizhouense* Bk41 and *M. novozealandicum* F99 at 10^5 conidia/mL, while no surviving larvae were found in *M. anisopliae* F672 and *M. robertsii* F447 treatments at 10^7 and 10^9 conidia/mL or with *M. novozealandicum* F99 at 10^7 conidia/mL and *M. guizhouense* Bk41 at 10^9 conidia/mL ($p<0.01$). The highest mycosis percentages were found in *M. anisopliae* F672 and *M. robertsii* at 10^7 conidia/mL ($p<0.01$).

2.3.7..2 Mortality and mycosis due to Metarhizium *spp. and* Beauveria bassiana *in 30% (w/v) soil moisture*

The increase in moisture helped to accelerate the infection process for all the conidia concentration tested. At 10^5 conidia/mL, the treatment *M. guizhouense* F16 was the first to reach 50% mortality after 5 days post inoculation (Table 2.11). By 15 DPI isolates *M. anisopliae* A1080 and *M. guizhouense* F16 both had mortalities of 75% (LSD 5.2; Figure 2.13). The treatment *M. guizhouense* F16 was again the first isolate to reach a 25% mycosis of total larvae treated but, in this case, just after 10 DPI ($p<0.01$; LSD = 2.0). However, as previously noticed, no remaining cadavers showed mycosis for this isolate. At the end of the study, 35 DPI, the highest mycotic values were determined for isolates *M. robertsii* F447 (42%) and *M. guizhouense* BK41 (50%) ($p<0.01$; LSD = 2.2). *B. bassiana* Bb21 and *M. anisopliae* F672 were the only two treatments where no larvae with signs of mycosis were found (Figure 2.14).

At the intermediate conidia concentration of 10^7 conidia/mL, the treatment *M. guizhouense* BK41 was the first to kill all larvae just after 5 DPI (Table 2.11; $p<0.01$). The next treatment to kill all the larvae after 15 DPI was *M. anisopliae* F672 which was followed closely by *M. anisopliae* A1080 with 92% ($p<0.01$). Also, mycoses on the dead larvae were first detected at significant levels in *M. anisopliae* A1080 (25%) after just 5 days from inoculation ($p<0.05$). At 20 DPI isolates which had mycosis above 25% were *M. robertsii* F447, *M. anisopliae* A1080 and F672, and *M. guizhouense* F16 with 66.7% (Figure 2.13). This treatment, at the end of the study, was the only one to reach 83% of cadavers supporting sporulation followed by *M. anisopliae* A1080 and F672 with 42% and 50% respectively ($p<0.01$; Figure 2.14).

Table 2.11. *Costelytra giveni* mortality, in 2nd and 3rd instar larvae, at different conidia concentrations in 30% (w/v) soil moisture. All isolates belonged to different species of *Metarhizium* except for *Beauveria bassiana* Bb21. Larval survival was monitored every 5 days after inoculation.

$1x10^5$ (cond/mL)	**5**	**10**	**15**	**20**	**25**	**30**	**35**
A1080	33.3	55.5	75	100	-	-	-
Bb21	0	0	0	0	25	25	50
Bk41	25	50	50	75	75	100	-
F16	50	50	75	75	100	-	-
F447	25	25	50	75	100	-	-
F672	0	0	16.7	50	58.3	58.3	66.7
F99	25	25	25	25	50	58.3	100
LSD	23.1	18.4	17.8	5.2	9.2	12.6	9.2
$1x10^7$ (cond/mL)	**5**	**10**	**15**	**20**	**25**	**30**	**35**
A1080	83.3	83.3	91.7	100	-	-	-
Bb21	83.3	83.3	83.3	100	-	-	-
Bk41	100	-	-	-	-	-	-
F16	58.3	66.7	75	100	-	-	-
F447	50	50	58.3	100	-	-	-
F672	50	75	100	-	-	-	-
F99	66.7	66.7	75	75	83.3	83.3	83.3
LSD	23.1	18.4	17.8	5.2	9.2	12.6	9.2
$1x10^9$ (cond/mL)	**5**	**10**	**15**	**20**	**25**	**30**	**35**
A1080	38.9	52.8	69.5	91.7	100	-	-
Bb21	8.3	25	50	66.7	66.7	75	83.3
Bk41	25	75	100	-	-	-	-
F16	41.7	58.3	75	100	-	-	-
F447	25	58.3	91.7	100	-	-	-
F672	25	33.3	66.7	100	-	-	-
F99	33.3	58.3	83.3	100	-	-	-
LSD	23.1	18.4	17.8	5.2	9.2	12.6	9.2
Controls	6.7	6.7	10.0	10.0	11.7	13.3	13.3

At a concentration of 10^9 conidia/mL the treatment *M. guizhouense* BK41 after 10 days since inoculation was the first to 75% of larvae dead (Table 2.11). After 20 DPI almost all *Metarhizium* strains caused 100% mortality, but for the *B. bassiana* treatment mortality only reached 66.7% ($p<0.01$).

At 30% w/w moisture content and at 20 DPI, the highest larvae mortality was found in *M. novozealandicum* F99 at 10^9 conidia/mL ($p<0.01$), while mycosis was highest for *M. robertsii* F447 and *M. guizhouense* Bk41 at 10^9 conidia/mL, and for *M. guizhouense* F16 at 10^7 conidia/mL ($p<0.01$). After 35 DPI, alive larvae were only found in treatments *M. anisopliae* F672 and *B. bassiana* Bb21 at 10^5 conidia/mL ($p<0.01$), while the highest values of larvae with mycosis were found in *M. anisopliae* F672 and *M. robertsii* F447 both at 10^9 conidia/mL and in *M. guizhouense* F16 at 10^7 conidia/mL ($p<0.01$).

Figure 2.13 ***Costelytra giveni*** **survival after 20 days of inoculation with a conidial suspension and at 30% w/w humidity content.** Total larvae population in every treatment was evaluated as percentage of alive, dead without mycosis or dead with mycosis. Therefore, total dead is "dead" + "mycosis". A = 1 x 10^5 conidia/mL; B = 1 x 10^7 conidia/mL; C = 1 x 10^9 conidia/mL. p-values: Alive ($p<0.01$); Dead ($p<0.01$); Mycosis ($p=0.01$).

Signs of fungal infection, were detected in 33% of larvae after 10 DPI at 10^9 conidia/mL in *M. guizhouense* BK41 and *M. robertsii* F447. Both isolates increased mycosis on larvae to 58% after 20 DPI and were followed close by treatment *M. guizhouense* F16 with 42% ($p<0.01$; Figure 2.13). At the last evaluation day, the highest mycotic values were recorded for *M. robertsii* F447 and *M. anisopliae* F672 with 66.7%, while for *M. guizhouense* BK41 it was 58% ($p<0.01$; Figure 2.14). At this concentration, no larvae with signs of mycosis were found in the treatment with *B. bassiana* Bb21.

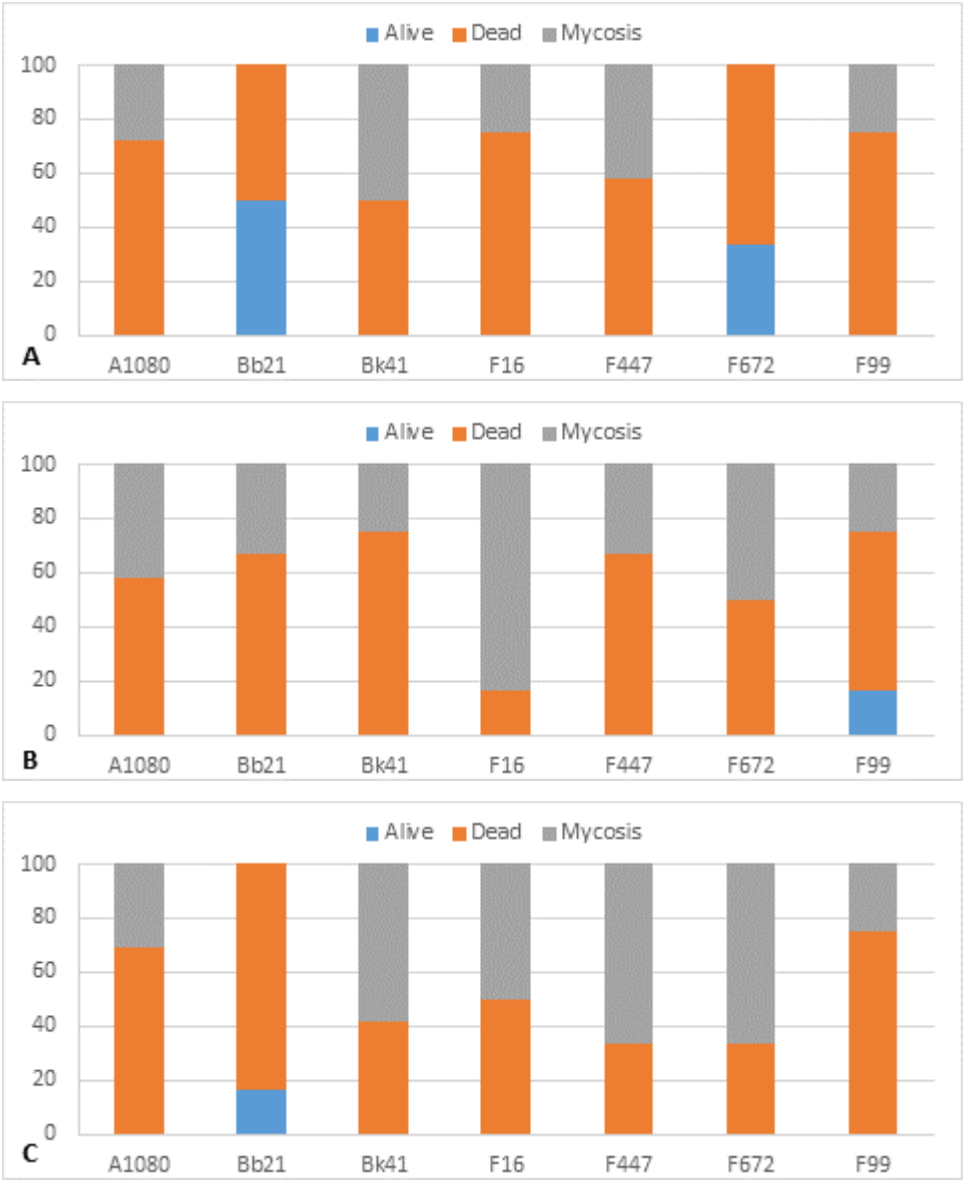

Figure 2.14 ***Costelytra giveni*** **survival after 35 days of inoculation with a conidial suspension and at 30% w/w humidity content.** Total larvae population in every treatment was evaluated as percentage of alive, dead without mycosis or dead with mycosis. Therefore, total dead is "dead" + "mycosis". A = 1 x 10^5 conidia/mL; B = 1 x 10^7 conidia/mL; C = 1 x 10^9 conidia/mL. p-values: Alive ($p<0.01$); Dead ($p<0.367$); Mycosis ($p=0.01$).

2.4 Discussion

Entomopathogenic fungi in the genus *Metarhizium* have proved useful for biological control programmes against economically important arthropod pests worldwide (Vega *et al*., 2008; St Leger *et al*., 2011; Glare *et al*., 2012; Moonjely, Barelli & Bidochka, 2016). An essential first step to elucidate the ecological use of species of *Metarhizium* as entomopathogens, endophytes and/or soil adapted fungi is to define the species (Rehner & Kepler, 2017). However, understanding the true diversity and ecology of these organisms is hampered by convergent morphologies between species (Kepler & Rehner, 2013). The application of molecular techniques has enabled greater resolution of species than possible by morphology alone (Driver *et al*., 2000; Bischoff et. al 2006, 2009; Kepler & Rehner, 2013). In particular, the commonly used biocontrol agent *M. anisopliae* was found to be a species complex composed of nine species.

The present work was conducted with a recently used marker in fungal phylogenetics: the EF-1α gene (Bischoff *et al*., 2006, 2009; Kepler *et al*., 2014; Rehner & Kepler, 2013). In previous studies, using this marker, the genus *Metarhizium* was characterized and new taxons were described. The taxon *M. frigidum* was separated from *M. flavoviride*, while *M. anisopliae* complex was recognised as containing the species: *M. anisopliae*, *M. guizhouense*, *M. pingshaense*, *M. brunneum*, *M. robertsii*, *Metarhizium acridum*, *Metarhizium lepidotae, Metarhizium globosum* and *M. majus* (Bischoff *et al*., 2006, 2009). In the present work, *Metarhizium* isolates from different locations and samples from around New Zealand, were identified to species level using the complete sequence of the EF-1α. The resulting phylogenetic tree placed most isolates originally identified as *M. anisopliae* in the *M. novozealandicum* clade highlighting the importance of this taxon in New Zealand. Other isolates were identified as belonging to *M. robertsii*, *M. brunneum* and *M. guizhouense*, which have agriculture potential (Kabaluk & Ericsson, 2007; Vega *et al*., 2009; Behle *et al*., 2013; Keyser *et al*., 2015; Moonjely, Barelli & Bidochka, 2016). The *Metarhizium* isolates originally obtained as endophytes belonged to *M. robertsii* (F447), *M. anisopliae* (F672) and *M. guizhouense* (Bk41). Only one isolate was grouped in *M. anisopliae* and none of the isolates were found in the *M. flavoviride* taxon. This is the first report about the presence of recently described new species of *Metarhizium* spp. in New Zealand.

The lack of presence of isolates of *M. flavoviride* is interesting since members of this species were described from Europe and Australia and grouped together with isolates adapted to cold regions (Driver *et al*., 2000). In this taxon were described isolates of the species-complex adapted to cold such as *M. flavoviride* var. *novozealandicum* (Driver *et al*., 2000) and later Kepler *et al*. (2014) defined a separated taxon for *M. novozealandicum*. A more exhaustive work with a higher number isolates from New Zealand would be necessary to determine whether the species *M. flavoviride* is present in New Zealand.

The presence of PARB clade in New Zealand, composed of *M. pingshaense*, *M. anisopliae, M. robertsii* and *M. brunneum* (Bischoff et al., 2009), is in agreement with previous research about the world-wide distribution of this clade. *M. robertsii* and *M. brunneum* had been described in America, Asia and Australia while *M. pingshaense* has been mentioned as the only member of the clade with narrow continental provincialism, being isolated mainly from Japan and Australia and a few isolates from North and South America (Rehner & Kepler, 2017). The absence of *M. pingshaense* in New Zealand supports the theory of its Asiatic origin and dispersion from Asia to the western hemisphere. On the other hand, the MGT clade described by Bischoff *et al*. (2009) included the species *M. guizhouense* and *M. majus*, so was partially represented with various isolates of *M. guizhouense*.

However, the classification of *Metarhizium* spp. seems still far from resolved. Previous work in fungal phylogenetics used common conserved genes (BTUB, RPB1, RPB2 and EF1a) which, according to Kepler & Rehner (2013), are likely under-represent in the *M. anisopliae* complex. The same authors in a recent publication described that the species in the PARB clade may encompass several phylogenetic linages. For example, they found that *M. brunneum* included seven well-resolves strongly supported terminal clades, each of which may represent distinct species (Rehner & Kepler, 2017).

The detection and distribution of the genes *mad1*, *mad2* and *mrt* in the isolates of this study was made possible by using degenerated primers that worked with the different species of *Metarhizium* spp. Species of this genus differentially express two adhesin proteins, MAD1 and MAD2, which are used for the adhesion to insect cuticles or plant cell walls, respectively (St. Leger, Wang & Fang, 2011; Sasan & Bidochka., 2012). The importance of determining their presence in the isolates resides in the main purpose of the selection of the isolates. The *mad1* gene is mainly related with the ability of *Metarhizium* to infect insects and is, therefore, a desirable attribute for control insect populations. The genes *mad2* and *mrt* play an important role in the ability of *Metarhizium* to survive in the rhizosphere of plants. Theoretically, isolates which present both genes would have better chance to survive and persist on the roots than isolates without these genes. In a previous work done with fungal mutants defectives in the *mad2* or *mrt* genes, the mutants were unable to promote plant growth and colonize roots, while the rhizospheric compatibility was not affected by mutants defectives in the *mad1* gene (Liao *et al*., 2014).

In this study, the PCR results revealed that the *mad1* gene was found only in 33.3% of the New Zealand isolates and its presence was homogenously distributed among the different species. The *mad2* gene, was detected in 31% of the isolates and particularly in those that also had the *mad1* gene. On the other hand, the gene associated with ability of fungi to assimilate sugars from roots exudates, *mrt*, was

widely distributed among the different species, being detected in 80% of the New Zealand isolates. This fact may support the hypothesis about the ancestral origin of *Metarhizium* as a plant associated fungus which later evolved into an insect pathogen (Spatafora *et al*., 2006; Vega *et al*., 2009; Moonjely, Barelli & Bidochka, 2016).

The *mrt* gene was detected in almost all *Metarhizium* isolates except for the *M. frigidum* MW#2 and MW#8 isolates. This gene was not found in other genera like in the *Beauveria* isolates or in the *Isaria* isolate, nor in other genera of known plant promotor capabilities like *Trichoderma.* Using degenerated primers for the *mrt2** it was possible to detect this gene in all *Metarhizium* isolates, but not in the isolates of the other genera. This might indicate that this is a highly specific and conserved gene in *Metarhizium*, supporting again the theory of *Metarhizium* lineage evolved from plant-associated fungi (Moonjely, Barelli & Bidocka, 2016). The *M. novozealandicum* clade was the only one that, even though all the isolates were from insects, (except for the isolate F133 obtained from soil), none of them amplified for the genes *mad1* and *mad2*, although they were positive for the *mrt1* and *mrt2*. Another two isolates obtained from insects that amplified for the *mrt* gene but not for *mad1* and *mad2* were those belonging to *M. frigidum*.

According to the results obtained with the Dot-blot technique, the *mad1* gene for adherence to the insect cuticle was present in the 79% of the samples. The gene *mad1* for insect adherence seems to be widely distributed among the *Metarhizium* spp. species but for *M. frigidum*. The *ma*d2 gene related to adherence to the plant surface, was also widely distributed among the different genera being found in 82% of the isolates. The gene for the *Metarhizium raffinose transporter* was present in all the isolates but not in *M. novozealandicum* F11. This method confirmed again that genes related to the association of *Metarhizium* to plants are present in almost all *Metarhizium* isolates. Also corroborate previous studies that demonstrated the absence of MRT homologs in *Trichoderma*, while their presence was determined in other genera like *Magnaporthe*, *Fusarium*, *Aspergillus* and *Epichlöe* (Fang & St Leger, 2010; St Leger *et al*., 2011).

The discrepancies between both molecular methods, may be associated with differences in specificity between both techniques. The PCR is highly specific and requires conserved primer sites to amplify a particular sequence. Whereas, dot-blot binds total DNA from a marked probe and it can detect more variable genes similarities, but also can bind to related genes. Additionally, in high DNA concentrations the rate of false positives increased. In comparison studies between the PCR and dot-blot for the detection of virus, it was concluded that the PCR was found to be more sensitive when compared to dot-blot technique (Shekhar, Azad & Ravichandran, 2006; Vassilakos *et al*.,2012).

Consolidating results from both techniques, PCR and DNA hybridization by Dot-blot, there is a high frequency of distribution of the *mrt* gene among the different *Metarhizium* isolates of this study. Additionally, the genes *mad1*, *mad2* and the *mrt* were uniformly distributed among the isolates in the PARB and MGT clades. The *M. frigidum* isolates were without the *mad2* gene while some isolates of the *M. novozealandicum* clade might not have the *mad2* gene. This in agreement with previous works where it was found that plant adhesins (*mad2*) had diverged among *Metarhizium* lineages, contributing to clade-and species-specific variation, while insect adhesins (*mad1*) had been largely conserved (Wyrebek & Bidocka, 2013).

The variability in the composition of *Metarhizum* genome has been noticed before. Previous research has shown that the genome of *M. robertsii* is larger than that of *M. acridum* and encodes more toxins and extracellular enzymes, which could explain the versatility of *M. robertsii* as an insect pathogen or a plant endophyte (St. Leger, Wang & Fang, 2011). In other studies, on the ecological significance of *Metarhizium* in agronomic soils, it was found that *M. flavoviride* was not associated with roots and had lower virulence toward *Tenebrio molitor* larvae than *M. brunneum* or *M. robertsii* isolates but was still pathogenic to above ground weevils (Keyser *et al*., 2015). These results suggested that certain species of *Metarhizium* not only play a role in the regulation of at least some insect populations but also have the capability to associate with plants, therefore have genetic adaptations that allow these ecological roles.

The molecular characterization mirrored the results observed when entomopathogenic fungal isolates were grown in the presence of RE. In general, entomopathogenic fungal species belonging to the clades PARB or MGT grown in RE50% had kinetic parameters values corresponding to a high capability to establish and survive on the rhizosphere. Concordantly, in these group of isolates it was found that the genes *mad2* and *mrt* were present among the species in the clades. On the other hand, *M. novozealandicum* and *M. frigidum* were two species with the lowest performance in RE, and probably with poor rhizo-compatibility. Again, in some of the *M. novozealandicum* the *mad*2 gene was not found and, in both *M. frigidum* isolates was absent.

Based on the results obtained in RE the highest growth among the *Metarhizium* species was in *M. anisopliae*, *M. robertsii*, *M. brunneum* and *M. guizhouense*. Even *Trichoderma´*s growth was behind most of the isolates of these species. In the present work, when fungal growth was evaluated as quantity (change, slope or maximum value) the species M. *anisopliae*, *M. robertsii*, *M. brunneum* and *M. guizhouense* produced more biomass than *M. novozealandicum* or *M. frigidum*. When fungal growth was evaluated as speed (average rate), *M. anisopliae*, *M. brunneum* and *M. robertsii* were faster than *M. novozealandicum* and *M. frigidum*. Finally, when growth was evaluated as time-based, *M. anisopliae*, *M. guizhouense* and *M. brunneum* started to grow before than *M. novozealandicum* or

M. frigidum, while *M. anisopliae*, *M. guizhouense* and *M. robertsii* reached a maximum rate faster than *M. novozealandicum* and *M. frigidum*. These results agreed with reported ability of certain species of *Metarhizium*, like *M. robertsii*, *M. brunneum*, and *M. guizhouense* to associate with roots plants, or even more to become endophytic (Liao *et al*., 2004; Keyser *et al*., 2014; Krell *et al*., 2017).

The spectrophotometric determination of entomopathogenic fungal growth in RE as a method to ascertain rhizosphere competence has not been studied before. Clearly, the method showed that using the kinetic parameters was possible to discriminate the isolates according to their growth in RE which reflected their individual abilities to metabolize nutrients present in the rhizosphere and so, highly probable to stablish them on the roots. Given the complexity of the nutrients presents on the RE that varies between plant species, it would be necessary to evaluate each situation in particular when selecting entomopathogenic isolates with rhizo-compatibility for a certain type of plant.

When fungi were grown in PDB, *T. harzianum* F327 had the best performance, and higher growth, than the entomopathogenic fungi. This highlights the ability of *T. harzianum* to metabolize energy sources like dextrose better than entomopathogenic fungal species. Also, the average rate was higher in PDB than in RE for most fungal isolates. The slower growth in RE50% than in PDB might be based on the complexity of its composition with different sugars, amino acids, and carbonic acids which require the adaptation and use of several metabolic pathways. Isolates with higher adaptability to utilise these nutrients sources would growth faster in RE than isolates with lesser metabolic adaptability or genetic traits like *M. novozealandicum* or *M. frigidum*.

The *B. bassiana* isolates also showed high rate growth in RE. Species of *Beauveria* spp. are considered poor competitors for organic resources against other ubiquitous saprophytic soil fungi (Ownley *et al*., 2010). The capacity of *B. bassiana* to metabolize roots exudates may provide it with an ecological advantage that leads *Beauveria* to root colonization and endophytism. *Beauveria* spp. have been found associated to the rhizosphere of wheat (Renwick *et al*., 1991) and as endophyte in roots and aerial parts of the plants (Vega *et al*., 2009; Bruck, 2010; Ownley *et al*., 2010). Tefera & Vidal (2009) also reported to have found *Beauveria* as endophyte in sorghum after soil inoculations what might indicate a certain level of rhizocompatibility. However, previous research had also demonstrated that *Beauveria* colonization levels in cassava roots decreased with time (Greenfield *et al*., 2016).

Finally, the kinetic values obtained in RE allowed the ranking of the isolates accordingly to their growth in presence of RE. Those isolates with the best performance were originally from plant organs as endophytes: *M. robertsii* F447, *M. anisopliae* F672 and *M. guizhouense* Bk41. In this group were also those with proven plant promotion capacities, like *M. anisopliae* A1080 and *T. harzianum* F327 (Hu & St. Leger, 2002; Chirino-Valle *et al*., 2016). This suggests that fungal growth characterization in root exudates might indicate isolates or species with high rhizo-compatibility.

The mortality in *C. giveni* was, as expected, depended on moisture. Increasing mortality was determined with higher water content demonstrating the importance of moisture for conidia germination and fungal infection (Bruck, 2005). Mycosis also increased with the concentration of the conidial suspension. This might indicate that the lowest conidial concentration, 10^5 conidia/ml, could be the lower threshold for a succesful biological control of *C. giveni* with entomopathogenic fungi, and it may be necessary to use conidial suspension around or above 10^7 conidia/mL.

On average, production of new conidia on cadavers (mycosis) was higher at 10^7 than at 10^9 conidia/ml, except for larvae treated with *M. guizhouense* F16. There are reports indicating that high conidia densities can negatively affect germination by increasing the concentration of fungal volatile compounds, like 1-octen-3-ol, which is produced by numerous species (Nassr, 2013; Wyatt *et al*., 2013). The inhibitory effect of this volatile compound on the germination rates of the entomopathogenic fungus *Lecanicillium fungicola* was between 55 – 98% reduction (Roeland *et al*., 2013). The inhibitory effect on germination, because of a high density of *Metarhizium* conidia, could be potentiated since the confined space where these studies were done, reducing the fungal infection or mycosis percentages determined on *C. giveni* larvae.

M. guizhouense F16, in the three conidial suspensions applied, was the fastest to cause fungal infection on larvae of *C. giveni* at a soil-moisture content of 20%. This fact might indicate that this isolate is able to cope with the drier conditions better than the remaing isolates. The increase in moisture helped to accelerate the infection process for all the conidia concentration tested and improved the infection performance of the isolates in general. *M. guizhouense* F16 at 10^5 conidia/mL was also one of the isolates fastest to cause mycosis and that might be indicative of the virulence and velocity of this isolate to infect larvae of *C. giveni*.

Not all cadavers supported fungal sporulation. This fact was also observed in previous work by Glare (1994). There is a high probability that the fungal infection would be incomplete or affected by other microbes. The mortality levels observed in *B. bassian*a Bb21 and *M. novozealandicum* were high with conidia concentrations of 10^7 and 10^9, but the mycosis was absent in *Beauveria* or just 25% for *M. novozealandicum*.

Chapter 3

Fungal interactions with Maize

3.1 Introduction

Maize is one of the most important food crops in the world, followed by rice and wheat. In 2016, global maize production from 168 different countries was 1060 million tonnes produced from 187 million ha (FAO, 2016). Total maize production in Australia and New Zealand was 610,357 tonnes harvested from 71,274 ha, of which 18,000 ha were from New Zealand, and Uruguay had a total production of 471,000 tonnes harvested from 66,000 ha (FAO, 2016). Maize is a staple food for animal and human consumption in Africa, South and North-America, Pacific islands, Asia and Europe (FAO, 2016). As an important food crop there is an increasing requirement for the use of sustainable practices for production and this includes a reduction in chemical use.

Entomopathogenic fungi can contribute to sustainable production practices and their use in biological control programmes against plant pests and diseases has been the topic of several recent reviews (Moonjely, Barelli & Bidochka, 2016; Parsa *et al*., 2016; Vega 2018; Jaber & Ownley, 2018). Despite the fact that known entomopathogenic fungi encompass more than a 1000 species distributed in approximately 90 genera, only a few species, belonging to the genera *Beauveria*, *Lecanicillium*, *Isaria* and *Metarhizium* have been developed as bioinsecticides (Vega *et al*., 2009; Moonjely, Barelli & Bidochka, 2016). Among these genera, *Metarhizium* has been the most intensively used (Vega *et al*., 2008; Glare *et al*., 2012; Keyser *et al*., 2014; Moonjely, Barelli & Bidochka, 2016).

Species of the genus *Metarhizium* have a world-wide distribution and a broad range of insect hosts making this genus suitable as a biological control agent for different plant pests (Zimmermann, 2007; Keyser *et al*., 2014). *M. anisopliae* in particular has been developed as bioinsecticides for the control of insects that affect field crops. However most of the research in this area has been focused on mass production and little attention has been paid to ecological aspects of the relationship between the fungus and its host (Bruck, 2005; Meyling & Eilenberg, 2007; Vega *et al*., 2009). Since the discovery that *Metarhizium* has the ability not only to infect insects but also to be rhizosphere competent, and probably become endophytic, expectations about the potential of use of *Metarhizium* as a biological control agent has dramatically increased.

Recent studies have shown that different genotypes of *Metarhizium* are closely associated with habitat and plant species (Bidochka *et al*., 2001; Wyrebek *et al*., 2011). The ability of *Metarhizium* to

proliferate on the rhizosphere has been observed for different isolates and this fungal association with the root system benefits the plant host by providing nutrients from sources inaccessible for plants (Behie *et al*., 2012). This suggests that the potential of entomopathogenic fungi have been under-estimated and that these fungi have more complex niches than previously thought (Selosse, Schnedier-Maunoury & Martos, 2018). However, what are the reasons that lead to plants and fungi to associate with each other?

Soil dwelling fungal entomopathogens persist in the soil, and the search for resources in this environment in the presence of other microorganisms can be ferocious and competitive (Inglis *et al*., 2001; Moonjely, Barelli & Bidochka, 2016). Nevertheless, if the fungus has the ability to establish on the plant root surface, the surplus of carbohydrates from the plant should be, in ideal conditions, unlimited in a mutualistic relationship. Thus, the entomopathogenic fungus *M. robertsii* was recently been shown to provide to the plant host with insect-derived nitrogen and in return the fungus receives plant-derived carbohydrates (Fang & St. Leger, 2010; Behie & Bidochka, 2014; Moonjely, Barelli & Bidochka, 2016; Behie *et al*., 2017). Additionally, fungi have the ability to access to nutrients and mineral resources that plants cannot reach, therefore, while extending and reaching into small pores and other regions in the soil that would otherwise be inaccessible to roots, they assist the plants to absorb nutrients from the soil (Hillel, 2008; Hodge, 2017).

Another reason that partially supports the hypothesis of a favourable entomopathogenic fungus – root association is that, as plant symbionts, the fungi might also gain mobility increasing their chances of finding susceptible insect hosts (Keyser, Thorup-Kristensen & Meyling, 2014). The fact that *Metarhizium* can retain its pathogenicity to insects while associated with plant roots could support this hypothesis. In previous studies, Bruck (2005) found that root cuttings inoculated with *M. brunneum* conidia resulted in successful infections of root feeding black vine weevil (*Otiorhynchus sulcatus*) larvae. Kabaluk and Ericsson (2007) discovered *Metarhizium* infected wireworm larvae (*Agriotes obscurus*) after sowing *M. brunneum* treated maize seeds in a field trial. Keyser, Thorup-Kristensen & Meyling (2014) also found that different *M. brunneum* isolates used to coat wheat seeds maintained their pathogenicity towards *T. molitor* larvae after being dispersed by roots.

Entomopathogenic fungi may become integrated into plant tissues. The process of plant penetration has been described in detail for *Beauveria* in maize leaves, starting with a conidium germinating and forming a germ tube. This gradually elongates with hyphae travelling a short distance before entering the plant surface via either natural openings or directly through the epidermal cell walls with the aid of enzymes and mechanical pressure (Wagner & Lewis, 2000; Vidal & Jaber, 2015). The penetration site(s) were randomly located, indicating that *B. bassiana* does not require specific topographic signals

for an appropriate entry site as do some phytopathogenic fungi. Appressorium-like structures at the penetration site of the maize leaves were not found, although these structures have been observed when penetrating the insect cuticle (Wagner & Lewis, 2000). In contrast, it has been observed that, after germinating, conidia of *M. anisopliae* can penetrate some leaf surfaces by forming appressoria (St. Leger, 2008). Whether specific compounds on the plant surface also contribute to the incidence of endophytic colonization of entomopathogens in fungus–plant interactions has so far not been investigated in detail. Inside the maize plants, the hyphae grow through the air spaces between parenchyma cells, and sometimes also within xylem vessels (Wagner & Lewis, 2000; Vidal, 2015). In another work with sorghum plants, it was determined that leaves and stems were colonized by *B. bassiana* to a greater extent than roots, and that the fungus moved to different plant parts through the vascular system (Tefera & Vidal, 2009). However, *Metarhizium* spp. have been found only associated with roots (Bruck, 2005; Hu & Leger, 2002; Keyser *et al*., 2014; Moonjely, Barelli & Bidochka, 2016; Krell *et al*., 2017).

Different studies with several plants after rhizosphere or endophytic colonization with entomopathogenic fungi have revealed that in many cases the association is beneficial for the plant (Vega *et al*., 2009; Behie, Zelisko & Bidochka, 2012; Sasan & Bidochka, 2012; Moonjely, Barelli & Bidochka, 2016; Greenfield *et al*., 2016; Zitlalpopoca-Hernández *et al*., 2017). The benefits gained by the plant host depend on the fungal species involved, and can be as varied as increase in plant biomass and productivity, alleviation of abiotic stresses (i.e., drought, salinity, temperature fluctuations) or improved resistance to biotic stress (i.e, herbivory and fungal disease). As indicated before, increased plant growth might be directly as result of the acquisition of growth-limiting nutrients (i.e., nitrogen and phosphorus) or indirectly through alleviation of stress that would otherwise limit growth (Moonjely, Barelli & Bidochka, 2016; Greenfield *et al*., 2016). For instance, in studies done by Kabaluk & Ericsson (2007) to protect maize plants from *A. obscurus* (wireworm) and increase yields, seeds were coated with conidia of *M. brunneum* strain F52 (originally identified as *M. anisopliae*). The maize plants from seeds with the fungal treatment produced higher yields and fresh weight than untreated seeds, while wireworm cadavers infected with the strain F52 were found in the plots (Kabaluk & Ericcson, 2007). In a different study that coupled both *in vitro* and *in vivo* bioassays, endophytic *M. robertsii* conferred protection against root rot of bean caused by *Fusarium solani* (Sasan & Bidochka, 2013).

A question that remains is whether a continued symbiotic relationship between a plant and an entomopathogenic endophytic fungus is specifically reliant on nutrient exchange. That is, if the fungus were to suddenly stop transferring nitrogen, would the plant perceive the fungus as pathogenic and reject the fungal partner? In mycorrhizal symbioses between *Medicago* and various species of *Glomus*, the exchange of phosphorus and carbon was shown to be bidirectionally controlled (Moonjely, Barelli

& Bidochka, 2016). In previous studies it was proven that both *Medicago* and *Glomus* were able to detect the nutrients provided by the partner and allocate resources based on the most rewarding root/hyphae (Kiers *et al*., 2011). Therefore, plants have the ability to shift resource allocation to reciprocating hyphae and thus, away from less-cooperative hyphae. As a consequence, the fungal symbiont survivability and growth would be compromised in the absence of nutrients provided by roots (Moonjely, Barelli & Bidochka, 2016). For example, in recent studies with drench inoculation of *M. anisopliae* on cassava, Greenfield *et al*. (2016) concluded that *M. anisopliae* colonization rates remained relatively constant or increased over time, whereas *B. bassiana* colonization rates decreased. This conclusion was supported by previous studies showing *M. anisopliae* as rhizosphere competent and persisting well in the soil environment. In contrast, *B. bassiana* did not persist well in the soil which may be explained by the fungus being more commonly found above ground than below ground (Greenfield et al., 2016). However, this may also be the consequence of a less plant-cooperative *B. bassiana* contrasting with a good plant-partner, *M. anisopliae*.

3.2 Plant immune defence in the presence of endophytic fungi

In the presence of plant pathogens or insect herbivores, plants will allocate resources to the synthesis of defence compounds, and as a consequence plant growth may decrease. On the other hand, plants form associations with non-pathogenic root-associated microbes such as mycorrhizae, rhizobia, and rhizobacteria that can promote plant growth by increasing their access to soil minerals (Pangesti *et al*., 2013). The phytohormones salicylic acid (SA) and jasmonic acid (JA) function as important compounds in coordinating the complex signalling pathways involved in *Induced Resistance* (IR) to repel enemies and attract mutualistic organisms above- and below-ground (Dicke & Baldwin, 2010; Pieterse *et al*., 2012; Pangesti *et al*., 2013). Additionally, previous work has shown that different strains of root-colonizing microbes can mediate IR via SA and JA interference (Pangesti *et al*., 2013). For instance, the strains *Pseudomonas fluorescens* WCS417r and SS101 decreased the performance of the generalist leaf feeder *Spodoptera exigua*. However, whereas strain WCS417r does it via JA- and ET-dependent signalling pathways (Pieterse *et al*., 1998), strain SS101 acts via the SA-pathway (van de Mortel *et al*., 2012). These facts support the hypothesis that the combined application of root-associated microbes acting via different phytohormonal signalling pathways may enhance plant defence to either pathogens or insect herbivores (Pangesti *et al*., 2013).

3.2.1 Plant defence manipulation by microorganisms

Some root endophytes are able to evade or manipulate plant hormone signalling in different ways. For instance, some endophytes syntheses auxins and auxin analogues along with gibberellins (GA) which may attenuate SA signalling; others use effectors which modify the hormonal signalling pathways, as is the case for mycorrhizal fungi, while others by transient accumulation of JA at an early

stage of mycorrhiza formation and root-nodule formation, supposedly to bypass the SA-triggered response (Vandenkoornhuyse *et al*., 2015). In a recent study with the root endophyte *Piriformospora indica* it was demonstrated that when endophytic in rice, this fungus was able to elicit the GA pathway supressing the stimulation of JA provoked by root herbivory (Cosme *et al*., 2016).

Entomopathogenic fungi as endophytes can induce systemic resistance in plants (Vega *et al*., 2009). Proteomic analysis of *Phoenix dactylifera* infected with *B. bassiana* or *Lecanicillium* spp. demonstrated the induction of proteins related to plant defence or stress response, while proteins involved in energy metabolism and photosynthesis were also affected (Gómez-Vidal et al., 2006; Ownley *et al*., 2010). Furthermore, endophytic colonization may cause plants to enter a "primed state", which could contribute to the antagonist effects observed against plant pathogens (Ownley *et al*., 2010). In this state, when plants are challenged by biotic stress, the defence response is accelerated by the strongly potentiated gene expression (Van Wees *et al*., 2008).

3.2.2 Plant response to the presence of root herbivory

Root feeder's life stages are in general longer than those of foliage feeders and therefore interact for a longer time with the host plant. The soil dwelling insects are not always in physical contact with a host plant and can graze and detach different parts of the root system which are not necessarily connected. Unlike aerial parts of the plant, roots are protected from abiotic mechanical stresses like wind or rain, and root wounding may be a more reliable indicator of an herbivore attack (Johnson, Erb & Hartley, 2016). Additionally, an open wound in the soil matrix would provide entry for a large number of root-associated microorganisms, many of which produce elicitors that trigger pathogen-related responses (Berendsen *et al*., 2012; Johnson, Erb & Hartley, 2016). In this way, following herbivore recognition, plants reconfigure their metabolism through changes in phytohormonal networks. Jasmonates, which are widely viewed as the master regulators of plant responses to herbivores, are less inducible in the roots than the leaves. Nevertheless, roots respond to herbivore attack by increasing their jasmonate production regulating root resistance (Pierre *et al*., 2012). SA signalling for instance, can buffer the JA response aboveground, and in contrast to leaf herbivore attack, root herbivore attack does not seem to induce SA signalling, which again may boost JA signalling (Johnson, Erb & Hartley, 2016).

3.3 Opportunity

The endophytic capabilities of entomopathogenic fungi have the potential to enhance biocontrol of soil-dwelling pests or plant pathogens which cannot be easily controlled by pesticides (Moonjely, Barelli & Bidochka, 2016).

Numerous species of insects are pests of maize crops worldwide (CSIRO, 2007). The main pests of maize are *Helicoverpa armigera* (corn earworm) and a number of soil-dwelling insects. Soil-dwelling insects can seriously reduce plant establishment, plant populations, plant growth, and subsequent yield potential. Maize seed treatments are a method to deter feeding and prevent damage. *Costelytra giveni* larvae usually inhabit the soil for 6 - 9 months of the year feeding on roots of maize or other plant species (Jackson & O´Callaghan, 2006). *C. giveni* is mainly controlled by the application of pesticides to the seed or through biological control agents like *Serratia entomophila* (Jackson, Huger & Glare, 1993; Glare, 1994; Jackson & O´Callaghan, 2006).

Fusarium graminearum is a common maize pathogen in many areas of the world that often causes economically devastating diseases at different stages of plant development, such as seedling blight, maize root rot, wheat head blight (FHB or scab), and maize ear rot, leading to a reduction in the quality and yield of the crop (Duan *et al*., 2016). The fungus colonises the maize plant becoming endophytic/pathogenic but can also switch from a biotrophic to a necrotrophic life style where it produces mycotoxins like trichothecenes which cause serious health problems in animals and humans when consumed through contaminated maize grains (Pan *et al*., 2007; Oldenburg *et al*., 2017). There are different strategies to reduce the incidence of *F. graminearum* but further work is required to eradicate the disease from maize crops and reduce the production of mycotoxins (Pan *et al*.; 2007; Oldenburg *et al*., 2017). The development of a seed coating with the ability to both prevent feeding by insect pests and infection by *F. graminearum* is highly desirable.

3.3.1 Objective of this chapter

The hypothesis tested in this study was to assess whether maize plant development in the presence of *C. giveni* and *F. graminearum* could be improved by coating entomopathogenic fungi onto the maize seeds and whether the endophyte elicits induced resistance in the plant. To fulfil this goal there were four main activities. First, the effect of entomopathogenic fungal isolates applied to maize seeds as a conidial coating on seed germination and plant growth, was determined. Second, maize plants treated with selected entomopathogenic fungal isolates were grown in the presence of *C. giveni* and *F. graminearum* and subsequent plant performance was evaluated. Third, the ability of the fungal isolates coated on maize seeds to form an association with roots or to become endophytic, was determined. Finally, the contents of salicylic acid and jasmonic acid in maize plants grown from fungal coated seeds were determined, to evaluate any changes in the induced response in the plants compared to plants without fungal treatments.

3.4 Material and methods

3.4.1 Maize seeds

The seeds used were of the hybrid maize, *Zea mays*, designated as 34H31 (Pioneer) which was produced by crossing two Pioneer Hi-Bred International, Inc. proprietary inbred maize lines (US patent 6,897.360 B1). Hybrid 34H31 is characterized by stable yield performance across a wide range of environments, early flowering, good drought tolerance and excellent stalk strength through mid to late season.

3.4.2 Fungal cultures and conidial suspensions

Fungal isolates were obtained from the collections of AgResearch, the Bioprotection Research Centre, Landcare Research fungal collections (International Collection of Microorganisms from Plants) and from the ARS-USDA entomopathogenic fungal collection (Table 2.1). Isolates used are described in the following results section. Conidial suspensions were obtained with the following methodology: PDA plates were inoculated by spreading conidia of the fungal isolate onto the surface and incubating at 22°C in light:dark conditions (12:12 h). After 14 days conidia were harvested from the PDA plates with 3 - 5 mL of 0.01% Triton X-100 and a sterile hockey stick was used to help dislodge the conidia from the fungal colony. The suspension was transferred from the Petri plate to a 15 mL Falcon tube containing 5 glass beads of 0.5 mm diameter. Conidia concentration was determined using a haemocytometer (Neubauer chamber) and conidial suspension was adjusted to the required concentration using a solution of 0.01% Triton X-100 when necessary.

3.4.3 Seed coating

Maize coating was achieved in two steps. As a first step (pre-coating), the seeds were covered with a thin layer of a solution of methylcellulose in water (1% w/w). This first coating smoothed the seed from irregularities, and homogeneously covered the hydrophobic surface in preparation to receive a second polymer containing the conidia in suspension. For the precoating 76.2 g of methylcellulose solution was slowly applied to 350 g of maize seeds by spraying onto the seeds contained in a rotary pan coater (ERWEKA, AR403) at 200 rpm. During pre-coating the methylcellulose film was dried with the assistance of a hand dryer at low temperature (Figure 3.1).

After pre-coating, seeds were left to rest overnight in a laminar flow cabinet. A second coating with a polymer gel containing the conidia was applied to the pre-coated seeds. The polymer was composed of xanthan gum (0.1 g), canola oil (0.1 g) and 10 mL of the spore suspension in 0.01% Triton X-100 (Figure 3.2).

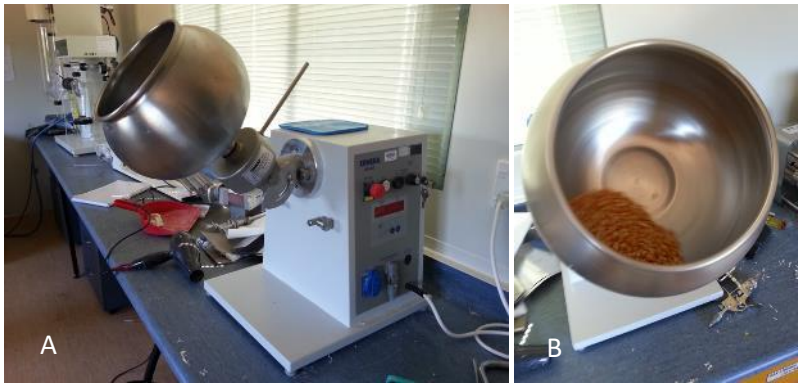

Figure 3.1 Seed pre-coating. A seed coater (A) with a rotary pan (B) was used for the precoating of the maize seeds with a solution of methylcellulose (1%).

Subsequently, 7.8 g of the biopolymer was added to 90 g of pre-coated maize seeds and mixed by hand. Finally, in two consecutive steps with mixing, 7.8 g of bentonite and 7.8 g of talc were added to the coated maize (Figure 3.3). For the control seeds, the second coating was made with the polymer gel, omitting the conidia.

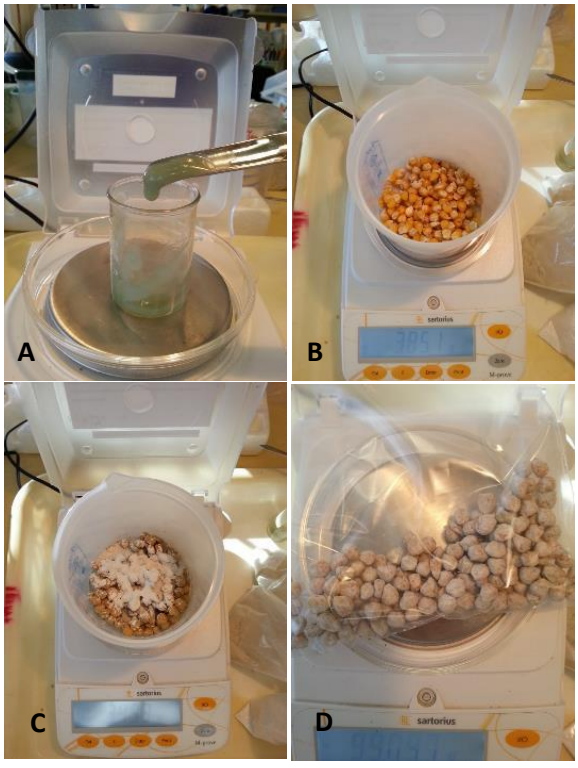

Figure 3.2 Maize seeds coating. A biopolymer containing conidia, xanthan gum and canola oil (A) was added to the maize seeds pre-coated with methylcellulose (B). After the biopolymer was mixed with the seeds, bentonite and talc were added (C). These two ingredients help to set the coating and at the same time prevent conglomeration of the seeds (D).

3.4.4 Determination of the density of conidia coated onto the seeds

The final quantity of conidia coated onto the seeds was evaluated through the determination of colony forming units (Petrikkou *et al*., 2001). After coating, approximately 10 g of maize coated seeds were weighed accurately and transferred to a Falcon tube (50 mL) containing 30 mL of a solution of 0.01% Triton X-100. The seeds and solution were shaken for 30 min in a flask-shaker at maximum speed (Stuart). Dilutions (1:10; 1:100 and 1:1000) of the conidial suspensions were prepared in 0.01% Triton X-100 and 100 µL aliquots of dilutions were plated onto Petri plates containing water agar. Inoculum was spread across the plate using a glass hockey stick. Conidial suspensions were inoculated in triplicate plates and incubated at 20 ± 2°C in light:dark conditions (12:12 h). After 5 days, the number of colony forming units (CFU) per dilution was determined and the number of conidia/g of maize seed was determined. The efficiency of the coating was calculated as the percentage of the original number of conidia coated onto the seeds and the final number of CFU/g of maize seeds.

3.4.5 Seed germination tests

Two germination tests were used: the *between paper method* and the *sand germination method* to determine the effect of coating components and coating with fungi on maize seed germination, respectively. The seeds were arranged in replicates and tested under favourable moisture conditions in accordance with the methods and procedures recommended on the International Rules for Seed Testing, ISTA (2017).

3.4.5..1 Between paper method

Coated seeds were evenly spaced 2 cm from the top edge of a double layer of germination paper, (Anchor; 40 x 29 cm) which had been moistened to saturation with distilled H_2O. The row of 25 maize coated seeds was secured by placing a third layer of paper on top and then rolled up into a tube, which was placed standing upright in a container with seeds in the top. Each roll was sealed in a plastic bag to keep moisture and reduce evaporation (Figure 3.3) and then placed for 7 days in a plant growth chamber (Panasonic MLR 352H-E) with a light:dark regime (16:8 h) and alternating temperatures 20:30°C, respectively. The resulting data from this setup will be referred to as the between paper method (BPM) with three treatments: coated seeds (CS), pre-coating seeds (PC) or pure seeds (PS). Control seeds were pure uncoated maize seeds. The setup was repeated on eight separate occasions to complete four repetitions of 50 maize seeds per treatment. Statistical design was a randomised block design with eight blocks of the three treatments. Germination was assessed after 7 days of incubation. Statistical analysis was carried out using an analysis of variance with eight blocks (= assays) and three treatments, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

Figure 3.3 Between paper method germination test. A row of 25 maize seeds was evenly spaced 2 cm from the top edge of a double layer of germination paper moistened to saturation with distilled H_2O. Seeds were then secured by placing another layer of paper on top (A). The three layers of paper with seeds were rolled up into a tube and then placed standing upright with seeds in the top. Each roll was sealed in a plastic bag to keep moisture and reduce evaporation (B).

3.4.5..2 Sand germination method

Maize seeds were coated with two different conidia concentration, 1 x 10^5 and 1 x 10^8 conidia/mL, obtained from *M. novozealandicu*m (F11, F99 and F133); *M. robertsii* F138; *M. guizhouense* F16 and *M. anisopliae* F672. Coated-fungal seeds were evenly spaced 4 cm apart from each other in a tray containing 6 cm depth of sand moistened to saturation with distilled H_2O (Figure 3.4). The seeds, 100 per tray, were later covered with 2 cm layer of sand and incubated in a glass house at 20°C ± 2 for 7 days.

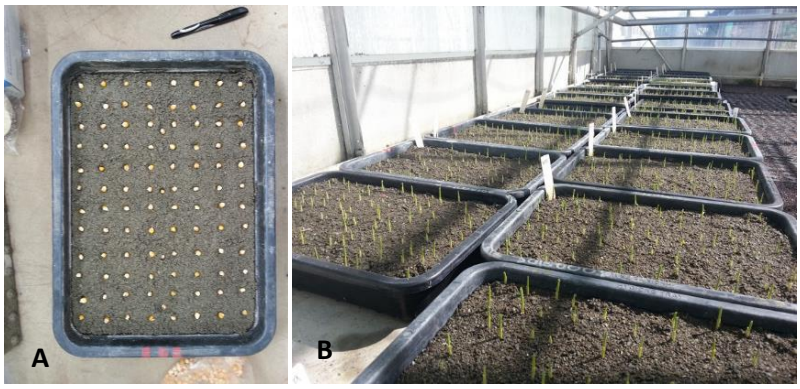

Figure 3.4 Sand germination method. Maize fungal-coated seeds were evenly spaced 4 cm apart from each other in a layer of sand moistened (A). The seeds, 100 per tray, were later covered with sand and incubated in a glass house at 20°C ± 2 for 7 days.

Control seeds were maize seeds only with pre-coating and seeds with the complete coating but without any fungi. The resulting data from this assay will be referred to as the sand germination method (SGM) with treatments: pre-coating seeds (PC), coated seeds (CS), and the fungal coating treatments at the two conidial concentrations. Each randomised assay consisted of a single replicate tray of six isolates at two conidial concentrations, and two seed controls without a fungal treatment, pre-coating (PC) and complete coating (CS). During this study seedling shoot length was also determined. The assay was repeated on separate occasions to complete two repetitions of 100 maize seeds per treatment. Statistical analysis was carried out using an analysis of variance for a randomised block design with two blocks (= assays) and 14 treatments in a 6x2+2 factorial design, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

3.4.6 Evaluation of maize plant performance after coating with conidia from entomopathogenic fungi

Maize seeds were coated with two different conidia concentration, 1 x 10^5 and 1 x 10^8 conidia/mL, obtained from *M. novozealandicu*m (F11, F99 and F133); *M. robertsii* F138; *M. guizhouense* F16 and *M. anisopliae* F672. Coated seeds were sown in pots of 1 L of capacity containing 920 g of potting mix without additional nutrients. The potting mix consisted of 80% bark, 20% pumice and horticultural lime 1 g/L of soil. Soil was watered with 400 mL of tap water and transferred to a glasshouse were medium temperature and relative humidity were 22°C and 60%, respectively. For three weeks plants were grown and watered every seven days with 400 mL of tap water. Controls included pre-coating (PC) and complete coating (CS) seed treatments without fungi. Each trial consisted of three randomised blocks, with four pots for each treatment in each block. Treatments were 6 isolates at two conidial concentrations, plus a pre-coating (PC) and complete coating (CS). Experiments were repeated twice. After one-week emergence was quantified and at the end of the study shoot height was determined. To combine data over the two trials, treatment means were input into an analysis of variance which treated trials as “blocks”, with treatments having a 6x2 +2 factorial structure, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

3.4.7 Effect of fungal seed-coating on maize growth in the presence of *Costelytra giveni* and *Fusarium graminearum*

Based on the results obtained of the effect of the conidia concentration on seed germination and plant development, together with the final results on quantity of viable conidia coating onto the seeds (CFU/g), it was decided to use a conidia concentration of 1 x 10^6 conidia/g of seed, intermediate between the two previously used coatings. For this purpose, maize seeds were coated with 1 x 10^8 conidia/mL obtained from the *M. anisopliae* F672; *M. guizhouense* Bk41 and F16; *M. novozealandicum* F99, *M. robertsii* F447 and *B. bassiana* Bb21, isolates. Controls included two fungal strains known as

plant growth promotors, *M. anisopliae* A1080 (Hu & St. Leger, 2002) and *Trichoderma harzianum* F327 (Chirino-Valle *et al*., 2016), and maize seeds coated but without fungal agents (control seeds). Fungal-coated maize seeds, and control coated seeds (without fungi), were sown in 1 L pots capacity containing 920 g of potting mix. Maize growth was also evaluated in the presence of two larvae of C*. giveni*, and of *F. graminearum* (the biomass of the fungal pathogen was incorporated in potting mix at 0.5% w/w) per pot. The experimental design was a randomised split plot with a single replicate (for each repetition in time) of four main-plots with 2 x 2 factorial main-plot treatments of only potting-mix; potting-mix challenged with two larvae of *C. giveni*; potting-mix where plants were challenged with *F. graminearum* (0.5% w/w); and potting-mix challenged with both *C. giveni* and *F. graminearum* (0.5% w/w). After sowing four seeds per pot in each block, pots were watered with 400 mL of tap water (Figure 3.5). After four days emergence was quantified, and in blocks with *C. giveni* challenge, all but one maize plant per pot were gently pulled out. Subsequently, two larvae of *C. giveni* were added to the pot containing only one maize plant.

Figure 3.5 Maize seeds were coated with 1 x 10^8 conidia/mL from entomopathogenic fungi were sown in pots containing potting mix and grown at 24.3 ± 2.5°C with light:dark conditions (16:8 h) and 65 ± 10 RH% (A). Plants were challenged with biotic treatments which included two larvae of *Costelytra giveni* per pot or *Fusarium graminearum* incorporated into the potting mix (0.5% w/w), both challengers together or none of them. After 4 – 5 days plant emergence was checked (B). Plants after one week (C) and maize plants after two weeks (D).

Plants were allowed to grow for two weeks in the Biotron (Lincoln University, control temperature facility), at 24.3 ± 2.5°C, (referred as 25°C) in 16:8 h light:dark conditions, 65 ± 10 RH% and watered each 4 days with 300 mL of tap water. Two weeks after sowing, plants were harvested, and dry weight determined. Maize plants were pulled gently from the soil in order to not disrupt the roots, soil was

shaken off, and then washed off thoroughly with tap water. Stem and shoots were split from roots and plant samples placed individually in brown paper bags and incubated at 65°C to dry until constant weight (Contherm Thermotec 2000). Experiments were repeated three times. To combine data over the three trials, data were input into a split plot analysis of variance which treated trials as "blocks", with main-plot treatments having a 2 (*C. giveni*, no *C. giveni*) by two (*F. graminearum*, no *F. graminearum*) factorial structure, and with sub-plot treatments being the fungal treatments. The latter were compared using an unprotected least significant difference (LSD) procedure (Saville, 2015).

Fusarium graminearum inoculum production

F. graminearum 13083 was obtained from Landcare Research had been isolated from a root rot sample of maize. *F. graminearum* 13083 was grown in PDA Petri plates of 9 cm of diameter at 25°C with light:dark conditions (12:12 h). After 14 days 10 plugs of approximately 1.0 cm diameter were taken from the edge of the fungal colony grown on PDA and transferred to 1 L Erlenmeyer flasks containing 500 mL of malt extract broth (MEB). The flasks were closed with a cotton plug to allow the exchange of gases and incubated in an orbital shaker incubator (CoconoTU 4540) at 180 rpm and 25 ± 2°C. After 7 days, biomass was harvested by centrifugation for 5 minutes at 400 rpm at 4°C. Using this methodology, it was possible to obtain up to $1.3 \times 10^8 \pm 5.0 \times 10^7$ blastospores/mL and 69.7 ± 3.4 g/L of *F. graminearum* biomass. In the studies of the induction of resistance challenged by *F. graminearum* 13083, the biomass of the phytopathogen was incorporated in potting mix at 0.5% w/w and mixed by hand to homogenize. The mix was used to fill pots and then coated maize seeds were sown and grown for two weeks at 25°C. At the end of this period the average recovery of *Fusarium graminearum* after plating onto *Fusarium* selective medium (FSM) was 396.0 ± 224.4 CFU/g of potting mix.

Costelytra giveni collection and processing for maize plant trials

Grass grubs of *C. giveni*, 2nd and 3rd instar, were collected from the field and individually kept in a 24 well-plate with a small piece of carrot at 20 ± 2°C in the dark. After 72 h, larvae were checked for feeding and natural mortality (Figure 3.6). Only healthy feeding larvae were selected for the maize challenging experiments. Only larvae that actively buried themselves in the first 2 minutes were used in the experiments, otherwise they were removed and replaced by new larvae. For the next week larvae were in contact with maize roots from fungal-coated seeds. At the end of the experiment, when plants were harvested, larvae of *C. giveni* were recovered and individually placed in a well of a 24-well plate. Plates with larvae were put in a humid chamber to promote fungal development and incubated in the dark for 24 days at 20 ± 2°C. Larvae were periodically monitored for signs of fungal infection, and those with mycosis of *Metarhizium* or *Beauveria*, fungi were isolated and colonies observed macro and microscopically to confirm infection from the fungi used in the seed coating.

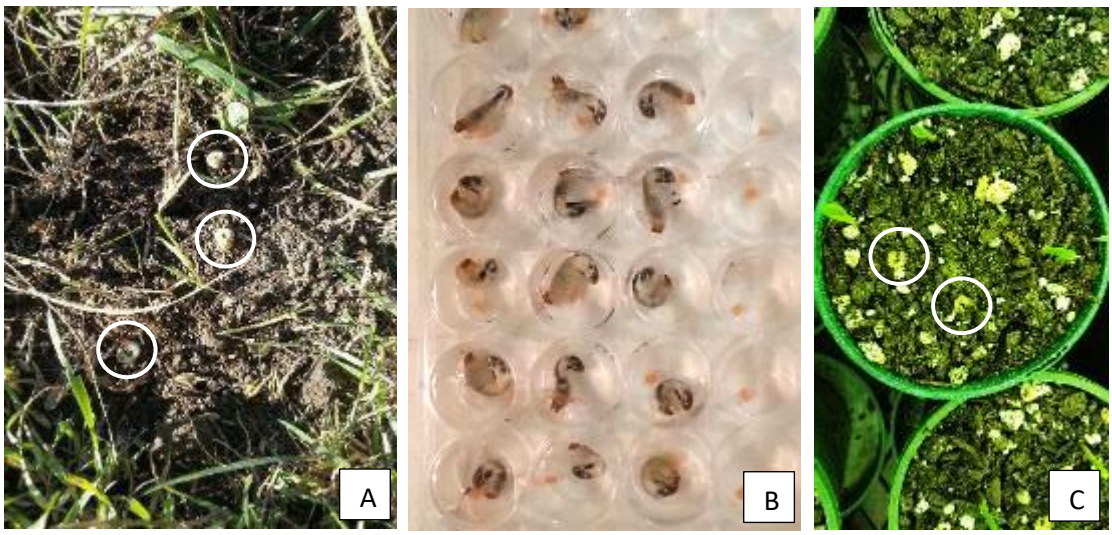

Figure 3.6 ***Costelytra giveni*** **collection and processing.** *C. giveni*, 2nd and 3rd instar, collected from the field (A) and individually kept in a 24 well-plate with a small piece of carrot at 20 ± 2°C in the dark to checked feeding habit and natural mortality (B). After 72 hrs, two larvae of *C. giveni* were added to each pot. Only one plant was left per pot (C).

3.4.7..1 Maize plants from fungal-coated seeds: performance evaluation

Two weeks after sowing, plants were harvested to determine length and dry weight. Maize plants were pulled gently from the soil in order to not disrupt the roots, soil was shaken off, and then washed off thoroughly with tap water. Root and shoot length were then recorded, after which plants were processed for determining dry weight. Shoots were split from roots and placed individually in brown paper bags (20 x 12 cm). Later, paper bags containing shoots and roots were placed in an oven tray and then in an oven at 65°C (Contherm Thermotec 2000). Maize plants were left to dry until a constant weight. Additionally, one maize plant per pot was also taken for the evaluation of fungal performance (rhizosphere competence and endophytic colonization). In the block with *C. giveni* challenge there was only one plant per pot to use for the determination of length and dry weight. Eventually, from this one plant, rhizosphere soil was collected for the analysis of fungal rhizosphere competence. Root fragments were collected from the potting mix, because of the presence of the grass grub, and used as samples for fungal endophyte isolation. Plants were visually assessed for symptoms of *Fusarium* rot root and percentages of infected plants determined.

3.4.8 Rhizospheric and endophytic colonization of maize plants by entomopathogenic fungi at 25°C and 28°C

3.4.8..1 Isolation of entomopathogenic fungi from rhizosphere soil: rhizosphere competence

Entomopathogenic fungi were isolated from the rhizosphere following the methodology of Wyrebeck *et al*. (2011) with modifications. Maize seeds coated with the fungal treatments were sown in potting mix and grown in controlled conditions with light:dark conditions (16:8 h), 65 ± 10 RH% and at two

temperatures: 24.3 ± 2.5°C (referred to as experiments at 25°C) and 28.2 ± 3.2°C (referred to as experiments at 28°C). Plants were watered every 4 days with 300 mL of tap water. Two weeks after sowing plants were harvested. Roots were split from the rest of the plant and shaken gently to release the excess of soil. Roots with adhering soil were weighed and then transferred to a 50 mL Falcon tube containing 10 mL of a solution of 0.01% Triton X-100 and chloramphenicol (0.5 g/L). After vortexing, soil was left to precipitate and dilutions (-1, -2 and -3) were prepared. Subsequently, 100 µL of each soil suspension were plated per duplicate, in 9 cm diameter Petri plates containing selective media appropriate for the corresponding fungal coating. Treatments with *Metarhizium* spp. were plated onto *Metarhizium* selective medium (MSM), *B. bassiana* treatments were in *Beauveria* selective medium (BSM) while *T. harzianum* were in *Trichoderma harzianum* selective medium (THSM). Control seeds, without a fungal coating, were plated onto plates containing each of the selective media. Inoculated plates were incubated at 22 ± 2 °C with light:dark conditions (12:12 h). Plates were observed after five days for emerging colonies and those corresponding to the morphology of the genus of interest were transferred to MSM or BSM plates. Entomopathogenic isolates were identified based of growth on the selective media, colony morphology and microscopic examination of the conidia as described in Sasan & Bidochka (2012). Quantification was done as colony forming unit (CFU) of only the genera of interest. Two petri plates of each fungal treatment and control plants (CS) were averaged. To combine data over the three trials, CFU means were input into an analysis of variance which treated trials as "blocks", with treatments being fungal treatments, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

3.4.8..2 Isolation of entomopathogenic fungi from surface sterilized maize tissues: endophytism

Endophytic fungi were isolated from leaves and stems from treated maize plants following the methodology applied by Govinda Rajulu *et al*. (2011) and Wyrebeck *et al*. (2011) with modifications. Two weeks old maize plants from coated seeds with entomopathogenic fungi and controls, grown at 25°C and 28°C, were processed within 24 h. after harvested. Roots were separated from the rest of the plant and processed for rhizosphere studies as detailed above before surface sterilization. Leaves and stems were washed thoroughly in running water and air dried before surface sterilization. For sterilization, leaves and stems, or roots, were rinsed with 70% ethanol for 5 s, immersed in 4% (w/v) sodium hypochlorite for 90 s and finally rinsed in sterile distilled water for 10 s, then let to dry in a laminar flow cabinet between sterile paper towels. Later, organs were cut in segments between 0.3 – 0.5 mm size, and 25 segments from each organ (leaves, stems or roots) were plated, per quadruplicate, in 9 cm diameter Petri plates containing selective media appropriate for the corresponding fungal coating as detailed above. Inoculated plates were incubated at 22 ± 2°C with light:dark conditions (12:12 h). Plates were inspected each day for 30 days for emerging colonies and those corresponding to the morphology of the genus of interest were transferred to MSM or BSM plates. Entomopathogenic isolates were identified based on growth on the selective media, colony

morphology and microscopic examination of the conidia (Sasan & Bidochka, 2012). For quantification purposes, when more than one isolate of a genus of interest emerged from the same segment, only one was considered. The efficacy of the surface sterilization technique was confirmed from random samples by pressing gently sterilized segments of each organ on the surface of corresponding selective media in Petri plates and removing them. Petri plates were then incubated at the same conditions for 10 days. No colonies were obtained from print impression of surface sterilized plant segments. A total of 100 segments from the corresponding plant organ, and from each fungal treatment and control plant (CS) were averaged. To combine data over the three trials, CFU means were input into an analysis of variance which treated trials as "blocks", with treatments being fungal treatments, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

3.4.9 Effect of entomopathogenic fungal seed-coating on jasmonic acid and salicylic acid content in maize in the presence of *Costelytra giveni*

3.4.9..1 Maize plants

Maize seeds coated with entomopathogenic fungi and the untreated controls were sown in 1 L pots containing 600 g of vermiculite (fine grade 2) and watered with 400 mL of tap water. Plants were grown in the Biotron, at 20 ± 0.1°C with light:dark conditions (16:8 h), 69.1 ± 5.4 RH% and watered each 4 days with 300 mL of tap water (Figure 3.7). After one week, plant emergence was checked and three grass grubs of *C. giveni* per pot were added. After 15 days from seed sowing maize plants were harvested and roots washed thoroughly with tap water. Plants were split into roots and shoots, wrapped in aluminium foil, immediately frozen in liquid N_2 and stored at -80°C till sample processing for phytohormone extraction. *C. giveni* larvae were checked for health before use as described above.

Figure 3.7 Plant maize growth for phytohormones extraction. Maize seeds were coated with conidia of *M. anisopliae* A1080 and F672 isolates and sown in pots containing vermiculite. One week after sowing three grass grubs of *C. giveni* per pot were added. Plants were grown for two weeks in the Biotron, at 20 ± 0.1°C, light:dark conditions (16:8 h), 69.1 ± 5.4 RH% and watered each 4 days with 300 mL of tap water.

3.4.9..2 Sample preparation

Maize roots and shoots were frozen in liquid N_2 to halt enzymatic activities and stored at -80ºC prior to use. While samples were still frozen, roots and shoots were freeze-drye (Thermo Micro Modulyo 23C) to extract all water before tissue pulverization. A coffee grinder was used to pulverize samples to a fine powder (Figure 3.8). Pulverized samples were weighed to approximately 25 mg and transferred to 2 mL screw cap FastPrep® tubes containing 1 g Zirmil® beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA). Plant samples were kept at -80°C during the different stages of sample preparation and until the final phytohormone vapour-phase extraction.

Figure 3.8 Sample processing for salicylic and jasmonic acid extraction. After maize plants were harvested roots and shoots were frozen in liquid N_2 and stored at -80ºC. Plant samples, roots and shoots, were freeze-drying and samples ground to a fine powder. Left: roots. Right: shoots.

3.4.9..3 Phytohormone vapour-phase extraction

Root or shoot samples were added to 20 µl of the internal standard mix, dihydrojasmonic acid and D6-salicylic acid in methanol (10 µg/mL) and 600 µl of 70°C preheated extraction buffer water:1-propanole:HCl (1:2:0.005). Samples were vortexed and then vigorously agitated in a tissue homogenizer at 1500 RPM for 45 seconds. After agitation, 1 mL of methylene chloride (DMC) was added, samples were vortexed and centrifuged for 1 minute at 10.000 g for phase separation. The lower organic phase was transferred to 4 mL glasses and dried over Na_2SO4. In order to increase the volatility of jasmonic acid and enable separation by gas chromatography, samples were treated with 2 µl of 2 M trimethylsilyldiazomethane (TMS) in hexane (Sigma-Aldrich) for seven minutes at room temperature. TMS is a methylating agent that converts carboxylic acids into methyl esters. The methylation reaction was stopped by adding 2 µl of 2M acetic acid in hexane. After this step the samples were subjected to vapour-phase extraction, according to Schmelz *et al*. (2004) and Mishina and Zeier (2006) with minor modifications as follows.

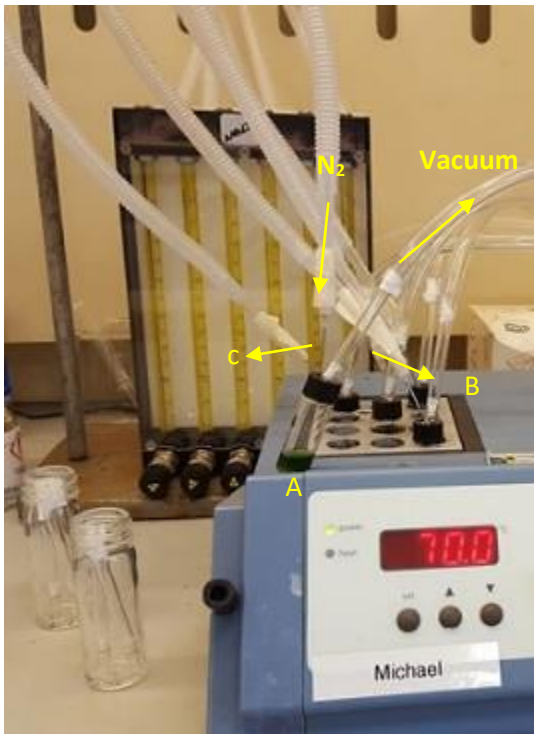

Figure 3.9 Phytohormone vapour-phase extraction. Through the septum of a tightly closed 4 mL vial (A) inserted into a glass column with a Super Q filter (B). The glass column was connected to a vacuum pump at a flow rate 0.4 L min^{-1}, and a needle (C) suppling a stream of N_2 was also inserted through the septum-lid at a flow rate 0.2 L min^{-1}. The vial was placed in a block heater at 70 °C till samples had completely evaporated.

First, the 4 mL vial was stoppered with an open cap fitted with a high temperature septum (Schott, Germany). A glass column with a Super Q filter (Altech, IL, USA) was inserted through the septum and connected to a vacuum pump at a flow rate 0.4 L min^{-1}. Finally, a needle that supplied a gentle stream of N_2 was also inserted through the septum-lid at a flow rate of 0.2 L min^{-1}. The vial was placed in block heater (Stuart SBH 200DC) at 70°C till samples had completely evaporated (Figure 3.9). Volatiles and phytohormones were trapped in the Super Q adsorbent filter and were eluted from the filter with 100 µl of DMC into a reaction vial. Extracted samples were kept at -80°C till determination through chemical ionization gas chromatography - mass spectrophotometry (Schmelz *et al*., 2004). The sample mixtures were analysed using a Shimadzu GC-MS-QP2010 gas chromatograph-mass spectrometer fitted with a Restek Rxi-1ms fused silica capillary column (30.0 m x 0.25 mm i.d. x 0.25 µm, Bellefonte). Three µL of each sample was injected in pulsed splitless mode at a temperature of 220ºC and with a pulse of 168 KPa for 40 s. Oven temperature was held at 50ºC or 3 min and then raised to 320ºC at 8ºC min^{-1} and held at this temperature for 8 min. Helium was used as carrier gas at a constant flux of 1.5 ml min^{-1}. All mass spectra were recorded in selected ion monitoring (SIM) mode to increase the

detector's sensitivity. The recorded spectra were analysed using GCMS Postrun in LabSolutions, Version 2.5 (Shimadzu Corporation, Japan). The peaks of the quantifying ions for the two compounds of interest and for the two internal standards, respectively, were integrated, and JA as well as SA contents were calculated according the following formula:

$$Ph\ (ng.g^{-1}) = \frac{area_{m/z}\ (Ph) x\ 200\ x\ correction\ factor}{area_{m/z}\ (Ph\ internal\ standard)\ x\ DW(g)}$$

where, Ph = phytohormone (SA or JA); Correction factor (SA = 1.1; JA = 3.5); DW = dry weight of plant sample (roots or shoots)

Each trial consisted of two randomised blocks with one pot in each block with the corresponding fungal treatments (*M. anisopliae* F672 and *M. robertsii* F447) and control seeds (CS) without a fungal treatment. Additionally, there were four randomised blocks with one pot in each block with the corresponding fungal treatment (*M. anisopliae* F672 and *M. robertsii* F447), and control seeds (CS) without a fungal treatment, but all these pots containing larvae of *C. giveni*. To combine data over the two trials, SA and JA concentration means from roots and shoots were input into an analysis of variance which treated trials as "blocks", with treatments being fungal treatments, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

3.5 Results

3.5.1 Effect of conidia coated onto maize seeds on germination

Germination was defined as the stage where essential structures of the plant indicate whether or not it is able to develop further into a satisfactory plant under favourable conditions in the field (ISTA, 2017). In this study, seed coating was elaborated in two stages, a first stage or pre-coating (PC), where the seed was homogenously covered with methyl cellulose (MC) to change the hydrophobicity of the seed surface, and a second stage or complete coating seeds (CS) where a biogel containing conidia, bentonite and talc was added to the pre-coated seed surface. In order to determine if any of these stages could reduce the germination performance of the seeds, or if the concentration of conidia could be harmful for the developing seedlings, two different germination tests were used.

The BPM germination test was used to determine the effect of the PC or the CS on the ability of the seeds to germinate. There were no significant differences among the different coatings applied to seeds when compared to pure seeds (PS) without any type of coating (Table 3.1).

Table 3.1. Percentage seed germination between paper method.

Treatment	Normal Seedlings	Abnormal Seedlings	Ungerminated Seeds
PC	83.0	13.5	3.5
CS	87.0	11.0	2.0
PS	88.5	11.5	0.0
$LSD_{5\%}$	8.1	4.7	4.7

PC, pre-coating; CS, complete coating; PS, pure seeds

Once it was determined that inert components of the coatings had not effects on seed germination, the possible effect of the biological component when incorporated in the coating and then onto maize seeds were evaluated (Table 3.2). Average germination was 93.1 ± 2.4% with no significant differences between isolates or conidia concentration. This result suggests that at least at both conidia concentrations and conditions, the *Metarhizium* isolates used in this study have no impact on seed germination when applied onto the seeds as a coating (Table 3.2).

Maize seedlings also had similar development, but differences were found in the length of the shoots ($p<0.01$). Shoot length in maize seedlings from the *M. anisopliae* F672 and *M. novozealandicum* F11 and F99 coatings were significantly higher than shoot length in the CS seeds treatment. Only seedlings shoots from treatments with *M. anisopliae* F672 and *M. novozealandicum* F11 at 1 x 10^5 conidia/mL were longer than the seedling shoot registered in both controls, PC and CS (Figure 3.10).

Table 3.2. Percentage seed germination evaluation on sand.

Treatment	Conidia/mL	Normal Seedlings	Abnormal Seedlings	Ungerminated Seeds
F11	1 x 10^5	93.5	2.0	4.5
F11	1 x 10^8	94.5	3.0	2.5
F99	1 x 10^5	95.0	3.0	2.0
F99	1 x 10^8	89.0	4.0	7.0
F138	1 x 10^5	95.5	2.0	2.5
F138	1 x 10^8	91.5	3.5	5.0
F16	1 x 10^5	90.5	4.5	5.0
F16	1 x 10^8	95.0	1.0	4.0
F133	1 x 10^5	91.0	2.5	6.5
F133	1 x 10^8	94.0	3.5	2.5
F672	1 x 10^5	94.5	3.0	2.5
F672	1 x 10^8	93.0	4.0	3.0
PC	0	97.0	2.5	0.5
CS	0	89.5	1.5	9.0
$LSD_{5\%}$	-	6.5	3.6	5.6

PC, pre-coating; CS, complete coating

Using the CFU method, it was possible to determine the amount of conidia coated onto the maize seeds and to estimate the efficiency of the coating process. In general, the final concentration of viable conidia coated onto the seeds was exponentially two orders lower than the original conidial suspension used in the biopolymer (Table 3.3).

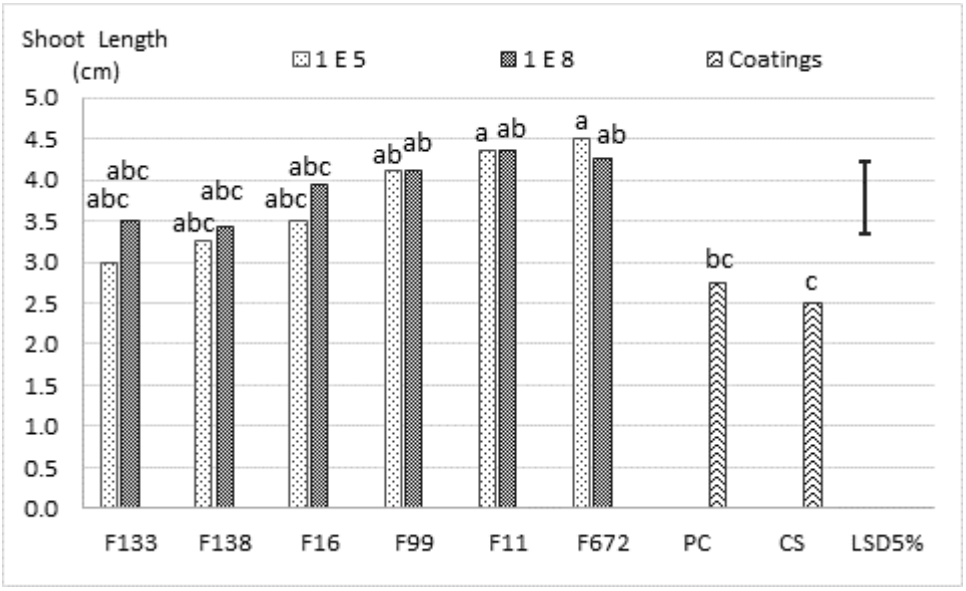

Figure 3.10 Shoot length in maize seedlings grown in sand. Maize seeds were coated with entomopathogenic fungi at two conidial suspensions, 1 x 10^5 and 1 x 10^8 conidia/mL. Seeds were sown in sand for the germination test on sand. After 7 days, seedling shoot length was determined. *Controls: PC, pre-coating, CS, control seeds complete coating.* Bar = $LSD_{5\%}$.

The efficiency of the coating process was calculated on average to be 30.6 ± 8.5 %. Main loses of conidia were probably due to the biogel retained on the walls of the vessel where the seeds were mixed with the other components of the coating.

Table 3.3. Quantity of conidia recovered from maize seeds after coating and efficiency of the process.

Treatment	Genera	Cond./mL (initial)	CFU/g (final)	Efficiency (%)
F11	*M. novozealandicum*	1×10^5	4.4×10^3	39.7
F133	*M. novozealandicum*	1×10^5	3.6×10^3	32.1
F138	*M. robertsii*	1×10^5	3.7×10^3	33.4
F16	*M. guizhouense*	1×10^5	2.6×10^3	23.5
F672	*M. anisopliae*	1×10^5	4.9×10^3	44.1
F99	*M. novozealandicum*	1×10^5	3.5×10^3	31.1
F11	*M. novozealandicum*	1×10^8	3.1×10^6	28.1
F133	*M. novozealandicum*	1×10^8	3.2×10^6	28.4
F138	*M. robertsii*	1×10^8	2.6×10^6	23.6
F16	*M. guizhouense*	1×10^8	2.0×10^6	17.8
F672	*M. anisopliae*	1×10^8	4.8×10^6	43.3
F99	*M. novozealandicum*	1×10^8	2.4×10^6	21.9
PC	-	0	0	-
CS	-	0	0	-
$LSD_{5\%}$				20.3

3.5.2 Evaluation of maize plant performance after coating with conidia from entomopathogenic fungi

3.5.2..1 Effect of conidia concentration on maize plant performance

The average plant height was 24.3 ± 1.2 cm for seedlings grown from coated seed. Differences in maize plant height among the different seed coating treatments were found ($p<0.01$). The ambient conditions in the glasshouse during the growth of the plants are detailed in Table 3.4.

Table 3.4. Average temperatures, humidity (%) and dew point registered in the glasshouse during the maize fungal-seed coating experiments.

	Temperature (°C)	Humidity (%)	Dew Point (°C)
Max.	45.0	86.5	20.3
Med.	21.9	60.8	12.9
Min.	12.0	19.3	6.2

The highest value was in plants from maize seeds with only the pre-coating treatment (PC), which were statistically different to the other coating treatments (Figure 3.11 - A). The entomopathogenic fungal seed coating treatments did not statistically differ or with the complete coating treatment (CS), except for the *M. novozealandicum* F133 treatment which had the lowest plant height (Figure 3.11 - A).

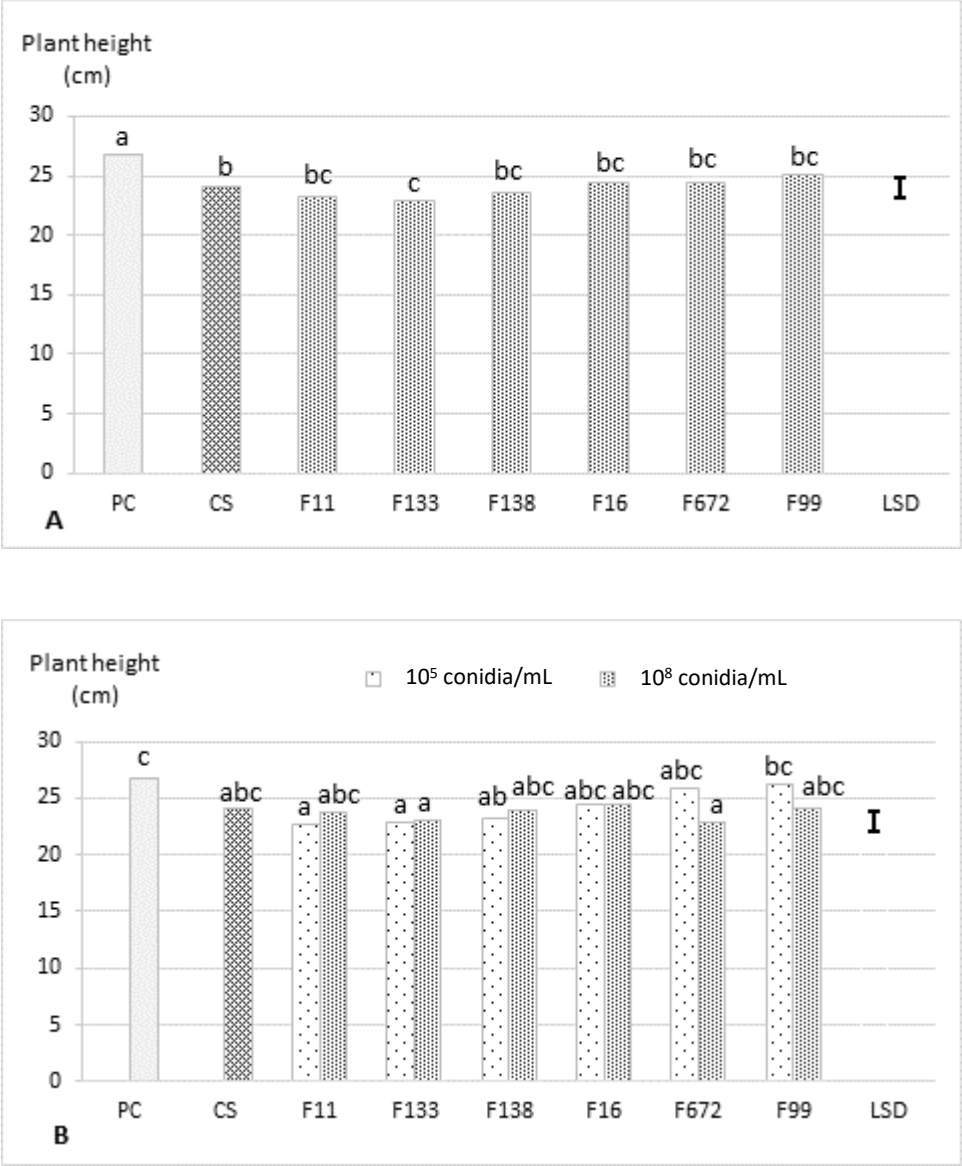

Figure 3.11 Evaluation of different maize seed coatings treatments with entomopathogenic fungi. Average plant height values obtained with the different treatments (A) Plant height using different conidia concentration in the seed coating (B). *Controls: PC, pre-coating, CS, control seeds complete coating.* Different letters above average values indicate significant differences ($LSD_{5\%}$) at $p<0.05$. Bar = $LSD_{5\%}$.

The conidia concentration and the fungal isolate used in the coating had an effect on plant height ($p<0.01$). Only maize height in the PC treatment was significantly different to some of the fungal treatments, while plant height with the CS treatment was similar to the rest of the entomopathogenic fungal treatments (Figure 3.11 – B).

Additionally, plant height in some isolates had differences to the height determined in the PC treatment depending on the conidia concentration used in the coating (Figure 3.11 – B). For example, the *M. novozealandicum* F11 and *M. robertsii* F138 treatments were different to the PC treatment only when the coating used was at a conidial concentration of 10^5 conidia/mL. On the other hand, the *M. anisopliae* F672 treatment was smaller than that in the PC treatment, when the conidia concentration in the seed coating was 10^8 conidia/mL (Figure 3.11 – B).

In summary, maize plant height was not affected by the PC or CS treatments, however, depending on the fungal isolate and the conidia concentration used for the coating, maize plant growth may be affected.

3.5.3 Effect of fungal seed-coating on maize plant growth

Maize plant general performance

On average, when maize plants were grown at 25°C under controlled conditions in the Biotron, shoot and root length had similar values and no significant differences were found among the treatments, or with the controls, except for *B. bassiana* Bb21 ($LSD_{5\%}$ = 0.03; Figure 3.12). This was the only entomopathogenic fungal coating which reduced shoot length ($p<0.01$).

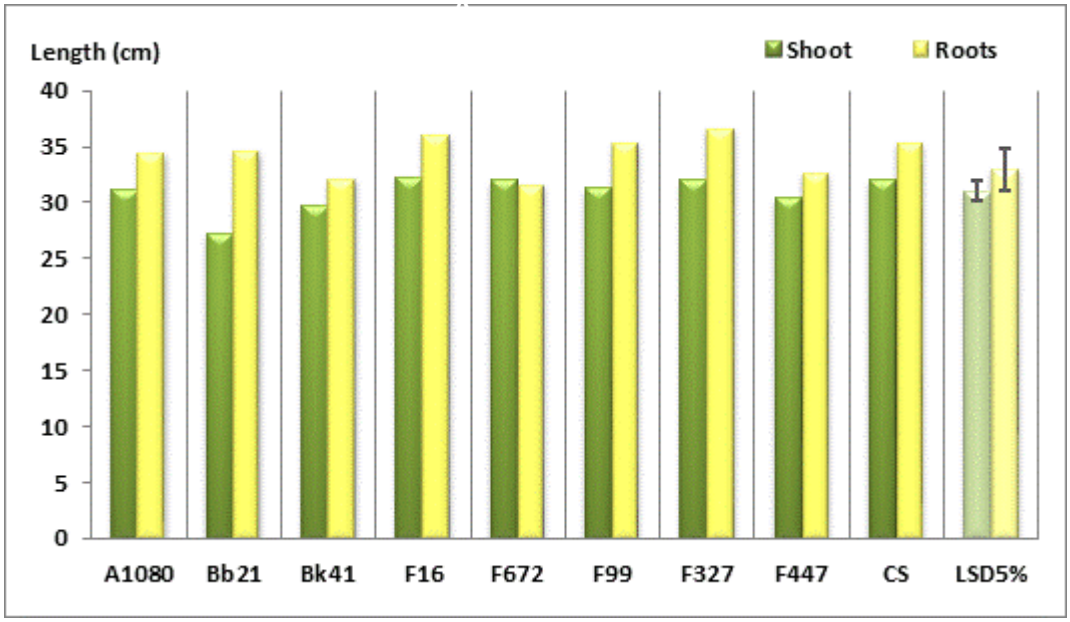

Figure 3.12 Shoot and root length of maize plants. After seeds were coated with a conidia suspension from entomopathogenic fungi, plants were grown at 25°C under controlled conditions. After two weeks length of shoots and roots was determined. Error bars = $LSD_{5\%}$.

Dry weight of control plants, CS, or of controls treated with the plant growth promoters *T. harzianum* F327 or *M. anisopliae* A1080, on average had similar values to maize plants with the entomopathogenic fungal treatments (Figure 3.13).

Both results, plant length and dry weight, indicated that the entomopathogenic fungi did not have a negative effect on plant growth.

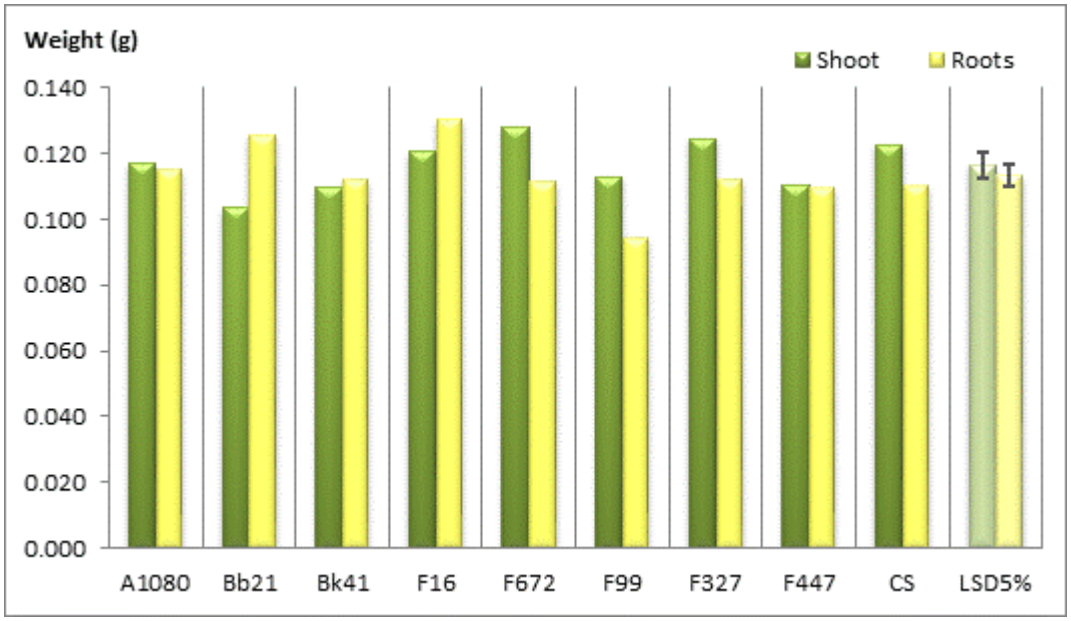

Figure 3.13. Shoot and root dry weight of maize plants. After seeds were coated with a conidia suspension from entomopathogenic fungi, plants were grown at 25°C at controlled conditions. After two weeks plants were recovered and processed to determine dry weight of shoots and roots. Error bars = $LSD_{5\%}$.

However, there were observed differences in plant weight at the 5% LSD level, indicating that plant growth was affected by the coating treatment. For example, shoot weight in controls, CS or the *T. harzianum* F327 treatment ($LSD_{5\%}$ = 0.065), were significantly higher than the shoot weight determined in plants with the *B. bassiana* Bb21 treatment (Figure 3.13). Also, shoot weight in the *M. anisopliae* F672 and *M. guizhouense* F16 entomopathogenic treatments ($LSD_{5\%}$ = 0.079) were higher than shoot weight in the *B. bassiana* Bb21 treatment. Although the average root weight between the treatments were similar, root weight in the *M. novozealandicum* F99 treatment was 16% lower than those recorded in CS or in plants with the *T. harzianum* F327 treatment ($LSD_{5\%}$ = 0.082). Plants with the *M. novozealandicum* F99 were 27% smaller than maize plants with the *M. guizhouense* F16 treatment (LSD = 0.100).

3.5.4 Effect of the entomopathogenic fungal isolates in maize plant performance in the presence of *C. giveni* and *F. graminearum*

Using the combined analysis, the effect of grass grub and disease could be determined through their effect on shoot and root length. The longest total length (shoot + root) was in the absence of *C. giveni* and *F. graminearum* while the lowest was found when both were present (Figure 3.14; $LSD_{5\%} = 0.051$). The main factor which negatively impacted total maize length was *C. giveni* ($p<0.01$; Figure 3.14). Root length was significantly reduced in presence of *C. giveni*, while in shoots it was determined as a trend where shoots were also reduced ($p< 0.06$). The average root length, in the absence of *C. giveni*, was 37.6 ± 2.7 cm, but when present, average root length was 31.2 ± 4.4 cm, representing a 16.6% reduction in root length ($p<0.01$; Figure 3.15). *F. graminearum* did not have any effect on maize root or shoot length (Figure 3.14).

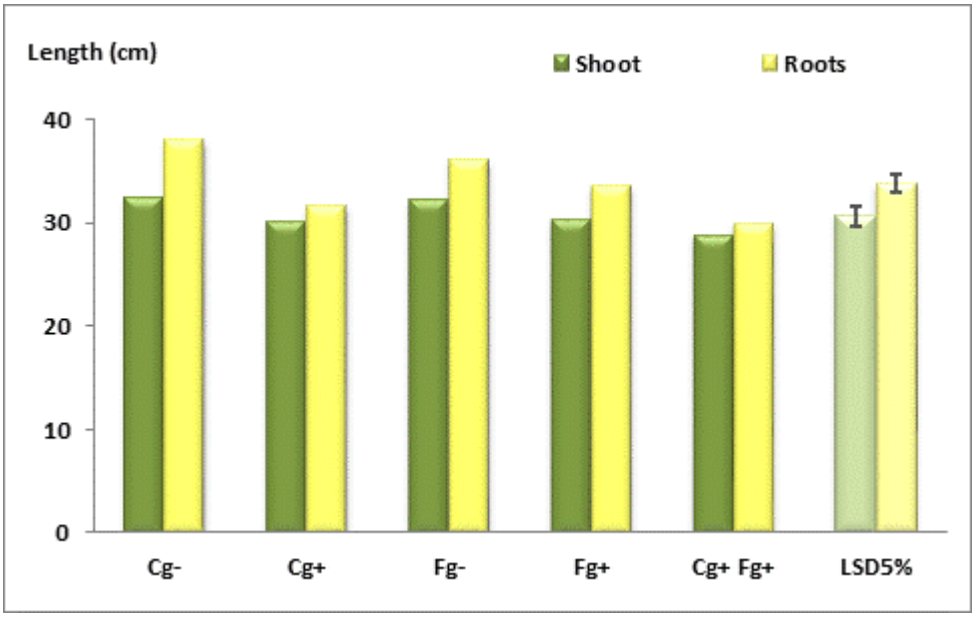

Figure 3.14. Combined effect of all coating treatments on maize growth in the presence of *Costelytra giveni* or *Fusarium graminearum*. Shoot and roots length were determined in maize plants after seeds were coated with entomopathogenic fungi and grown at 25°C for two weeks. Conditions: Cg^- / Cg^+: absence/presence of *C. giveni*; Fg^-/ Fg^+: absence/presence of *F. graminearum*. Error bars = $LSD_{5\%}$.

When maize growth was evaluated as dry weight, again the highest value was found in absence of *C. giveni* and *F. graminearum*, while the lowest maize dry weight values were found when *C. giveni* was present (Figure 3.15; $LSD_{5\%} = 0.104$). As consequence of the feeding activity of *C. giveni* there was an average 19% decrease in dry root weight in comparison to roots in absence of the insect pest (Figure 3.15; $p < 0.01$). *F. graminearum,* apparently, had no effect on maize root dry weight ($p = 0.982$).

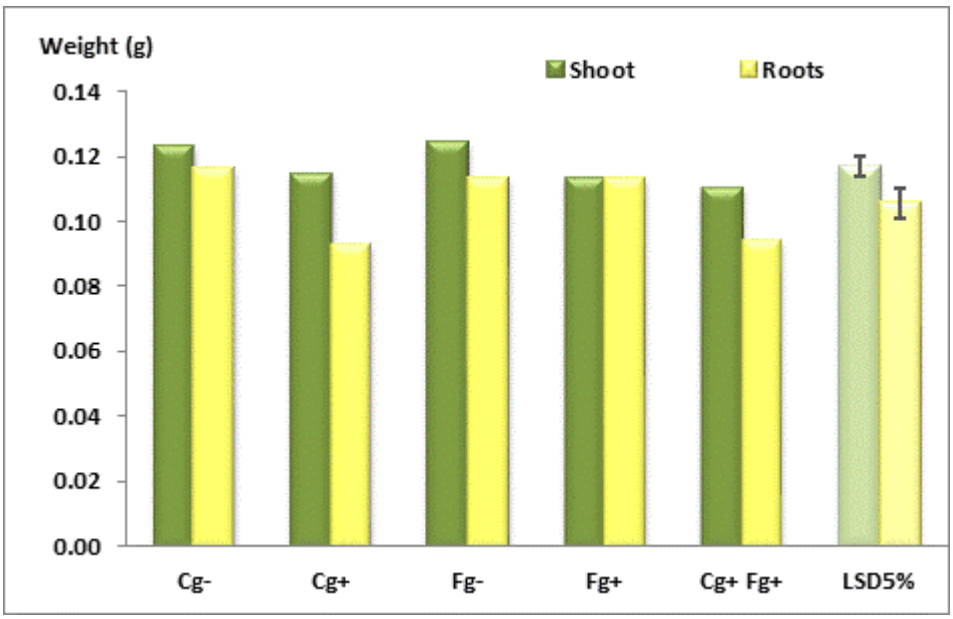

Figure 3.15. Combined effect of all coating treatments on maize dry weight in the presence of *Costelytra giveni* or *Fusarium graminearum*. Shoot and roots dry weight were determined in maize plants after seeds were coated with entomopathogenic fungi and grown at 25°C for two weeks. Conditions: Cg^- / Cg^+: absence/presence of *C. giveni*; Fg^-/ Fg^+: absence/presence of *F. graminearum*. Error bars = $LSD_{5\%}$.

There were significant differences between the treatments at the LSD (5%) level. These differences are explained for each case below.

Maize total length

Approximately 16% total maize length reduction was observed in plants from control seeds ($LSD_{5\%}$ = 0.046), *B. bassiana* Bb21 ($LSD_{5\%}$ = 0.080) and *T. harzianum* F327 ($LSD_{5\%}$ = 0.046) with the presence of *C. giveni,* compared to no larvae (Figure 3.16-A). In the presence of *Fusarium* there was observed trend suggesting that also the fungal isolates may decrease total plant length (p=0.06). For example, plants with the *B. bassiana* Bb21 and *M. robertsii* F447 treatments ($LSD_{5\%}$ = 0.080) were 28 and 17% smaller respectively, when compared to total plant length without *F. graminearum* (Figure 3.16-A). The most growth, in presence of *Fusarium*, was determined in *M. guizhouense* F16 which was significantly different to the lowest growth, which was in *M. robertsii* F447 and *B. bassiana* Bb21 ($LSD_{5\%}$ =0.077) treatments.

Also, in the presence of both challengers, plants in CS and with the *T. harzianum* F327 treatment had a 18% and 24% decrease in total plant length, respectively ($LSD_{5\%}$ = 0.06) in comparison to maize grown in the absence of *C. giveni* and *F. graminearum* (Figure 3.16 – A). In this situation, maize total length was also affected in some of the entomopathogenic fungal treatments where there was reduced of 42% in the *B. bassiana* Bb21 ($LSD_{5\%}$ = 0.113), 28% in the *M. robertsii* F447 ($LSD_{5\%}$ = 0.113) and 26% in

the *M. anisopliae* F672 treatments ($LSD_{5\%}$ = 0.113; Figure 3.16 - A). This may indicate that, in the presence of *C. giveni* and *F. graminearum*, there is an interaction between the plant and the entomopathogenic fungi which is reflected in plant growth. This effect seemed to be the strongest in *B. bassiana* Bb21.

Maize shoot length

There were differences in shoot length between the CS treatment and the *B. bassiana* Bb21 treatment which had the shortest value of all the treatments ($p<0.01$, Figure 3.16 -C). On the other hand, maize plants with the *Metarhizium* coating treatments had similar shoot length as CS plants indicating that these fungal seed coatings did not have a negative effect on shoot development. There was an observed trend related to the presence of *C. giveni* where shoots lengths were shorter than those in the absence of the grass grubs ($LSD_{5\%}$ = 0.07). For example, in *T. harzianum* F327 where shoots were reduced by 10% with *C. giveni* compared to no larva while the remaining fungal treatments did not differ in shoot length with or without the insect pests (Figure 3.16 - C). This indicates a better performance of plants with the fungal entomopathogens than with the *T. harzianum* F327 control treatment.

In the absence of *F. graminearum* all treatments had similar shoot lengths, except for the *M. robertsii* F447 treatment which had a significantly greater shoot length than *M. guizhouense* Bk41 ($p<0.01$). On the other hand, the presence of the plant pathogen resulted in a reduction in shoot length in some of the fungal coatings ($p<0.01$). For example, shoot length with the *M. robertsii* F447 or *B. bassiana* Bb21 treatments were 19 and 29% respectively, smaller than in the absence of the plant pathogen ($p<0.01$; Figure 3.16-C).

When both *C. giveni* and *F. graminearum* were present, shoot length from maize seeds coated with the plant promoter *T. harzianum* F327 ($LSD_{5\%}$ = 0.070) and *B. bassiana* Bb21 ($LSD_{5\%}$ = 0.098) were 16% and 33.5%, respectively, smaller than in the absence of these challengers (Figure 3.16-C). The remaining treatments did not experience variation in maize shoot length.

Maize root length

Roots length were negatively affected by the presence of the insect pest ($p<0.01$) and also there was a trend indicating that root lengths decreased in the presence of *F. graminearum* ($p=0.08$). Roots in control plants, CS and *T. harzianum* F327, had about a 22% decrease in length in the studies where *C. giveni* was present compared to roots in the absence of the insect pest ($LSD_{5\%}$ = 0.077). On the other hand, roots in plants with the *M. anisopliae* A1080 control treatment ($LSD_{5\%}$ = 0.077), or with the *M. guizhouense* Bk41 and F16 (LSD=0.134) treatments, did not show any variation in root length, indicating that these fungal seed coatings decreased the negative effect of *C. giveni* (Figure 3.16-E).

However, some of the entomopathogenic seed coatings were not as successful and roots were also significantly smaller with *C. giveni* compared to no larvae, i.e. 24% in the treatments with *B. bassiana* Bb21 ($LSD_{5\%}$ = 0.134), 23% in the *M. anisopliae* F672 ($LSD_{5\%}$ = 0.134) and 21% in the *M. robertsii* F447 treatment ($LSD_{5\%}$ = 0.134).

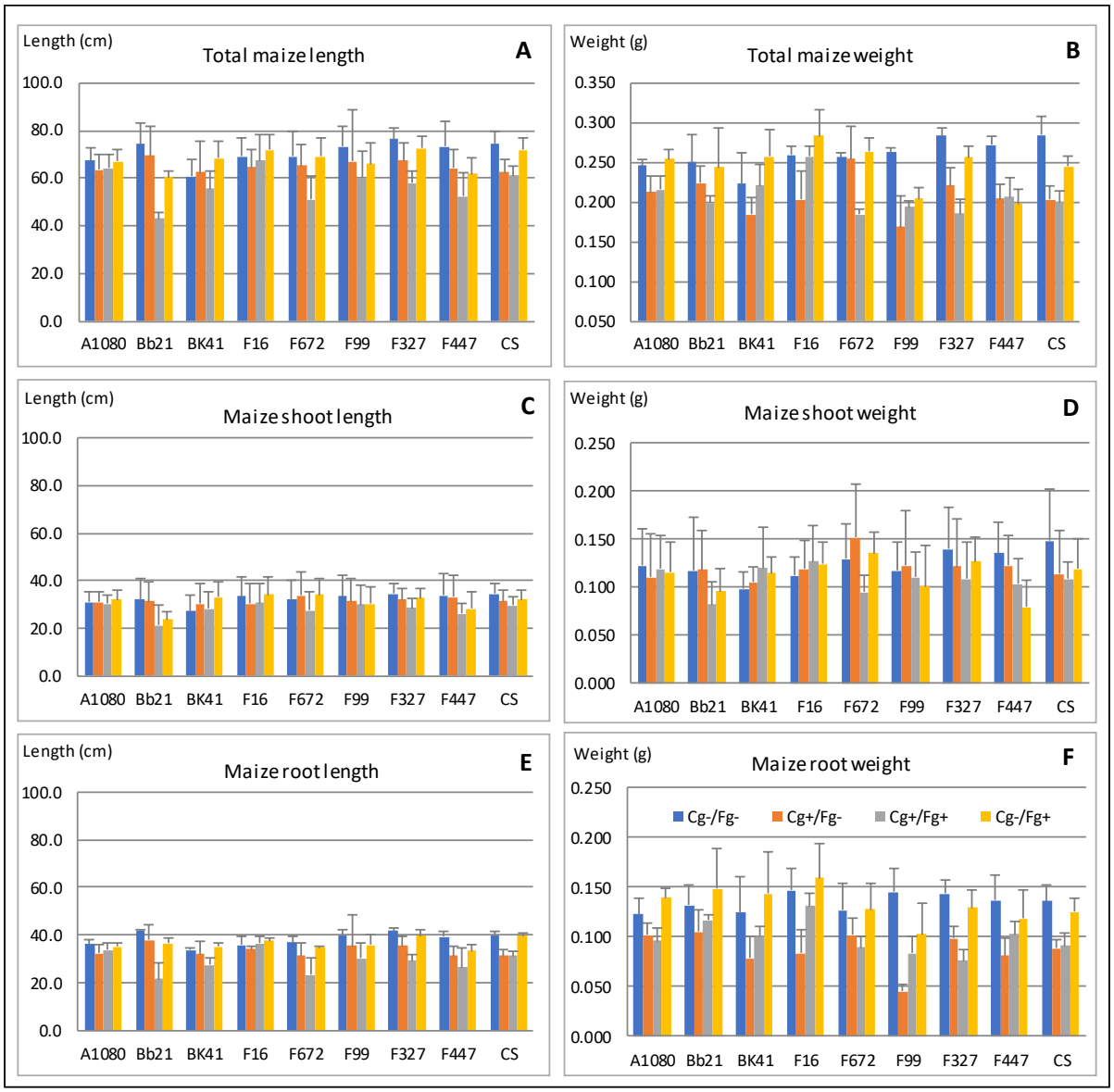

Figure 3.16. Plant maize length and dry weight after seed coating. Maize seeds were coated with different fungi and sown in potting mix in the presence of *Costelytra giveni* and *Fusarium graminearum*. Plants were grown at 25°C, with light:dark conditions (16:8 h) and 65 RH% After two weeks length and weight were recorded. Conditions: Cg^- / Cg^+: absence/presence of *C. giveni*; Fg^- / Fg^+: absence/presence of *F. graminearum*. Error bars = standard errors of means. A. Total maize length, B. Total maize dry weight, C. Maize shoot length, D. Maize shoot dry weight, E. Maize root length, F. Maize dry root weight.

F. graminearum had a significant effect on root length only in the *B. bassiana* Bb21 treatment where roots were 27% decreased in comparison to length in absence of the disease ($LSD_{5\%}$ = 0.134, Figure

3.16-E). In presence of the plant pathogen all the *Metarhizium* coatings treatments had similar root development to the control plants treatments, except for the *M. anisopliae* F672 where roots were significantly shorter than the CS treatment ($LSD_{5\%}$ = 0.109). The fact that the entomopathogenic isolates had similar growth to that recorded in control plants indicate the compatibility with this maize variety.

Control plants (CS) grown with *C. giveni* and *F. graminearum* at the same time had similar root length to those determined in absence of the challengers, while some of the entomopathogenic fungal treatments also did not experience variation. However, the *B. bassiana* Bb21 ($LSD_{5\%}$ = 0.189), *M. anisopliae* F672 ($LSD_{5\%}$ = 0.189) and *M. robertsii* F447 ($LSD_{5\%}$ = 0.189) treatments had significant reductions in root length (between 33 and 49%), when both *C. giveni* and *F. graminearum* were present in comparison to its absence (Figure 3.16-E). The interaction with the challengers and these fungal treatments may have had an effect on plant growth.

Maize total dry weight

Plants grown in the presence of *C. giveni* had a reduction in maize total weight ($p<0.01$). This decrease was significant in control plants, CS and *T. harzianum* F327, with approximately a 24% ($LSD_{5\%}$ = 0.080). On the other hand, maize plants with the entomopathogenic fungal treatments had not significant variation in total weight, except plants with the *M. novozealandicum* F99 treatment which also had a 23% decrease in dry weight in the presence of *C. giveni* ($LSD_{5\%}$ = 0.115; Figure 3.16-B) compared to without the insect. This result confirmed that seed coating with some of the entomopathogenic fungal isolates alleviate plants from the negative effects caused by the grass grubs.

In absence of *F. graminearum* all plant treatments had similar dry weights, but when the plant pathogen was present the highest plant dry weight was in plants with the *M. guizhouense* F16 treatments (Figure 3.16-B). This treatment was significantly different to control treatments, CS and *T. harzianum* F327 ($LSD_{5\%}$ = 0.082).

The simultaneous presence of *C. giveni* and *F. graminearum* had only a negative impact on plant weight of control plants, CS and *T. harzianum* F327 ($LSD_{5\%}$ = 0.118) where weight was reduced a 30 and 35% in comparison to the absence of the challengers (Figure 3.16-B). In this condition, where pest and pathogen were present, the highest plant dry weight was again in the *M. guizhouense* F16 treatment, which was significantly different to the lowest recorded in the control *T. harzianum* F327 treatment ($LSD_{5\%}$ = 0.142).

Maize shoot dry weight

Control plants, CS and *T. harzianum* F327, in presence of *C. giveni* were the only treatments which had a reduction in shoot growth, in comparison to the shoot dry weight obtained in the absence of insect pests, 17 and 14% less shoot growth, respectively ($LSD_{5\%}$ = 0.082). Plants with the entomopathogenic fungal treatments did not vary in shoot dry weight in presence or absence of *C. giveni*, which may indicate that the entomopathogenic fungal seed coating had a benefit on shoot growth (Figure 3.16 - D).

There was a trend indicating an interaction between the treatment and *F. graminearum* (p=0.100). In absence of the plant pathogen, all fungal entomopathogenic treatments and control plants had similar shoot dry weight, while in presence of *F. graminearum* the *B. bassiana* Bb21 and *M. robertsii* F447 treatments had significantly smaller plants than control plants ($LSD_{5\%}$ = 0.092; Figure 3.16 - D). This result suggests that some of the entomopathogenic fungal isolates changed their interaction with the plant in presence of an external factor and this change may have an effect on maize plant growth (p=0.01).

When *C. giveni* and *F. graminearum* were present at the same time, an observed reduction was only found in the CS treatment ($LSD_{5\%}$ = 0.115), with a 27% reduction in shoot dry weight in comparison to shoot weight in the absence of the challengers (Figure 3.16 - D). However, in presence of both challengers maize shoots with the *B. bassiana* Bb21 treatment had lower dry weight than CS ($LSD_{5\%}$ = 0.130) and that of the *M. guizhouense*, Bk41 and F16, treatments ($LSD_{5\%}$ = 0.159).

Root dry weight

The insect pest negatively affected root dry weight (p<0.01) and also a trend was observed in the interaction between the isolates, *C. giveni* and *F. graminearum* (p=0.06).

In absence of *C. giveni* there were no differences in root dry weight among the different coating treatments (Figure 3.16 – F). This observation indicates that the entomopathogenic fungi did not affect root development. However, the presence of *C. giveni* resulted in a reduction in plant root weight in all the treatments (p<0.01). This suggests again that in presence of a challenger, such as the grass grub, the association between the plant and the fungus may change. The decrease in root dry weight in presence of the grass grubs compared to without grass grub was 31% in CS and 36% in the *T. harzianum* F327 treatment ($LSD_{5\%}$ = 0.106), while in the entomopathogenic fungal treatments it was between 24 and 48% (Figure 3.16 - F). Only in the *B. bassiana* Bb21, *M. anisopliae* F672 and *M. robertsii* F447 treatments differences were not observed in root weight regarding the presence or absence of the pest or the disease ($LSD_{5\%}$ = 0.155).

The trend observed indicated that in presence at the same time of the insect pest and the plant pathogen root weight decreased in maize plants and this effect was related also to the coating treatment (p=0.06). In control treatments, CS and *T. harzianum* F327, root weight significantly decreased in presence of the challengers, 33 and 47% respectively ($LSD_{5\%}$ = 0.150), whereas most of the entomopathogenic fungal treatments had no variation in root weight in absence or presence of the challengers (Figure 3.16 - F). The *M. novozealandicum* F99 treatment was the only entomopathogenic fungal coating where there was also an observed significant reduction (43%) in root weight associated with the presence of the pest and the disease ($LSD_{5\%}$ = 0.219). In this condition, plants with the *M. guizhouense* F16 treatment had the highest root dry weight, significantly higher than the control plants, CS and *T. harzianum* F327 ($LSD_{5\%}$ = 0.164).

3.5.5 Effect of the entomopathogenic fungal seed coating on incidence of *Fusarium* root rot in maize plants

Plants infected with *Fusarium* were identified by the presence of a necrotic area affecting the mesocotyl, close to the root crown, where the root was dark brown to black, discoloured, decaying or completely rotted (Figure 3.17).

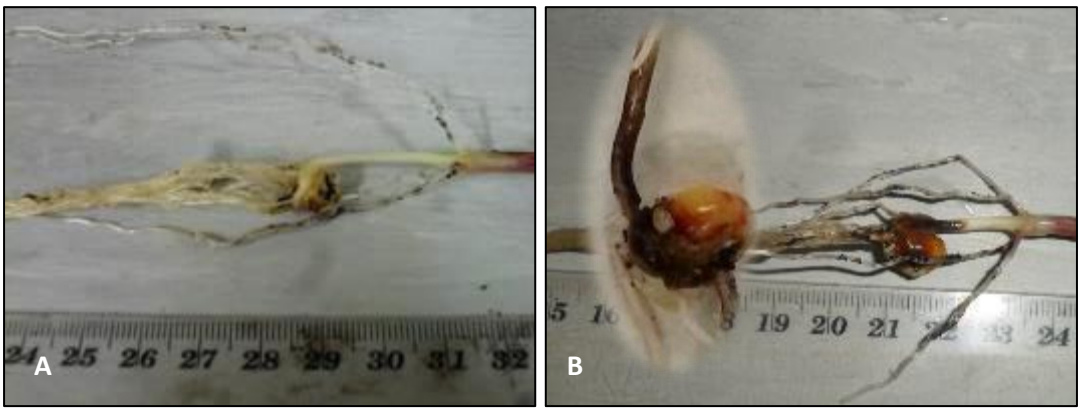

Figure 3.17 ***Fusarium graminearum*** **maize root rot.** Maize seeds coated with the fungal treatments were sown in potting mix containing *F. graminearum* (0.5% w/w). After two weeks plants were harvested and maize plants with symptom of *Fusarium* root rot were recorded. Plants infected with *F. graminearum* exhibited a brown to black rotten mesocotyl below the root crown. A. Healthy maize plant. B. Maize plant and seed with rot root.

Affected plants, at this stage, did not present signs of the disease in the aerial part of the plant. In general, symptoms of *Fusarium* infection were higher in plants from seeds without the fungal coating (control seeds), while *Fusarium* symptoms were lower in plants from seeds coated with the different fungal treatments (p<0.01; Figure 3.18). The controls *T. harzianum* F327 and *M. anisopliae* A1080 had

a lower incidence of infection than CS and also most of the entomopathogenic fungal coatings except, *M. anisopliae* F672 and *M. robertsii* F447 (Figure 3.18).

Symptom percentages similar to those observed in coated seeds were found also in *M. anisopliae* F672 and *M. robertsii* F447 (Figure 3.18). The lowest percentage symptoms were observed in maize roots from the *M. guizhouense* F16, *B. bassiana* Bb21 and *M. novozealandicum* F99 treatments. These were followed by *M. guizhouense* Bk41 and *M. anisopliae* A1080 (Figure 3.18). These levels were all significantly lower than the symptoms determined in CS ($LSD_{5\%}$ = 19.5; Figure 3.18).

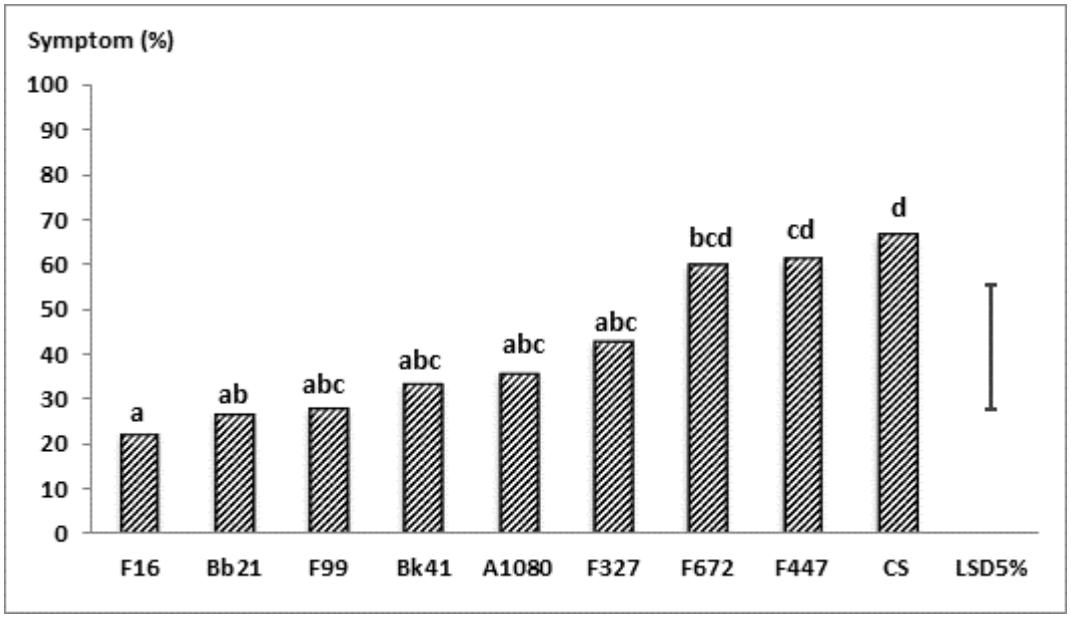

Figure 3.18 Maize root symptom of *Fusarium graminearum* infection. After seeds were coated with a conidia suspension from entomopathogenic fungi, plants were sown in potting mix containing *F. graminearum* 0.5% (w/w). Plants were grown at 25°C in light:dark conditions (16:8 h) and at 65 RH%. After two weeks, plants were harvested, and roots were assessed for *Fusarium* root rot symptoms. Error bars = $LSD_{5\%}$. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

No symptoms were found in the absence of *F. graminearum* ($p<0.01$), while *C. giveni* reduced the rate of symptom expression in roots ($p<0.01$) when present. Lesion symptoms were determined in roots from all plants when grown in presence of *Fusarium* and symptom percentage was different between the coating treatments ($p<0.01$).

In absence of *C. giveni* only plants with the *B. bassiana* treatment had lower *Fusarium* symptoms than CS ($LSD_{5\%}$ = 40.3), while the remaining seed coating treatments had similar rates of symptoms (Figure 3.19). The presence of grass grubs, in general, reduced symptom rates ($p<0.05$). The levels of *Fusarium*

root rot in CS and in plants with the *M. anisopliae* F672 treatment were statistically higher than in plants with *M. anisopliae* A1080 and with *M. guizhouense* Bk41 and F16 (Figure 3.19).

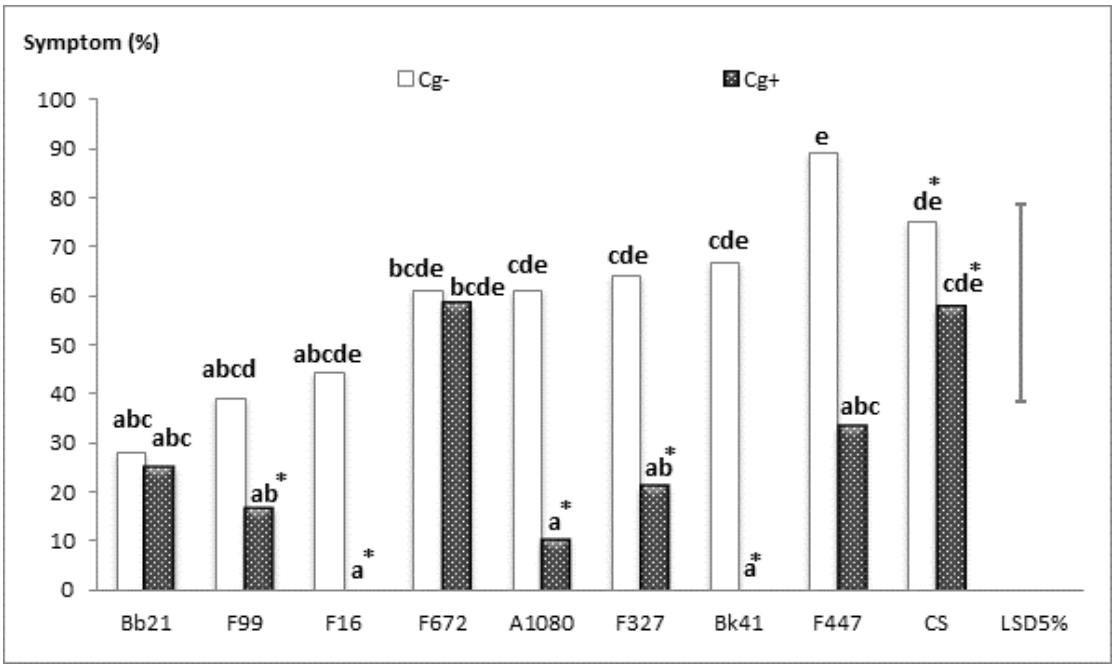

Figure 3.19 Maize root symptom from *Fusarium graminearum* infection in the presence of *Costelytra giveni*. After seeds were coated with a conidia suspension from entomopathogenic fungi, plants were sown in potting mix containing *F. graminearum* 0.5% (w/w) and two larvae of *C. giveni*. Plants were grown at 25°C in light:dark conditions (16:8 h) and at 65 RH%. After two weeks, plants were harvested, and roots were assessed for *Fusarium* root rot symptom. Conditions: Cg^- / Cg^+: absence/presence of *C. giveni*. Error bars = $LSD_{5\%}$. Different letters above average values represent statistical significant differences ($LSD_{5\%}$) at $p<0.05$.

In comparison to without *C. giveni*, only plants with *M. anisopliae* A1080, *M. robertsii* F447 and *T. harzianum* coating treatments had decreased *Fusarium* symptoms of 83, 63 and 67%, respectively, in the presence of the insect pest (Figure 3.19).

3.5.6 *Costelytra giveni* mortality in presence of maize plants treated with entomopathogenic fungal isolates

After destructive harvesting of the maize plants 145 larvae of *C. giveni* were recovered from the originally 180 placed in the pots (Figure 3.20). There were differences in the percentage mortality caused by the different entomopathogenic fungal coatings (Figure 3.21; $p<0.01$).

All *Metarhizium* isolates were equally pathogenic to *C. giveni* with similar mortality rates while *B. bassiana* was unable to infect grass grubs. The highest mortality was found in *M. anisopliae* F672 with 66.7% mortality.

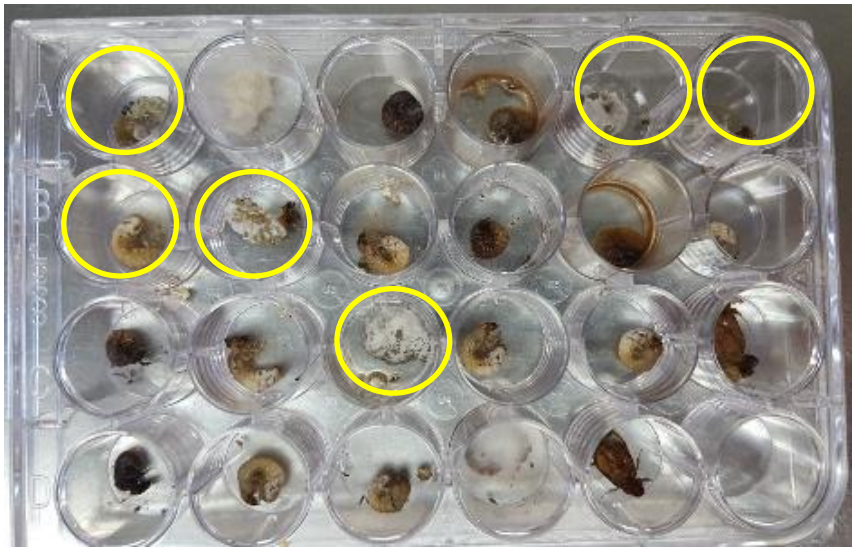

Figure 3.20 *Costelytra giveni* infection from seeds coated with entomopathogenic fungi. Grass grubs recovered from pots were kept in a humid chamber at 20 ± 2°C until showed symptoms of either, bacterial or fungal infection. In the wells marked in yellow are larvae infected with *Metarhizium*.

Metarhizium anisopliae F672 was the only isolate significantly different to the control *M. anisopliae* A1080 ($LSD_{5\%}$ = 18.4). No grass grubs died with signs of mycosis among the treatments with *T. harzianum* or the treatment with control seeds without a fungal coating (Figure 3.21).

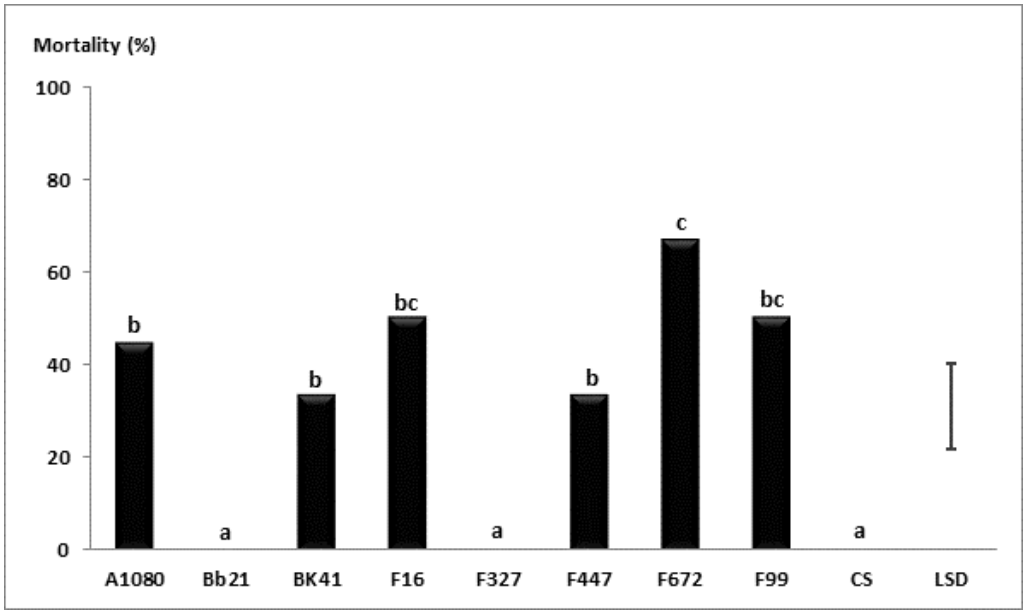

Figure 3.21 Percentage *Costelytra giveni* mortality. After plants were harvested, *Costelytra giveni* were recovered from pots, corresponding treatment identified and individually located in a well of a 24-well plate. Larvae of grass grub were incubated at 20°C and periodically inspected for symptoms of fungal infection and mycosis. Fungi from infected larvae were isolated and were identified to genera by microscopic and macroscopic observation of the colonies. Error bars = standard deviation. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

The presence of *F. graminearum* seemed to influence the fungal-induced mortality of *C. giveni* and on average lower rates were determined in the presence of the plant pathogen ($p<0.06$). Additionally, in some entomopathogenic fungal isolate treatments different mortality rates were found when *F. graminearum* was present or absent (Figure 3.22; $p<0.01$). In general, the presence of the plant pathogen reduced the ability of the fungi to infect *C. giveni*, except for *M. guizhouense* Bk41 and *M. anisopliae* F672 where no differences were determined whether *Fusarium* was present or not. On the other hand, *M. robertsii* F447 was the only isolate which increased the mortality with the presence of *F. graminearum* ($LSD_{5\%}$ = 30.9). This treatment, together with *M. anisopliae* F672, had the highest mortality levels, statistically different to the other treatments (Figure 3.22).

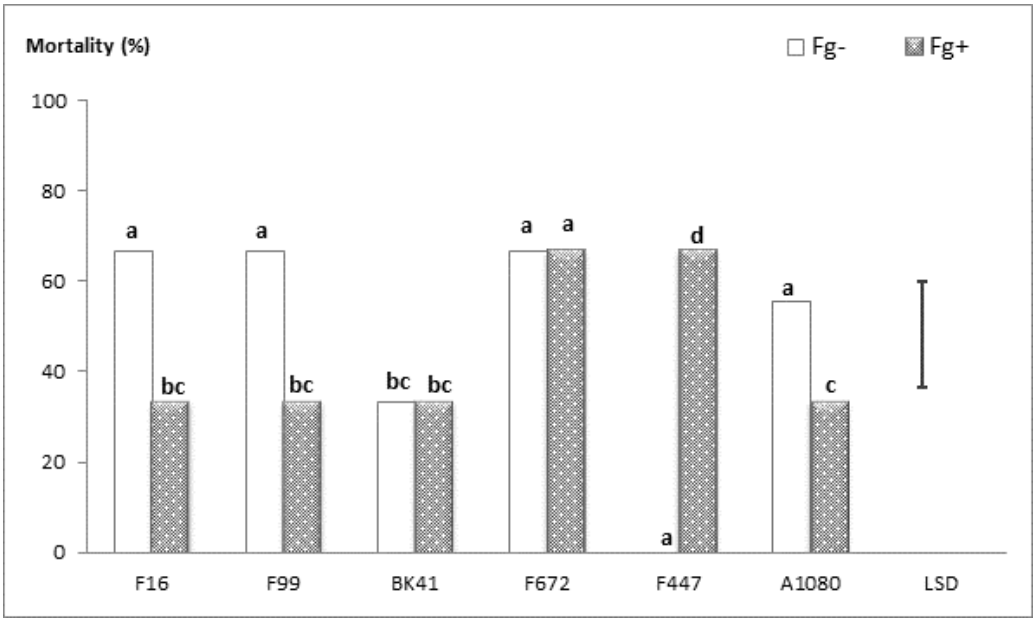

Figure 3.22 ***Costelytra giveni*** **mortality in presence of** ***Fusarium graminearum*****.** After plants were harvested, *Costelytra giveni* were recovered from pots, the corresponding treatment identified, and larva individually located in a well of a 24-well plate. Larvae of grass grub were incubated at 25°C and periodically inspected for symptoms of fungal infection and mycosis. Fungi from infected larvae were isolated and were identified to genera by microscopic and macroscopic observation of the colonies. Conditions: Fg-/ Fg+: absence/presence of *F. graminearum*. Error bars = standard deviation. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

3.5.7 Rhizosphere and endophytic colonization of maize plants by entomopathogenic fungi at 25 and 28°C

The average fungal colonies conforming to the applied isolates obtained from the rhizosphere soil at 25°and 28°C, was approximately 1.4 x 10^3 CFU/g. At both temperatures, differences were found among the isolates in rhizosphere colonization when determined as the average number of CFU/g of rhizosphere soil ($p<0.01$). There were no fungal colonies of *Trichoderma, Beauveria* or *Metarhizium*

isolated from the rhizosphere of control plants (CS). In samples of shoots and leaves, there were no colonies belonging to the fungi used in the seeds-coating found.

C. giveni had a significant effect on fungal rhizosphere colonization at 25°C, but *F. graminearum* did not. The average number of fungal colonies obtained from the rhizosphere, in the experiment at 25°C, dropped from 2.2 x 10^3 CFU/g in the absence of *C. giveni* to 6.7 x 10^2 CFU/g in the presence of the insect pest ($p<0.01$; Figure 3.23).

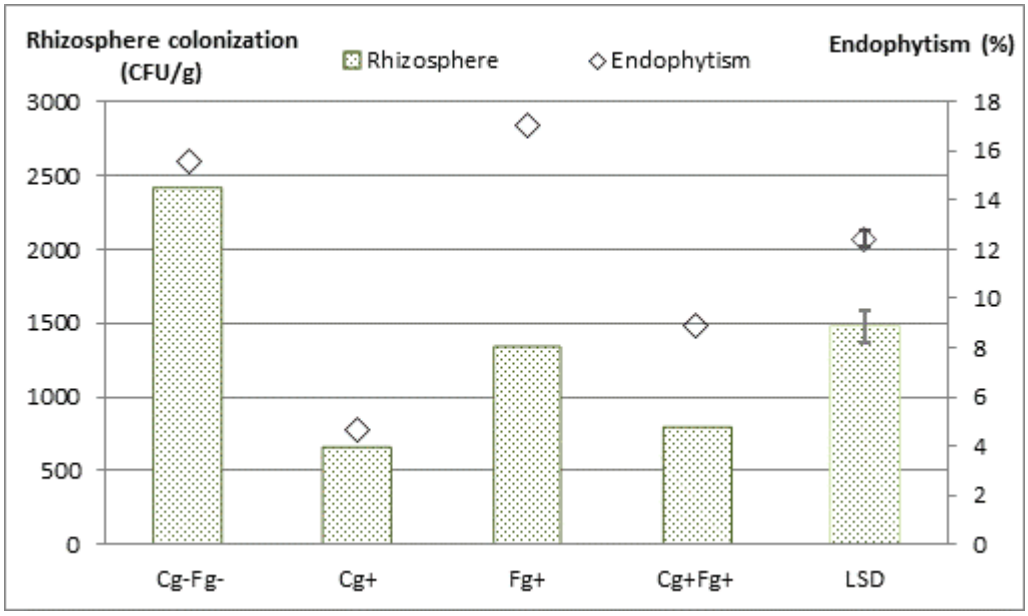

Figure 3.23 Overall rhizosphere and endophytic colonization by entomopathogenic fungi in presence of *C. giveni or F. graminearum* at 25°C. Values correspond to the number of colonies (CFU) obtained per gram of rhizosphere soil or to the average number of colonies as endophytes as a percentage of root segments colonized. Conditions: Cg-/ Cg+: absence/presence of *C. giveni*; Fg-/ Fg+: absence/presence of *F. graminearum*. Error bars represents $LSD_{5\%}$.

On the other hand, fungal rhizosphere colonization was stable in the presence or absence of *F. graminearum*, with an average value of 1.3 x 10^3 and 1.5 x 10^3 CFU/g, respectively ($p<0.192$). The fungal rhizosphere colonization by the entomopathogenic fungi was affected also by the simultaneous presence of *C. giveni* and *F. graminearum* ($p<0.01$; Figure 3.23). In general, when only one of the biotic factors is considered, the reduction caused by *C. giveni* on the number of fungal colonies per gram of rhizosphere soil was 78% of the number of colonies compared to no larvae, while when only *F. graminearum* is considered, this decrease was only 22% ($LSD_{5\%} = 314$). However, when both pathogens were present rhizosphere colonization by entomopathogenic fungi was reduced on average a 69%, compared to no larvae and plant pathogen ($LSD_{5\%} = 314$). The effect on rhizosphere colonization by entomopathogenic fungi of *C. giveni* at 28°C was not possible to evaluate since this temperature was

detrimental for the pest and only the effect of *F. graminearum* was determined. As before, the plant pathogen did affect the rhizosphere colonization by the entomopathogenic fungi. The average number of rhizospheric colonies of entomopathogens at 28°C was 2.8 x 10^2 cfu/g in the absence of *Fusarium* and 2.4 x 10^2 cfu/g with the plant pathogen.

After surface sterilization of roots, stems and leaves, fungi from coatings were only recovered from roots from experiments at 25 and 28°C. On average, at both temperatures, the number of endophytic colonies obtained from plant segments, corresponding to the fungal seed-coatings, was low with an average of 12 colonies per sample treatment (representing only root samples as the other plant parts were negative). At 25°C, the presence of *C. giveni* and *F. graminearum* influenced the ability of the entomopathogenic fungi to become endophytic but, whereas the insect reduced endophytic colonization, the fungal plant pathogen presence increased it (Figure 3.23). The decrease in endophytism because of the presence of *C. giveni* was determined to be around 77%, compared to no larvae whilst, the increase in endophytic colonization with the presence of *F. graminearum* was around 212% ($p<0.01$). At 28°C, no effect on fungal endophytism in the presence or absence *F. graminearum* was observed ($p<0.493$).

3.5.8 Ability of the entomopathogenic fungal isolates to colonize the rhizosphere at 25 and 28°C

All the isolates used in the seed coatings colonized the rhizosphere but the extent of colonization, expressed as CFU per gram of rhizosphere soil, differed ($p<0.01$). The highest CFU/g were from plants with the *M. robertsii* F447 and *M. anisopliae* A1080 and F672 treatments ($LSD_{5\%}$=1008; Figure 3.24). F447 had a higher colonization level than the plant growth promotor and rhizosphere colonizer *T. harzianum* F327 ($LSD_{5\%}$=504).

In the presence of *C. giveni*, fewer CFU/g were recovered from the rhizosphere than in the absence of grass grub for all the fungal treatments ($p<0.05$). *M. anisopliae* F672 ($LSD_{5\%}$ = 712), was the only treatment not affected by the presence of *C. giveni* where, in fact, there was an increase in the rhizosphere colonization of 49% (Figure 3.24).

The fungal plant pathogen*, F. graminearum*, affected, positively or negatively, the ability of some isolates to colonize the rhizosphere ($p<0.01$). When *Fusarium* was present, *M. robertsii* F447, *M. anisopliae* A1080 and F672, had reductions in rhizosphere colonization of 94, 61, and 29% respectively compared to when not present (Figure 3.24). Only the *T. harzianum* F327, *M. guizhouense* F16 and *M. novozealandicum* F99 treatments increased the number of fungal colonies per gram of rhizosphere with the presence of the plant pathogen compared to not present.

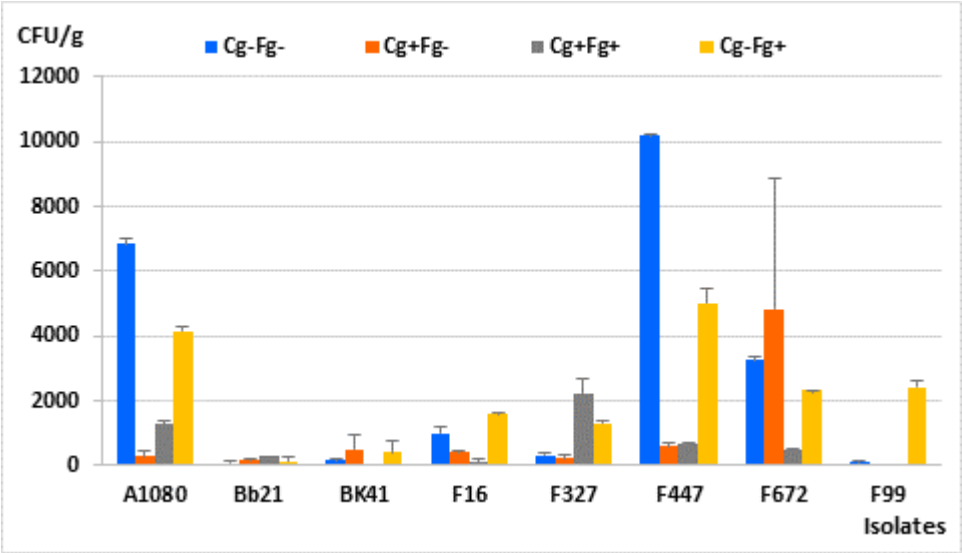

Figure 3.24 Fungal rhizosphere colonization at 25°C. Number of colony forming units (CFU) per gram of rhizosphere soil obtained from the fungal coatings treatments. Conditions: Cg-/ Cg+: absence/presence of *C. giveni*; Fg-/ Fg+: absence/presence of *F. graminearum*. Error bars represents standard deviation; $p < 0.01$.

The presence at the same time of both *C. giveni* and *F. graminearum* negatively affected the ability of *M. robertsii* F447 (93%), *M. anisopliae* A1080 (81%), and *M. anisopliae* F672 (84%) to colonize the rhizosphere ($p<0.01$; Figure 3.24). The plant promotor *T. harzianum* showed a significant increase in rhizospheric colonization from 312 CFU/g in the absence of *C. giveni* or *F. graminearum*, to 2.2 x 10^3 CFU/g when both were present which represents a 600% increase (Figure 3.24).

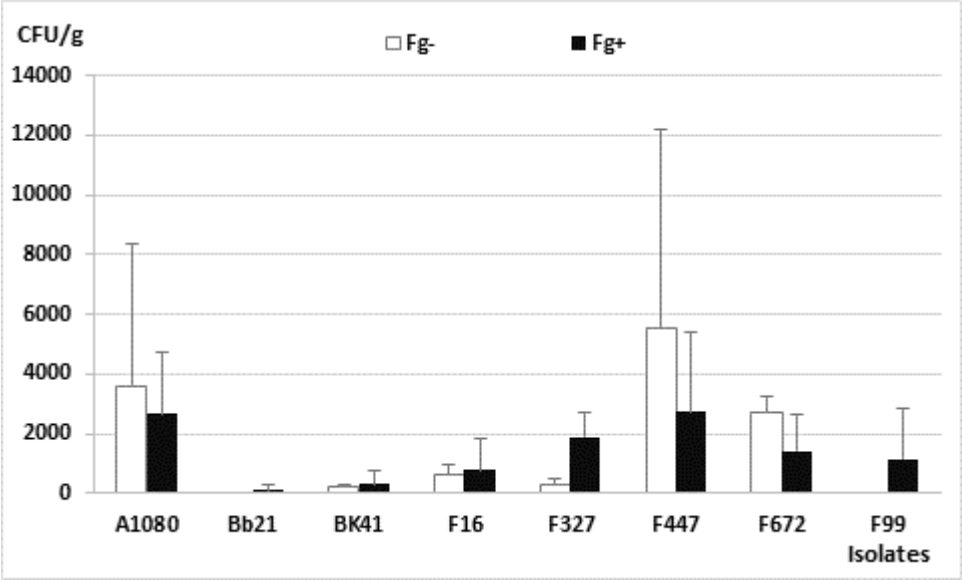

Figure 3.25 Fungal rhizosphere colonization at 28°C. Number of colony forming units (CFU) per gram of rhizosphere soil obtained from the fungal coatings treatments. Conditions: Fg-/ Fg+: absence/presence of *F. graminearum*. Error bars represents standard deviation; $p = 0.411$.

At 28°C the trend of rhizosphere colonization by the different fungal isolates was the same as observed at 25°C. The highest value of CFU/g was observed again in *M. robertsii* F447 and *M. anisopliae* A1080 and the colonization of the rhizosphere by these isolates was also significantly higher than the plant growth promotor *T. harzianum* F327 ($p<0.01$; Figure 3.25).

At 28°C, the presence *F. graminearum* did not affect the ability of the isolates to colonize the rhizosphere. There were no statistical differences among the fungal coating treatments whether *Fusarium* was present or not (Figure 3.25).

3.5.9 Ability of the entomopathogenic fungal isolates to endophytically colonize maize plants at 25 and 28°C

All the isolates, except for *B. bassiana* Bb21, were found to be endophytic in the roots, but with different endophytic rates. The endophytic capability was dependent not only on the fungal isolate ($p<0.01$), but also in the presence of *C. giveni* or *F. graminearum* ($p<0.01$).

At 25°C, in absence of the soil dwelling pest or the fungal plant pathogen, endophytic fungal colonies were only obtained from the plant growth promotors *T. harzianum* F327 and *M. anisopliae* A1080, and also *M. robertsii* F447 with the proportion of roots segments colonized of 35, 33 and 29%, respectively ($p<0.01$). For the remaining isolates, no endophytic colonies were obtained (Figure 3.26).

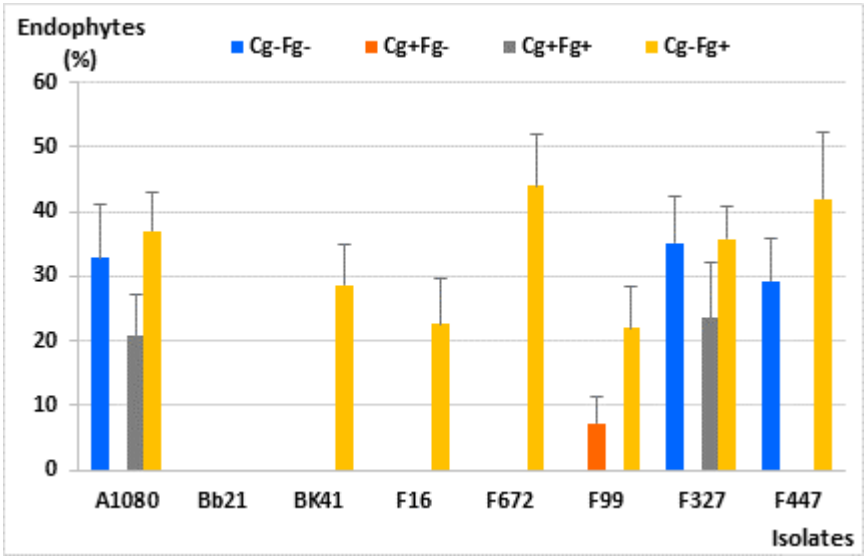

Figure 3.26 Fungal root endophytic colonization at 25°C. Percentage of root segments colonized endophytically for the fungal coating treatments after surface sterilization. Conditions: Cg-/ Cg+: absence/presence of *C. giveni*; Fg-/ Fg+: absence/presence of *F. graminearum*. Error bars represents standard deviation; $p < 0.01$.

In the presence of *C. giveni* no endophytism was recorded except for *M. novozealandicum* F99. However, the presence of *F. graminearum* favoured endophytic colonization ($p<0.01$). All the fungal isolates, except for *B. bassiana* Bb21, were found in root segments when *F. graminearum* was present in the soil. The highest percentage of endophytism, was found in *M. anisopliae* F672 and *M. robertsii* F447, and these were significantly greater than the values found in the plant promotors, *M. anisopliae* A1080 and *T. harzianum* F327 treatments ($LSD_{5\%}$ = 0.842). The endophytic colonization observed increase for the different *Metarhizium* isolates, with the presence of *F. graminearum*, was between 43 to 76% (Figure 3.26). The presence of both challengers suppressed the ability of the treatments to endophytic colonise the roots and only *M. anisopliae* A1080 and *T. harzianum* F327 were found as endophytes in 21 and 24% of the root samples ($LSD_{5\%}$ = 0.842; Figure 3.26).

Results for 28°C were similar to those described for the lower temperature, however the endophytic rates were even lower than those at 25°C. The highest endophytic colonization was found in the plant growth promotors *T. harzianum* F327 (23%) and *M. anisopliae* A1080 (22%), and in the *M. robertsii* F447 treatment (14%). In the remaining treatments, no endophytic colonies from the coating treatments were found (Figure 3.27).

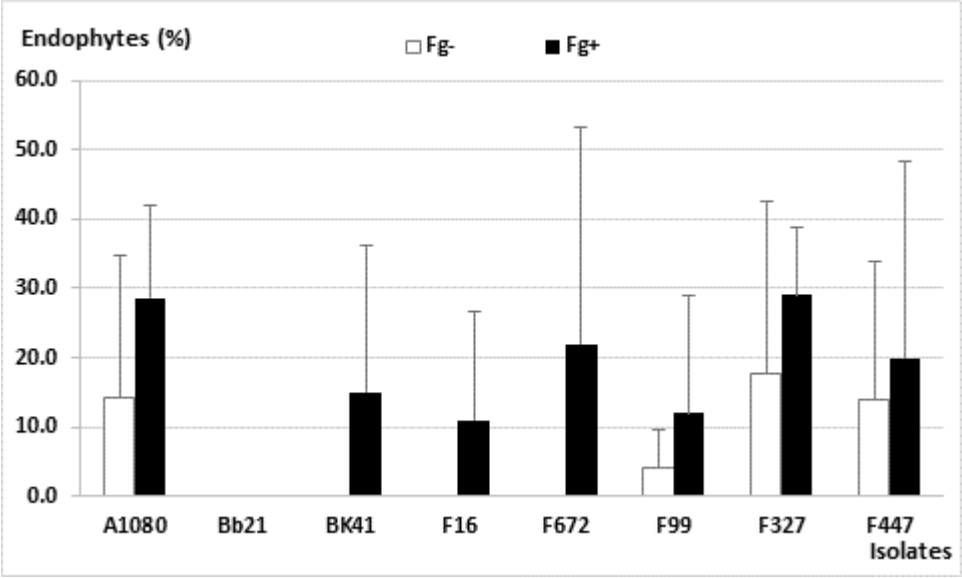

Figure 3.27 Fungal root endophytic colonization at 28°C. Percentage of root segments colonized endophytically by the fungal coating treatments after surface sterilization. Conditions: Fg-/ Fg+: absence/presence of *F. graminearum*. Error bars represents standard deviation; p = 0.535.

In the presence of *F. graminearum* endophytic colonies from root segments were obtained from all the fungal isolates except for *B. bassiana* Bb21, but it was not possible to determine an effect because the presence or absence of the plant pathogen (Figure 3.27).

3.5.10 Effect of *Metarhizium anisopliae* seed-coating on jasmonic acid and salicylic acid content in maize in the presence of *Costelytra giveni*

3.5.10..1 Total content of salicylic acid and jasmonic acid in whole maize plants from seeds coated with isolates of M. anisopliae.

The average concentration of salicylic acid (SA) in maize plants was 155.5 ± 67.5 ng/g while for, jasmonic acid (JA), it was 259.1 ± 174.6 ng/g. Both phytohormones were found in higher concentrations in roots than in shoots ($p<0.01$; Table 3.5).

Table 3.5. Average concentration of the phytohormones salicylic acid and jasmonic acid in maize plants

Phytohormone	Root (ng/g)	Shoots (ng/g)
Salicylic acid	183.5 ± 73.6	125.6 ± 44.1
Jasmonic acid	379.4 ± 159.6	130.4 ± 61.5

In general, in the absence of *C. giveni*, the concentration of SA in maize plants seeds coated with *M. anisopliae* A1080 and F672 did not differ from control plants, CS (Figure 3.28). However, maize plants with the treatment *M. anisopliae* A1080 had higher average SA concentrations, 190 ng/g, than with *M. anisopliae* F672 treatment, with 135 ng/g ($LSD_{5\%}$ = 0.079).

On the other hand, when *C. giveni* was present, *M. anisopliae* A1080 decreased SA by 23% compared to the condition without grass grub, while no variation was observed in the content of this phytohormone in plants from the *M. anisopliae* F672 treatment or in CS (Figure 3.28). It seems that the presence of *C. giveni* did not affect the overall concentration of SA in CS but the response was variable depending on the entomopathogenic fungal treatment ($p<0.01$).

In the absence of *C. giveni*, plants treated with *M. anisopliae* A1080 had the highest JA concentration, 259 ng/g, which was statistically greater than CS with 122 ng/g ($LSD_{5\%}$ = 0.205; Figure 3.29). CS had a 56% JA increment in the presence of *C. giveni* compared to no larvae, while no variation was determined in plants with both fungal treatments ($LSD_{5\%}$=0.159). In the presence of the grass grub all the maize plants had the same levels of JA, approximately 208 ± 22 ng/g (Figure 3.29).

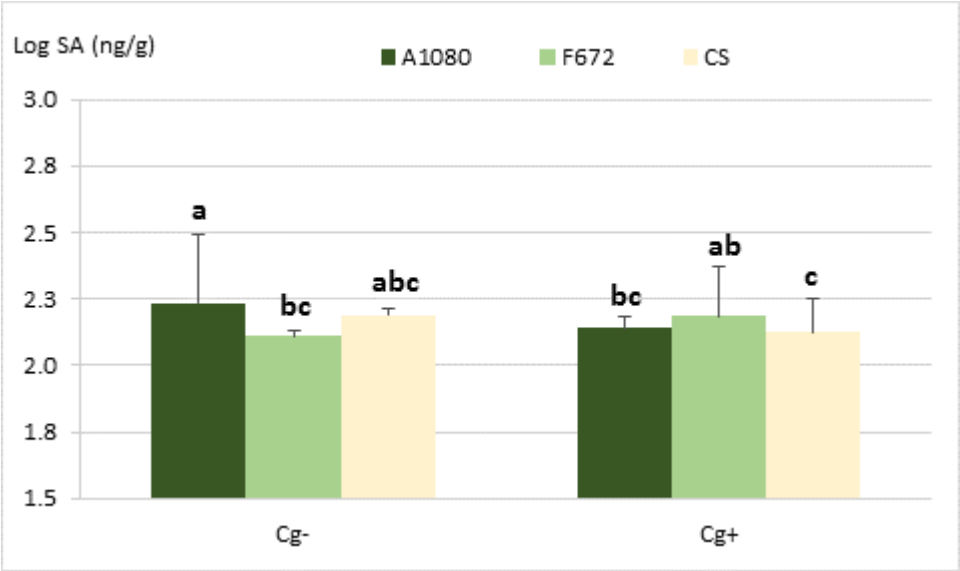

Figure 3.28 Salicylic acid (SA) concentration in maize plants from seeds coated with *Metarhizium anisopliae* (A1080 and F672) or without a fungal coating (control seeds, CS). Seeds coated with each treatment were sown in potting mix and grown for two weeks at under controlled conditions of temperature, light and humidity. After one week three grass grubs of *C. givenі* were added to each pot containing one plant. After 2 weeks the plants were immediately frozen in liquid nitrogen until phytohormones extraction. Cg-/Cg+: absence/presence of *Costelytra giveni*. Error bar indicate standard deviation, $p<0.01$. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

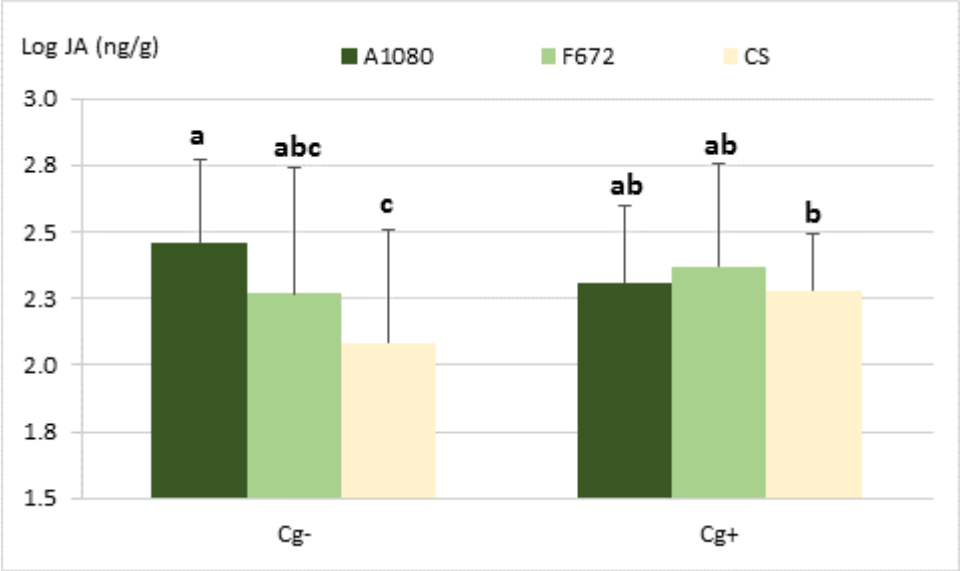

Figure 3.29 Jasmonic acid (JA) concentration in maize plants from seeds coated with *Metarhizium anisopliae* (A1080 and F672) or without a fungal coating (control seeds, CS). Seeds coated with each treatment were sown in potting mix and grown for two weeks at under controlled conditions of temperature, light and humidity. After one week three grass grubs of *C. givenі* were added to each pot containing one plant. After 2 weeks the plants were immediately frozen in liquid nitrogen until phytohormones extraction. Cg-/Cg+: absence/presence of *Costelytra giveni*. Error bar indicate standard deviation, $p<0.01$. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

3.5.10..2 Effect of M. anisopliae seed-coating on salicylic acid and jasmonic acid content in maize roots and shoots in presence of C. giveni

In general, there were more differences in the phytohormone SA between treatments in the roots than in the shoots in the presence of *C. giveni* (Figure 3.30; $p<0.01$). In contrast, the JA concentration in plants from the treatments with *M. anisopliae* did not have any significant change in concentration as result of grass grub presence, but did for CS in shoots (Figure 4; $LSD_{5\%} = 0.237$). On the whole, in roots or in shoots, at least one of the fungal treatments had a phytohormone concentration different to CS in presence or absence of the grass grubs (Figure 3.30 and 3.31).

In absence of *C. giveni*, roots from seeds coated with the *M. anisopliae* A1080 treatment had the highest concentration of SA (Figure 3.30-A). Under the same condition, shoots in CS had higher concentration of this phytohormone than *M. anisopliae* A1080 (Figure 3.30-B). The presence or absence of *C. giveni* resulted in a variation in the SA content in roots from plants treated with the entomopathogenic fungi ($p<0.01$). In comparison with the absence of the insect pest, plants with the *M. anisopliae* A1080 treatment had a 44% reduction in SA, while in the *M. anisopliae* F672 treatment the concentration was increased 66% in the presence of grass grub (Figure 3.30-A). There were no changes in SA concentration when CS plants grown in the presence or absence of *C. giveni* were compared. This fact could be indicative that the presence of the fungal treatments elicited a more rapid response in maize plants because of the grazing action of the grass grubs on the roots, but this response in the plant could be different depending on the isolate ($p<0.01$). On the other hand, only shoots from CS had a reduction in SA content with the grass grubs, while shoots in maize plants from the fungal coated seeds did not have any variation (Figure 3.30-B).

The scenario for the JA was similar to that observed with SA. In absence of the grass grubs, maize roots from the *M. anisopliae* A1080 had statistically greater JA concentration, 479.7 ng/g, than CS with 241 ng/g (Figure 3.30-A). In this condition, without *C. giveni*, the JA concentration in shoots in plants with the *M. anisopliae* A1080 treatment (171 ng/g) was statistically greater than CS (61 ng/g) and the *M. anisopliae* F672 treatment (86 ng/g) (Figure 3.30-B).

In presence of *C. giveni*, JA root´s content in the treatments did not change in comparison with values of this phytohormone in absence of the insect pest, however, in this condition, the concentration of the *M. anisopliae* F672 was higher to CS (Figure 3.31-B; $LSD_{5\%} = 0.290$).

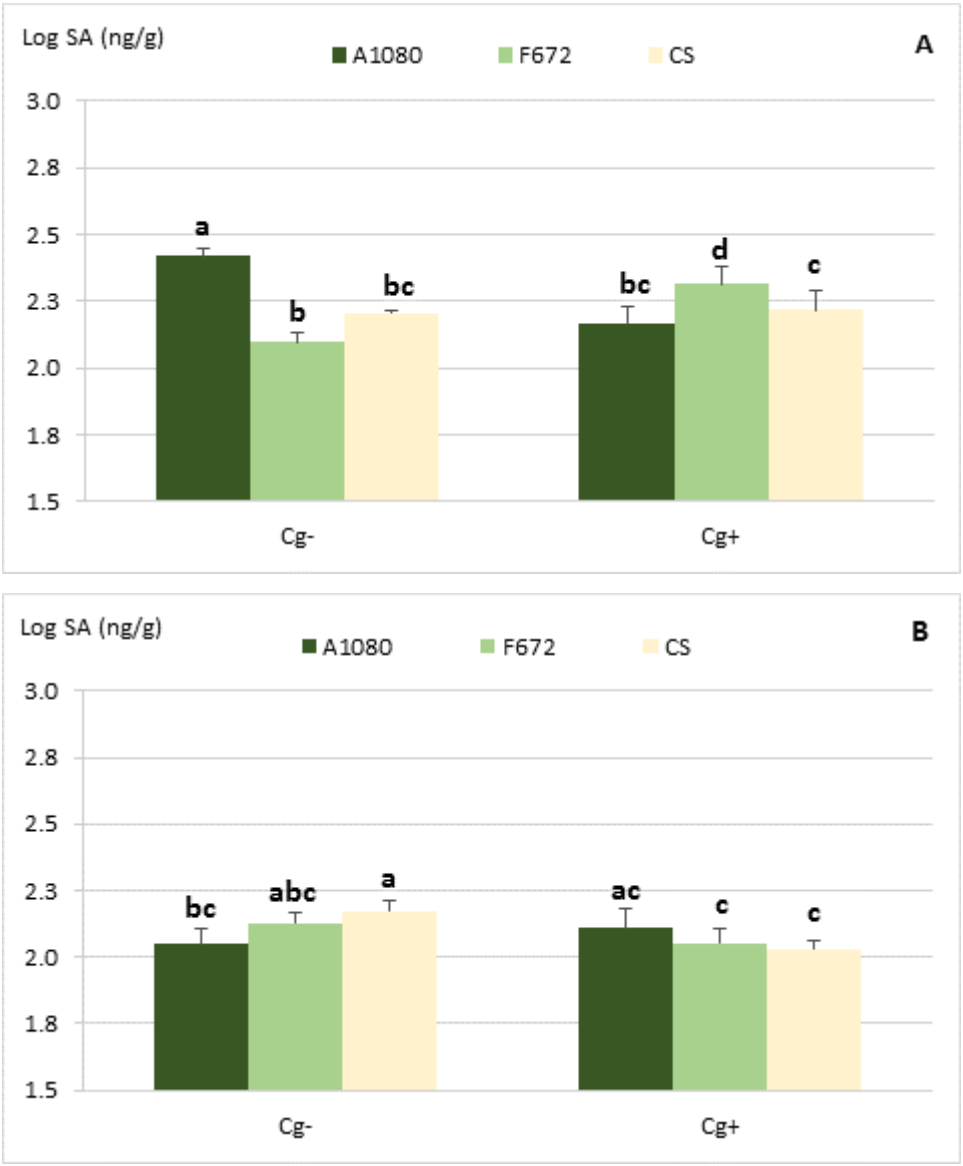

Figure 3.30 Salicylic acid (SA) concentration in maize plants from seeds coated with *Metarhizium anisopliae* (A1080 and F672) or without a fungal coating (control seeds; CS). Seeds coated with each treatment were sown in potting mix and grown for two weeks at under controlled conditions of temperature, light and humidity. After one week three grass grubs of *C. given*i were added to each pot containing one plant. After 2 weeks the plants were immediately frozen in liquid nitrogen until phytohormones extraction. A. SA concentration in roots. B. SA concentration in shoots. Cg-/Cg+: absence/presence of *Costelytra giveni*. Error bar indicate standard deviation, $p<0.01$. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

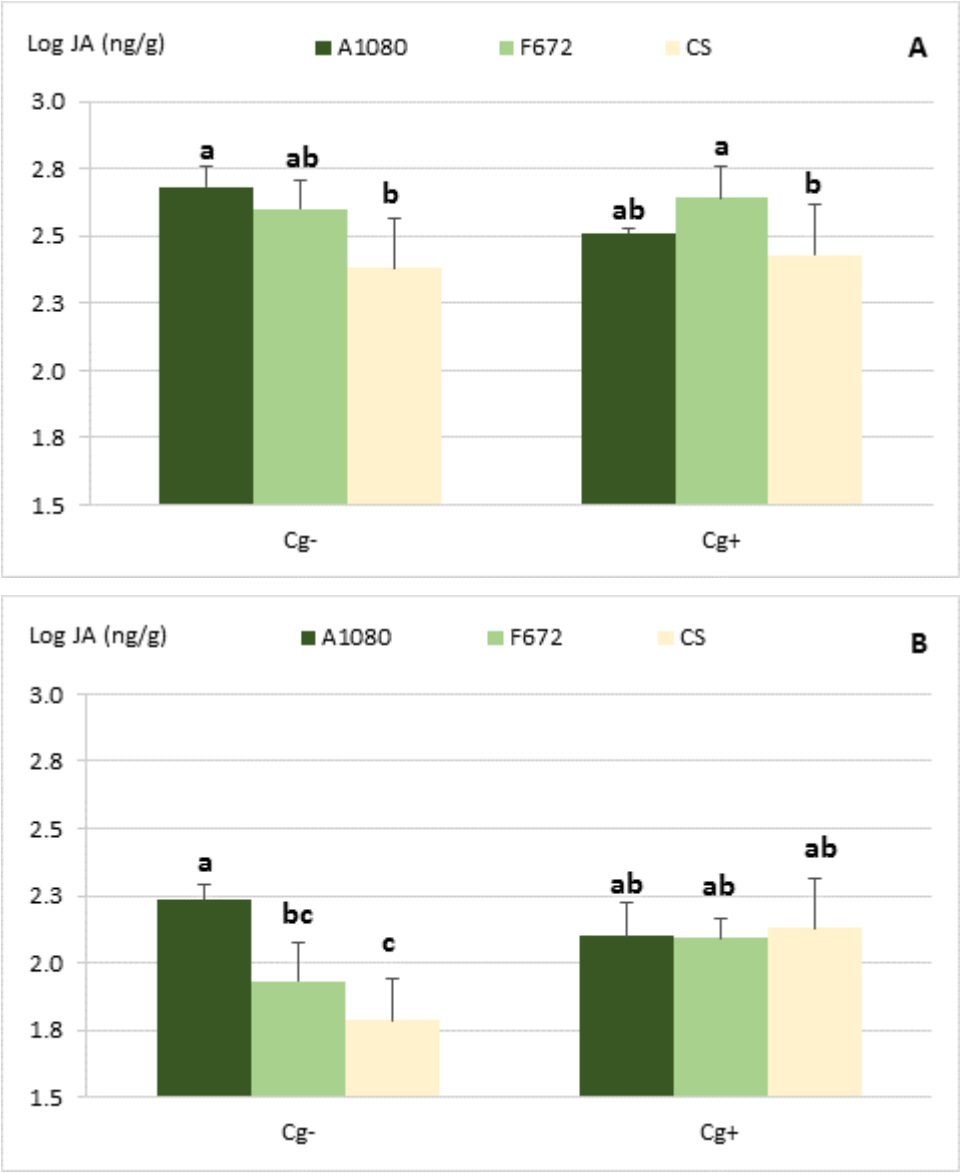

Figure 3.31 Jasmonic acid (JA) concentration in maize plants from seeds coated with *Metarhizium anisopliae* (A1080 and F672) or without a fungal coating (control seeds; CS). Seeds coated with each treatment were sown in potting mix and grown for two weeks at under controlled conditions of temperature, light and humidity. After one week three grass grubs of *C. giveni* were added to each pot containing one plant. After 2 weeks the plants were immediately frozen in liquid nitrogen until phytohormones extraction. A. JA concentration in roots. B. JA concentration in shoots. Cg-/Cg+: absence/presence of *Costelytra giveni*. Error bar indicate standard deviation, $p<0.01$. Different letters above average values represent statistically significant differences ($LSD_{5\%}$) at $p<0.05$.

In shoots, in absence of the insect pest, the treatment *M. anisopliae* A1080 had statistically greater content of JA than CS and the *M. anisopliae* F672 treatment ($LSD_{5\%} = 0.290$), while in presence of *C. giveni*, the JA content was similar among the seed coatings. In comparison between both conditions, CS was the only treatment to significantly increase (134%) the concentration of JA, from 61 ng/g to 134 ng/g (Figure 3.31- B).

3.6 Discussion

Understanding the response of entomopathogenic fungi to ecological variables is essential to improve their use as biocontrol agents. The work presented here links the effect of entomopathogenic fungal seed coatings (*Metarhizium* spp., *B. bassiana* and *T. harzianum*) on maize plant development and performance against two specific biotic factors. Additionally, the possible effects on the plant induced defence response due to the coating treatment in presence of grass grubs was also evaluated.

Seed coating with conidia of entomopathogenic fungi has been proposed as a feasible strategy for the delivery of these fungal biocontrol agents because, after conidia germination, the developing hyphae would survive on the exudates from growing plant roots (Bruck, 2005). However, the application of formulations based only on conidia seems not to be appropriate since most conidia are likely to lose viability fast in the environment and only minimal proportions will presumably succeed in infecting new hosts (Meyling & Eilenberg, 2007). The delivery of rhizosphere competent entomopathogenic fungi as a seed coating may improve survival on the roots, while at the same time, provide nutrients to the plant and protect against pest and diseases (Bruck, 2005; Ownley *et al*., 2010). Application of insecticidal microorganisms to seed is an ideal delivery system as it introduces the control agent into the plant rhizosphere where the target pests (in this case grass grub larvae) will be feeding, ensuring rapid contact between the pathogen and its host (Young, Townsend & O´Callaghan, 2009).

Seed germination was not affected by the components used for the coating or the entomopathogenic fungal isolates tested at both conidial concentrations, 10^5 and 10^8 conidia/mL. Kabaluk & Ericcson (2007) also found that *M. brunneum* F52 conidia had no effect on maize seed germination or on root growth at concentrations of up to 10^8 conidia/seed, but levels of conidia of 10^9 conidia/seed significantly reduced germination and also, root growth.

Fungi are able to grow over a huge number of substrates, even in limited quantities, and seeds could be seen from a fungus perspective as another nutrient resource. During germination in soil seeds are continuously exposed to microorganisms, either beneficial or pathogens, and may use different strategies to limit their growth during seedling development (Partida-Martínez & Heil, 2011; Pangesti *et al*., 2013). Plants succeed in this mission but also microorganisms establishing different relationships and associations with plants (Pangesti *et al*., 2013). There is likely to be a threshold for the amount of conidia of entomopathogenic fungi that seeds can tolerate, and beyond this limit, seed viability could be seriously jeopardized (Kabaluk & Ericsson 2007).

The isolates used in this study when applied as a coating to maize seeds at both concentrations, 10^5 and 10^8 conidia/mL, did not have any detrimental effect on seed viability or plant development, since

maize plant performance was comparable to that obtained with control seeds. However, the final concentration of conidia present on the seeds, or conidia viable after coating, was two orders lower than that originally calculated as applied (10^3 and 10^6 CFU/g), respectively, indicating either, that the viability of the conidia or the efficiency of the coating process, was low.

When maize plants were grown in the absence of biotic factors, in general there were no significant differences between the plants from the fungal entomopathogenic coated seeds and the control treatments. However, there was also no effect of plant growth promotion.

In this study, only maize plants coated with the *B. bassiana* Bb21 and *M. novozealandicum* F99 treatments had smaller shoot or root dry weight, respectively, than controls and other fungal coatings. Therefore, these isolates when interacting with the roots of maize plants might be a kind of "cheater" microorganism, operating as a sink, taking nutrients from the plant without adding benefits. It is also probable that the plant perceives this negative effect and uses more resources to limit fungal development, what in the end would affect plant growth. Another explanation could be the lack of specificity between these isolates and the maize plant used in the experiments. It has been suggested that certain species of entomopathogenic fungi are specific for particular plant species (Vega *et al*., 2009; Ownley *et al*., 2010; Pangesti *et al*., 2016). The lack of specificity could result in a higher rejection by the plant when limiting fungal colonization that in the end would also cause a reduction in plant growth.

Fungal entomopathogens in the soil can be exposed for long periods to specific plant communities which may work as a selection pressure on the fungi for those plants that can ''bridge'' the gap between insect hosts while persisting in the rhizosphere in that particular habitat (Kepler *et al*., 2015). As stated by Humber (2008) ''Natural selection may also lead a fungus to an increasing or decreasing level of nutritional and biological adjustment to its food source; such adjustments could move a fungus in any direction along the nutritional continuum from beneficial to commensal to saprobic to parasitic to pathogenic associations with the source of its nutrients''.

According to Partida-Martínez & Heil (2011) a plant that benefits from an associated microorganism would only be noticed in the presence of abiotic or biotic stress factors. In the absence of any stressor, the associated microorganism has a cost for the plant in terms of nutrients. Additionally, the plant will try to limit the growth of the microorganism, which might in the end reduce plant growth. However, in the presence of any external negative factor, if the associated microorganism provides the plant with an advantage to cope with this factor, the final outcome between the cost of harbouring the microorganism and the reduction in the incidence of the negative factor should result in no changes in

plant performance. This response can be also seen in the work of Kabaluk & Ericsson (2007) who concluded that the positive effect on maize plant growth was the result of the biocontrol activity of the entomopathogenic fungi on the wireworm pests. Cosme *et al*. (2016) also found no differences between fungal endophyte-free rice plants without root feeding larvae, and fungal endophyte-inoculated plants with or without larvae. In this context, the lack of plant growth promotion in the absence of plant-stressors should not be seen as a lack of efficacy of the fungal treatment. The possible benefits to the plant due to the presence of entomopathogenic fungi is more likely to be seen in the presence of pests.

Maize plant growth, as expected, was negatively affected by the presence of *C. giveni* or *F. graminearum*, and it was even worse when both were present simultaneously. *C. giveni* was the challenger with the major effect on plant growth since roots weighted on average 19% less when compared to plants in the absence of the pest. Plant growth performance varied with the isolates, and with the absence or presence of *C. giveni* and *F. graminearum*, or with their simultaneous presence.

Maize plants from seeds coated with *B. bassiana* in almost all the conditions tested had the poorest performance among the treatments and, in some cases, growth was worse than that of the control plants without a fungal treatment. Control plants (CS) also had a lower performance than some of the fungal treatments in the presence of one of the biotic factors, or with both at the same time. The effect of *T. harzianum* on plant growth was enough to counteract the negative effect of the biotic factors presence. Although plants treated with this isolate had 47% less root biomass when *C. giveni* and *F. graminearum* were present compared to not, the shoot dry weight was not affected, similar to CS. This effect on plant growth promotion, where no changes were recorded in shoot dry weight, was also observed with the entomopathogenic isolates *M. anisopliae* A1080 and *M. guizhouense* Bk41 and F16 where in the presence of *C. giveni* maize plants had root dry weight reductions of 24; 33 and 30%, respectively. However, plants with the *M. anisopliae* F672 treatments had no variations in root or shoot dry weight in any of the conditions which might indicate the ability of this isolate to promote maize growth and at the same time, reduce the possibly negative effect on growth associated with the presence of the biotic factors.

Metarhizium was first discovered to be an inhabitant of the rhizosphere more than a decade ago (Hu and St. Leger, 2002), although it was not until recently that the ability of *Metarhizium* spp. to transfer and exchange nutrients with plants was also confirmed (Behie *et al*., 2012; Kepler *et al*., 2015; Behie *et al*., 2017). In previous work, *B. bassiana* and *M. anisopliae* were re-isolated from surface-sterilized roots of cassava plants but never from the leaves or stems of those plants (Greenfield *et al*., 2016). This indicates that the fungi were not systemic within the plant, but rather remained localized in the

roots (Greenfield *et al*., 2016). This exclusive root localization is in contrast to studies that have found *B. bassiana* can establish as an endophyte throughout the entire plant, particularly after seed inoculation (Ownley *et al*., 2008; Tefera & Vidal, 2009). The results obtained herein with *Metarhizium* spp. isolates, which were only found as root endophytes, have been reported before. Species of *Metarhizium* are more often endophytes of roots and not of the upper parts of plants (Behie *et al*., 2017; Vega *et al*., 2009; Moonjely, Barelli & Bidochka, 2016).

The entomopathogenic fungal isolates when coated on seeds were all able to grow in the rhizosphere of the maize plants, but the presence of *C. giveni* or *F. graminearum* was detrimental for rhizosphere colonization. The insect pest had the strongest negative effect with a 78% decrease in rhizosphere colonization, while for *Fusarium* this was only a 22%. When both were present the rhizosphere colonization was reduced by 67%. The decrease in rhizosphere colonization when *C. giveni* was present is most likely associated with root loss because of the feeding behaviour, which can reduce root biomass but also separate sections of roots from the maize plant.

Entomopathogenic fungi like *M. robertsii* and *M. anisopliae*, when compared to *T. harzianum*, had higher percentages of rhizosphere colonization, while the lowest values were found in *M. guizhouense* Bk41, *M. novozealandicum* F99 and *B. bassiana* Bb21. These latter isolates may have a lower specificity with the maize plants used in this work when compared to the former isolates. Greenfield *et al*. (2016) also found that the levels of colonization and persistence on the rhizosphere were lower for *B. bassiana* than *M. anisopliae*.

In the presence of *C. giveni*, only for *M. anisopliae* F672 was rhizosphere colonization not affected, while for *M. robertsii* F447 and *M. anisopliae* A1080 it decreased by approximately 95%. In plant treatments with *M. anisopliae* F672 in the presence of this pest no variations in root dry weight were recorded, what might explain the constant rhizosphere colonization by the fungus. On the other hand, when plants were treated with *M. robertsii* there was a 28% decrease in root dry weight associated with *C. giveni*, which might explain the reduction in rhizosphere colonization by this fungus in the presence of the grass grubs. Conversely, in plants treated with *M. anisopliae* A1080 in the presence of *C. giveni* there were no variations in root biomass. Cosme *et al*. (2016) also found reductions in the percentage of root colonization by *Piriformospora inidica* in the presence of root feeding larvae. Zitapolca-Hernández *et al*. (2017) reported that maize root colonization by a native arbuscular mycorrhiza was reduced by the scarab *Phyllophaga vetula* herbivory. The decrease in rhizosphere colonization found might be linked to changes in root exudate composition triggered by the feeding insect. Previous research has demonstrated that plants can modify the microbiota present on the rhizosphere by altering the root exudates composition as a strategy utilized by the plant as

consequence of insect attack (Partida-Martínez & Heil, 2011; Pangesti *et al*., 2013). For example, Robert *et al*. (2014) showed that maize plants infested with root herbivores allocated carbon to the stems as a prelude to root regrowth, while in another work with the western corn rootworm the effect of the pest feeding on maize roots changed the microbiota on the rhizosphere through alterations in the roots exudates (Dematheis *et al*., 2012).

In this particular case, the decrease observed in rhizosphere colonization observed for isolates *M. anisopliae* A1080 and *M. robertsii* F447 might support the bodyguard hypothesis (Elliot *et al*., 2000). If the plant response to root-feeding larvae is to change roots exudate composition which reduces the ability of the entomopathogenic fungi to survive in the rhizosphere, this could be sensed by the fungus as nutrient deprivation. Nutrient deprivation will force fungi to initiate the conidiation process releasing infective conidia in the proximity of roots where the larvae are feeding. Previous authors have shown that fungi can initiate the conidiation process in response to environmental changes or to nutrient availability (Adams *et al*., 1998; Steyaert *et al*., 2010; Su *et al*., 2012). In addition, species of *Metarhizium* and *Beauveria*, in unfavourable conditions, can initiate the formation of conidia directly from the tips or sides of hyphae, in a process known as microcycle conidiation where the development of conidiophores is arrested (Jung *et al*., 2014).

All *Metarhizium* isolates were pathogenic to *C. giveni* when applied as seed coatings, and the highest mortalities were seen with *M. anisopliae* F672. The grass grubs were in contact with maize roots colonized by entomopathogenic fungi only for one week, and this time was enough to cause infection in the larvae. Bruck (2005) also found that colonization of the rhizosphere of *Picea abies* by a rhizosphere competent isolate of *M. brunneum* provided nearly 80% control of black vine weevil larvae within two weeks of exposure to inoculated roots. Kabaluk & Ericsson (2007) found that the application of *M. brunneum* as maize seed coating was infective to wireworms in field conditions, and the fungus was retrieved from the insect cadavers.

The effect of *F. graminearum* on rhizosphere colonization was variable depending on the entomopathogenic isolates used as seed coating. *M. robertsii* F447, *M. anisopliae* A1080 and F672 resulted in reductions in their ability to survive in the rhizosphere, while *T. harzianum* F327, *M. guizhouense* F16 and *M. novozealandicum* F99 increased their rhizosphere colonization. In the presence of a plant pathogen, the decrease in entomopathogenic fungi at rhizosphere colonization might be a consequence of a change in root exudate composition. However, whereas for some isolates rhizosphere competence was reduced, for others the ability to colonize the root was promoted and so probably competing with *F. graminearum* on root establishment. Again, this situation seems to fit with the bodyguard hypothesis, where the maize plant in the presence of *F. graminearum* responded by

increasing the ability of certain beneficial fungal species, like *T. harzianum*, which could compete with the plant pathogen. Species of *Trichoderma* are well known for their antagonistic capacity against plant pathogens (Steyaert *et al*., 2010). Entomopathogenic fungal isolates may have different roles for the plant, with some being able to better compete with *F. graminearum* than others (e.g. *B. bassiana* Bb21, *M. anisopliae* A1080, *M. guizhouense* F16 and *M. novozealandicum* F99) while other fungal isolates would be more suitable as insect pathogens (e.g. *M. robertsii* F447 and *M. anisopliae* A1080). It would still be necessary to determine which changes occurred in root exudates composition, if this is the reason, or if the changes in rhizosphere colonization was the result of plant hormonal regulation.

When the fungal isolates were applied in the absence of the pest or disease, the average recovery was 1.4×10^3 CFU/g of rhizosphere soil, within a range from 7.5×10^1 to 1.0×10^4 CFU/g. A previous report showed that *Metarhizium* species are among the most abundant fungi isolated form soils with levels reaching 10^6 conidia per gram in grassland soils (Gao *et al*., 2011). Hu & St Leger (2002) determined that the carrying capacity of *M. anisopliae* in the cabbage rhizosphere was around 10^5 propagules/g soil. A direct comparison of *Metarhizium* recoveries between this and other studies is difficult, owing (I) to the use of different field sampling and *in vitro* recovery methods for soil populations of entomopathogens, (II) the intrinsic characteristics of different isolates, and (III) plant different species which determine different rhizosphere colonization rates.

F. graminearum symptoms were higher on maize plants from the CS treatment, than on plants with the fungal treatments. Root symptoms were lower in treatments where there was an increase in rhizosphere colonization like with *M. guizhouense* F16 and *M. novozealandicum* F99, while *F. graminearum* symptom incidence was similar to CS in *M. anisopliae* F672 and *M. robertsii* F447 where a decrease in rhizosphere colonization was found. The presence of *C. giveni* reduced *Fusarium* symptoms in the *M. robertsii* F447; *M. guizhouense* Bk41 and F16 and *M. anisopliae* A1080 treatments.

F. graminearum decreased the ability of the entomopathogenic fungi to cause infection of *C. giveni*. Only in *M. anisopliae* F672 no differences were found whether *F. graminearum* was present or not, while *M. robertsii* F447 increased mortality on *C. giveni* in the presence of the plant pathogen.

Maize´s rhizosphere dominant species were *M. robertsii* F447 followed by *M. anisopliae* A1080 and F672. In a study by Kepler *et al*. (2015) in different crops and agricultural management practices they found that four *Metarhizium* species were present at the site, with *M. robertsii* being the dominant species, *M. brunneum* the second most abundant and both *M. pingshaense* and *M. lepidiotae* more rare species within the community. *M. robertsii* has been demonstrated to have a more generalist ability to colonize the plant rhizosphere when compared to *M. brunneum* and *M. guizhouense*

(Wyrebek & Bidochka, 2013). This ability may rely on its capacity to grow on a wide variety of carbon sources, including many sugars, amino acids, and organic acids that are present in root exudates (Fang & St Leger, 2010). Probably these characteristics are also shared with *M. anisopliae*, since isolates belonging to this genus have been found to be the second most common rhizosphere colonizers after *M. robertsii* F447 in the present study. Greenfield *et al*. (2016) also found that the level of colonization by *M. anisopliae* was explained as consequence of its competence in the rhizosphere.

Only some fungal treatments were able to become endophytic in maize plant tissues after they had been coated on seeds. The same effect of the presence of *C. giveni* or *F. graminearum* on rhizosphere colonization was observed for endophytism. The highest values for endophytism were found in *T. harzianum* F327, *M. robertsii* F447 and *M. anisopliae* A1080. However, these isolates were not found in the aerial parts of the maize plants, which may indicate that their association is exclusively with the roots or that the plants were still too young to have an extensive internal colonization from roots to stems and leaves.

There are several possible explanations for the lack of systemic colonization by the isolates used in this study. One is that endophytic colonization by the applied fungus is more likely in the plant part that was in direct contact with the inoculum and less likely, or not at all, in plant parts distant to the application site (Tefera and Vidal, 2009; Greenfield *et al*., 2016). Additionally, competition with other endophytes already present in the plant tissues is expected to be significant. Greenfield *et al*. (2016) found that approximately 40 other morphospecies were recovered from Cassava surface-sterilized root samples and the colonization rates of *B. bassiana* and *M. anisopliae* were reduced significantly in the presence of other endophytes. If host plant defence is induced by the presence of the entomopathogenic fungal endophyte, it may not be necessary for the fungus to be systemic (Jaber & Vidal, 2010; Greenfield *et al*., 2016).

A prior activation of plant defence that leads to resistance against pathogens is termed induced resistance (IR). IR has been studied extensively in the case of salicylic acid (SA)-mediated systemic acquired resistance (SAR) in dicotyledonous plants where it was determined that micro-lesions induced by necrotizing pathogens trigger a local accumulation of salicylic acid, with mitogen-activated protein kinases, H_2O_2 and other signals being involved. Another major type of IR is induced systemic resistance (ISR) which is triggered by non-pathogenic rhizobacteria. ISR depends on both NPR1 and the jasmonic acid/ethylene pathway, but not on SA (Waller *et al*., 2007).

As noted before, there wasn't a consistent response in plant phytohormone contents, salicylic acid (SA) and jasmonic acid (JA), in maize plants from seeds coated with the two isolates of *M. anisopliae*.

This fact highlights the importance of the isolate-plant effect compared with traits based on species or genera. Previous work has shown that the induction of ISR relies on specific plant-microbe interactions, and different strains of the same species can regulate the ISR via different signalling pathways (Vallad & Goodman, 2004; Pangesti *et al*., 2013).

Maize plants, treated with *M. anisopliae* A1080 and F672, had different levels of SA in absence of *C. giveni*, although, both levels were not significantly different from control plants. The higher content of SA in plants with the *M. anisopliae* A1080 treatment than in plants with the *M. anisopliae* F672 treatment may indicate that this particular isolate is an elicitor of SAR or also that this isolate is recognised by the plant as a potential pathogen. This was clearly observed in maize roots, where for the *M. anisopliae* A1080 treatment the levels of this hormone were the highest.

SA is a key hormone regulating plant defence against biotrophic pathogens and against insect herbivores with a piercing-sucking feeding mode (Pangaesti *et al*., 2013; Fernandez-Conradi *et al*., 2016). Also, the SA-dependent signalling is crucial in the interaction of plant roots with non-pathogenic microbes. It has been suggested that in the initial stage of symbiosis, non-pathogenic microbes are sensitive to SA-regulated defence responses (Pangesti *et al*., 2013). Initially the plant recognizes non-pathogenic microbes as alien organisms and, therefore, activates defence mechanisms via SA-dependent signalling pathways (Pangesti *et al*., 2016). The lower levels of SA in plants from the *M. anisopliae* F672 treatment could indicate that this isolate is not recognized by maize plants as an invader microorganism. The activation of the plant defence system has a significant physiological cost for the plant in terms of vegetative and reproductive growth (Vallad & Goodman, 2004).

The selection of entomopathogenic fungal strains that are not recognized by the plant as a potential invader could be another desirable characteristic when plant growth and yield is one of the aims. Whether or not the increase in the levels of SA induced by the *M. anisopliae* A1080 treatment is translated into an increase in the SAR in maize needs to be evaluated and so the cost associated with this phytohormone boost could be compensated with the additional control of above ground pests, which in the end may result in plant growth promotion.

The change in the SA profile was also distinctive among the control plants and plants with the *M. anisopliae* F672 treatment. The phytohormone in roots had a trend to increase in the fungal treated plants when *C. giveni* was added, while in CS it remained constant. This might indicate that plants with *M. anisopliae* F672 were in a primed state where the synthesis of the phytohormones was higher than in controls plants. From these results it is clear that the presence of entomopathogenic fungi was altering the phytohormone content in roots or shoots of the maize plants, while in control plants there

were no changes in the presence or absence of *C. giveni*. In contrast to leaf herbivore attack, root herbivore attack has not been seen to induce SA signalling (Johnson *et al*., 2016).

The JA profile was also distinctive between the different treatments. Maize plants with the *M. anisopliae* A1080 treatment, in the absence of *C. giveni*, had higher contents of JA in roots and shoots than control plants, while the only different was in leaves of the treatment *M. anisopliae* F672. These results suggest that the isolate *M. anisopliae* A1080 seemed not only to induce SAR in maize but also ISR. JA-signalling has also been described as the main pathway in ISR against aboveground herbivores and is stimulated by root-associated microbes (Pangesti *et al*., 2013). Several reports support JA/Ethylene signalling as the mechanism for induced systemic resistance where species of *Trichoderma* significantly increased the levels of JA but not SA (Ownley *et al*., 2010; Partida-Martínez & Heil, 2011; Vandenkoornhuyse *et al*., 2015; Cosme *et al*., 2016; Johnson *et al*., 2016). Induction of JA-signalling mainly occurs after attack by necrotrophic pathogens, tissue-chewing insects such as caterpillars and cell-content feeding insects such as thrips (Pangesti *et al*., 2016; Fernandez-Conradi *et al*., 2016). Another effect of JA is that it can also reduce root and shoot growth, both locally and systemically within the root system (Cosme *et al*., 2016).

Plant signalling pathways are also modulated by non-pathogenic microbes like rhizosphere entomopathogenic fungi that colonize roots without producing disease symptoms in the plant. During biotrophic root colonization it has been observed that JA signalling is required (Cosme *et al*., 2016). However, the activation of the JA-signalling pathway also affects the plant´s interaction with root-colonizing microbes (Pangesti *et al*., 2013; Cosme *et al*., 2016). In this study, while *M. anisopliae* F672 kept the level of JA close to those observed in control plants, plants with the *M. anisopliae* A1080 treatment had the highest levels of the hormone in roots and leaves. One ecological advantage of this for isolates of *Metarhizium* above other microorganisms is that the boost in the phytohormone content in the plant could impair root colonization by other microorganisms, like *F. graminearum*. Evidently, this entomopathogenic fungus is able to cope with high levels of the phytohormones, since has been found not only as a rhizosphere colonizer but also as an endophyte. This hypothesis would also explain why the symptoms of *F. graminearum* observed in plants treated with *M. anisopliae* A1080 were significantly lower than those observed in control plants, in the presence or absence of *C. giveni*.

Another clue for the distinctive hormone profile observed between both isolates might reside in the origin of these fungi. While *M. anisopliae* A1080 was obtained from an insect larva, *M. anisopliae* F672 was isolated as a Pine root endophyte. Further research would need to be done to determine if natural endophytic isolates are those with a less aggressive colonization behaviour than others, meaning that the plant might have not the ability to recognize them and so there is no induced resistance.

The presence of *C. giveni* in control plants resulted in a significant increase in the content of the JA hormone in leaves, when compared to control plants in the absence of the pest. This agrees with several lines of evidence which indicate that only root herbivory induces JA signalling in roots although roots commonly display a much weaker herbivore-induced JA burst than leaves (Pangesti *et al*., 2013; Cosme *et al*., 2016; Johnson *et al*., 2016). This increase in hormone level in leaves was observed also as a trend in maize plants from the *M. anisopliae* F672 treatment, while hormone levels in plants with the *M. anisopliae* A1080 treatment seemed to stay relatively constant. In the present study, the increase in the JA content observed in plants without the fungal treatment could be the normal response of maize plants when roots are under pressure by the grazing activity of the grass grub. On the other hand, the apparent lack of response in maize plants from coated seeds with entomopathogenic fungi might be the result of two factors, the already higher levels of this phytohormone that made unnecessary the *novo* synthesis of more molecules, or a protection effect, due to the presence of the fungi on the roots that could repel the feeding of *C. giveni*.

The distinctive phytohormone profile observed in the *M. anisopliae* A1080 treatment is further evidence to support the hypothesis about the costs and benefits for the plant of harbouring an associated microorganism, and that the positive outcome of the association would be only determined in the presence of biotic or abiotic factors (Partida-Martínez & Heil, 2011).

Chapter 4

Production of fungal microsclerotia and use in delivery of fungal biocontrol

4.1 Introduction

The commercial use of fungal entomopathogens to control insects is generally practiced using the inundation biocontrol approach where the environment harbouring the insect pest is inundated with high concentrations of infective fungal propagules (Jackson, Dunlap & Jaronski, 2009). Products developed for use in the inundative approach are often termed ‘‘mycoinsecticides’’ or ‘biopesticides’’ in reference to their similar usage pattern compared to chemical insecticides.

Fungal biopesticides for the control of soil dwelling insects are produced using aqueous suspensions of conidia, however, proper application with standard farming equipment, target access and homogenous distribution through the soil can be difficult (Jaronski & Jackson, 2008). A practical alternative for the application of fungal biopesticides is granular formulation. These granules are produced using an inert carrier with conidia incorporated or bound to the surface, or propagules with conidiogenic capability in or on a nutritive carrier (Burges, 1998; Jaronski & Jackson, 2008). Granular formulations are more practical for the standard at-planting application equipment, and once in the soil the granules place the fungus in the soil in the zone of the damaging pest and can be covered with large numbers of conidia.

The development of mycoinsecticides as conidiogenic granules is preferred since fresh conidia are produced *in situ* where the target pest is, and all the steps of harvesting, drying and formulation that can damaged the conidia are avoided. Soil application of biopesticides has some advantages, such as the lack of harmful effects from UV radiation and temperatures are moderated. Another advantage is that soil moistures are generally above the permanent wilting point of plants within the optimal range for survival and growth for fungi (Jackson & Jaronski, 2009).

Recently, it was found that entomopathogenic fungi belonging to *Metarhizium anisopliae* or *M. brunneum* were able to produce high concentrations of microsclerotia (MS) when grown in liquid media (Jackson & Jaronski, 2008; Vega *et al*., 2009; Jackson & Jaronski, 2012). MS were first found to be produced by many phytopathogenic fungi that need to survive in soil and from decaying plant materials and serve as persistent resting stages for the fungus when environmental conditions are unfavourable (Song *et al*., 2016). These resistant structures are desiccation tolerant, with excellent

storage stability, and have the potential to produce many infective conidia suitable to be used in mycoinsecticides to manage insect pest or as antagonist of plant pathogenic fungi (Vega *et al*., 2009; Song *et al*., 2016).

Microsclerotia are pseudoparenchymatal aggregations of hyphae that become melanised during development, reaching diameters between 50 to 300 µm. The developing of MS is initiated with the aggregation of compact hyphae. In the early stages of the formation of these structures, characteristically there is a rapid influx of nutrients into the sclerotia, and nutrient availability is the most important factor governing the growth and eventual size of these propagules. Then, during the maturation stage, nutrient influx stops and a complex of endogenous events such as dehydration, cell wall thickening, deposition of storage compounds and melanisation begins to occur to prepare the sclerotia for their survival role. These endogenous reserves provide to microsclerotia with the nutrients necessary for hyphal growth and the production of conidia simply when moisture is present (Jackson & Jaronski, 2009; Goble *et al*., 2016, Song *et al*., 2016).

In a study by Jackson & Jaronski (2009) the optimum conditions for the production of blastospores and MS from *M. anisopliae* was determined, as well as the performance of air-dried MS formulated in diatomaceous earth (DE). In general, MS–DE were obtained from 8 days old cultures of different strains of *M. anisopliae*. The MS-DE survived the drying process with no significant loss in viability except those MS obtained from cultures with a carbon-poor medium and low nitrogen content. In general, dried MS–DE preparations from carbon-rich media produced higher numbers of conidia than MS–DE preparations derived from media with lower carbon concentrations (Jackson & Jaronski, 2009). Viability was determined microscopically as hyphal germination after 24 h incubation. The conidia production by air-dried MS–DE preparations for all strains of *M. anisopliae*, regardless of media, was greater than 1 x 10^8 conidia/g dried formulate. The success of these formulated MS – DE was shown after incorporation to soil and obtaining mortality of 90 to 100% of *Tetanops myopaeformis* larvae in 14 days.

Another advantage of MS for use in biopesticides is that they can be mass produced using stirred-tank bioreactors (100 L) without reducing product quality or stability. Formulated MS granules in diatomaceous earth were stable for at least 12 months without significant viability losses (Jackson & Jaronski, 2012).

Once applied to the soil, fungal growth and conidiation will occur. Different studies have emphasized that the level of conidia production from MS depends on water availability, a critical abiotic parameter for hyphal growth and conidia production by these fungal resting structures. For instance, Goble *et al*.

(2016) formulated MS of *Metarhizium brunneum* (strain F52) as granules and conidia production of the granules was evaluated at different water activities (a_w) and temperatures. These authors found that, at 26°C, conidia production at high a_w (0.990) was 4.4 x 10^9 conidia/g of granules, while at lowers a_w (0.963 and 0.924), it was 3.6 x 10^7 and 9.0 x 10^6 conidia/g, respectively. The same trend was observed at a lower temperature, 15°C, with 1.9 x 10^9 conidia/g at a_w = 0.995 and less conidia production, 1.3 x 10^7 conidia/g, at a_w = 0.987. In a different study, Behle, Jackson & Flor-Weiler (2013) found that clay-based *M. brunneum* F52 MS containing granules produced a concentrated number of conidia (1.27 × 10^9 conidia/g) in 7 days when exposed to full moisture in Petri dishes lined with filter paper, and granules were able to produce even more conidia, e.g. 1.24 × 10^{10} conidia/g, in moist potting mix (Goble *et al*., 2016).

In summary, MS produced significantly more conidia at higher a_w values than at lower values, highlighting an important correlation between decreased moisture and decreased conidial production by the resistant fungal structures (Goble *et al*., 2016). Evidently, MS might have enough nutrients available for all the initial metabolic process involved in the reactivation of the MS and conidia production. However, availability of water is crucial since it is necessary as the main component in many biochemical reactions and also for the rehydration of dry MS. In conditions of unlimited moisture, MS will produce a full complement of conidia within 7 days. In these conditions, MS can continuously produce conidia until their endogenous reserves are depleted or until saprophytic survival is no longer possible (Jaronski & Jackson, 2008; Behle *et al*., 2011; Goble *et al*., 2016).

MS obtained from entomopathogenic fungi have been shown to be a viable alternative for formulation to conidia or other fungal propagules previously used in biocontrol programmes. The perfect environment to reactivate MS is soil, since the humidity conditions are favourable for the rehydration and germination process involved. Additionally, the capability of entomopathogenic fungi, like *M. anisopliae*, to form microsclerotia may be important for rhizosphere competence following a similar pattern to phytopathogenic fungi (Vega *et al*., 2009). An alternative to MS formulated as granules and incorporated into soils at planting is to formulate the MS directly as a seed coating. This strategy will provide to the seed, after MS germination and conidiogenesis, with both fresh conidia that would interact with the developing roots promoting rhizosphere competence, and biocontrol of soil dwelling insects in proximity to the seeds. In previous works with seed treatments with *M. pingshaense* had shown that 50% of larvae of *Anomala cincta*, a grass grub, were infected. Additionally, maize roots were endophytically colonized by the fungus (Peña-Peña *et al*., 2015). Keyser *et al.* (2014) also showed that after applying conidia of *M. robertsii* or *M. brunneum* to wheat seeds, the growing hyphae were associated with the roots. Larvae of *Tenebrio molitor* exposed to the roots from treated seeds were infected with *Metarhizium*. Thus, seed coating with entomopathogenic fungi has potential as delivery

strategy to cause infection in root-feeding insects, but also to promote the fungal colonization of roots and persist in the soil.

4.1.1 Objective of this chapter

The aim of this study is to evaluate MS production in different isolates of entomopathogenic fungi and determine the effect of MS coated onto maize seeds on plants grown in the presence of *F. graminearum*. The main goal of this objective is the mass production of MS of selected entomopathogenic fungal isolates with the aim to improve the survival of the biocontrol agents when coated to seeds during storage and delivery. The activities related with this objective were: firstly, to determine the ability of the selected entomopathogenic fungal isolates to produce MS. Second, MS viability was evaluated and MS coated to maize seeds. Third, plant growth performance after MS seed coating was evaluated in the presence of *Fusarium graminearum*. Lastly, the ability of the entomopathogenic fungal isolates, coated to seeds as MS, to associate with roots was determined through fluorescent and laser confocal microscopy.

4.2 Material and methods

4.2.1 Fungal isolates

The isolates selected for this study were the entomopathogenic fungi *M. anisopliae* A1080 and F672, *M. guizhouense* Bk41 and F16, *M. novozealandicum* F99 and *M. robertsii* F477, and the plant promotor *T. harzianum* F327. The isolates belong to the fungal collections of the BPRC, AgR and USDA as detailed in Table 2.1. For liquid culture fermentation studies, a monosporic culture of each isolate was grown on Petri plates containing potato dextrose agar (PDA, Difco) at 25 ± 2°C in light dark conditions (12:12). After 3 weeks, conidial suspensions were prepared from sporulated fungal colonies and stored in 10% glycerol at -80°C (stock cultures). Conidial inocula for liquid culture experiments were produced by inoculating PDA Petri plates with a conidial suspension from a frozen stock culture and growing the inoculated Petri plates at 25 ± 2°C in light dark conditions (12:12) for 2 weeks. Conidial suspensions were obtained from the fungi grown on PDA plates by rinsing colonies with 3 – 5 mL of a solution of 0.01% Triton X-100 and transferring the resulting conidial suspension to a 15 mL Falcon tube. For fermentation studies, all liquid cultures were inoculated with a conidial suspension of 5 x 10^6 conidia/mL.

4.2.2 Microsclerotia production from entomopathogenic fungal isolates in liquid fermentation conditions

4.2.2..1 Composition of liquid medium and fermentation conditions

Media composition and fermentation conditions were as described by Jackson & Jaronski (2009, 2012) with modifications only in carbon and nitrogen content. The liquid media used to produce hyphal inoculum and MS from the fungal isolates were composed of a basal salts solution with trace metals and vitamins. The defined basal salts solution used in all liquid cultures contained, per litre of deionised water: KH_2PO_4, 4.0 g; $CaCl_2{\cdot}2H_2O$, 0.8 g; $MgSO_4{\cdot}7H_2O$, 0.6 g; $FeSO_4{\cdot}7H_2O$, 0.1 g; $CoCl_2{\cdot}6H_2O$, 37 mg; $MnSO_4{\cdot}H_2O$, 16 mg; $ZnSO_4{\cdot}7H_2O$, 14 mg; thiamin, riboflavin, pantothenate, niacin, pyridoxamine, thiotic acid, 500 mg each; and folic acid, biotin, vitamin B12, 50 mg each. The pre-culture medium (C:N ratio of 33:1; [C] = 40 g/L) used for producing the hyphal inoculum of fungal isolates contained the basal medium supplemented with 80 g/L glucose and 15 g/L acid hydrolysed casein (Casamino acids†, Difco). Production medium for microsclerotia contained the same composition but with 25 g/L of hydrolysed casein (C:N ratio 23:1, [C] = 45 g/L). Carbon concentration and C:N ratio calculations were based on 40% carbon in glucose and 53% carbon, 8% nitrogen in acid hydrolyzed casein (Jackson & Jaronski, 2009). Glucose solutions (20% w/v) were autoclaved separately and added prior to inoculation to the basal salts solution with trace metals and vitamins. Pre-cultures were obtained by inoculating 90 mL pre-culture medium contained in baffled Erlenmeyer flasks (250 mL) with 10 mL conidial suspension of 5 x10^6 conidia/mL. Production cultures were obtained by inoculating 100 mL of

production medium contained in baffled Erlenmeyer flasks (250 mL) with 10 mL of pre-culture broth of 4 day-old. Production cultures were grown for 8 days at 28°C in an orbital shaker incubator (Cocono TU 4540) at 300 RPM. Samples for the determination of biomass, blastospore and MS were taken at days 3 and 4 after inoculation for pre-cultures and at days 3, 4, 6, 7 and 8 for production cultures. All pre-cultures and production cultures were inoculated by duplicated. Experiments were carried out at least three times (Figure 4.1).

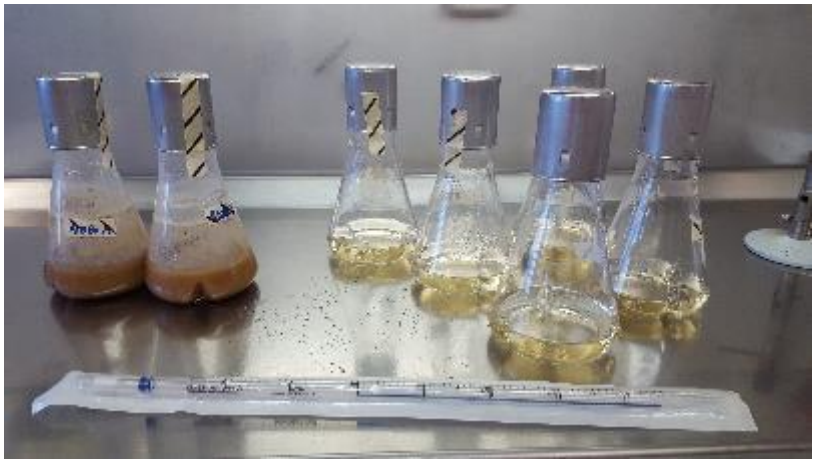

Figure 4.1 Precultures after liquid fermentation of entomopathogenic fungi in basal medium casamino acids (C:N ratio 33:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 4 days. Inoculum from fungi grown in precultures (in the left) was used to inoculate production cultures (in the right).

4.2.2..2 Quantification methods and sample processing

At each sampling day 5 mL of culture broth were taken from each isolate for quantification of biomass, blastospore and MS determination. Biomass was determined as dry weight after incubating 1 mL of culture broth in a 1.5 mL Eppendorf tube for 96 hr at 65°C. Blastospore concentration was determined by transferring 1 mL of culture broth to a tube containing 9 mL of distilled water and quantifying microscopically using a haemocytometer (Neubauer Improved). MS production was determined with 70 µL of sample placed onto a glass slide and covered with a large (50 x 24 mm) coverslip. All MS on the slide were counted. Only discrete hyphal aggregates larger than 50 µm in diameter and melanised were counted as MS. For each fermentation run, flasks were arranged in a randomized block design with two blocks per trial. In each block, there were the eight isolates and samples were taken at the corresponding day to determine biomass, blastospore and microsclerotia concentration. The fermentation runs were carried out three times. Determination of biomass was done taking two samples per flask, while for blastospore and microsclerotia, one sample was taken per flask. In order to combine data over the three trials for each assessment day, corresponding means were logarithm transformed and input into an analysis of variance which treated fermentation runs as “blocks”, with

eight treatments (isolates). Comparison of isolates employed an unprotected least significant difference (LSD) procedure (Saville, 2015). All quantifications were done in duplicate. Culture broth was diluted as appropriate for quantifications and broth suspensions were constantly vortexed to ensure homogeneity. Microscopic analysis was done using a Leica DM 2500 microscope and images were taken with the Celsens Standard software (Olympus).

4.2.3 Microsclerotia production for seed coating

MS were produced for maize seed coating as indicated above (section 4.2.2) using isolates *M. anisopliae* A1080 and F672, and *M. robertsii* F447. Pre-culture biomass and blastospore production were evaluated on day 4 after inoculation, and production cultures were evaluated for biomass and microsclerotia production on day 6 after inoculation. For each pre-culture fermentation run, flasks were arranged in a randomized block design with two blocks per trial. In each block, there were the three isolates and samples were taken at day 4 to determine biomass and blastospore concentration. The fermentation runs were carried out two times. Determination of biomass was done taking two samples per replicate flask, while for blastospores one sample was taken per replicate flask. In order to combine data over the two trials, corresponding means were logarithm transformed and input into an analysis of variance which treated fermentation runs as "blocks" with three (isolates) treatments. Comparison of isolates at day 4 employed an unprotected least significant difference (LSD) procedure (Saville, 2015). MS production cultures were done using six replicates per isolate. Harvest of microsclerotia for seed coating was made on day 6 after inoculation. For each production fermentation run, flasks were arranged in a randomized block design with three blocks per trial. In each block, there were the three isolates and samples were taken at day six to determine biomass and microsclerotia concentration. The fermentation runs were carried out three times. Determination of biomass was done taking two samples per replicate flask, while for microsclerotia one sample was taken per replicate flask. In order to combine data over the three trials, corresponding means were logarithm transformed and input into an analysis of variance which treated fermentation runs as "blocks", with three treatments (isolates). Comparison of isolates employed an unprotected least significant difference (LSD) procedure (Saville, 2015).

4.2.4 Microsclerotia harvest and drying process

After growing the cultures for six days, fungal biomass volume including MS was determined and 5% (v/w) of diatomaceous earth (DE, Hyflo Sigma-Aldrich) was added. The combined MS-DE were mixed and vacuum filtered with a Buchner funnel using Whatman N°54 filter paper (Figure 4.2).

Figure 4.2 **Harvesting and formulation of MS.** Fungal production from six day old cultures were combined with 5% (v/w) of diatomaceous earth (A). The combined MS-DE were vacuum filtered with a Buchner funnel using Whatman N°54 filter paper (B - C). The resulting filter cake (D) was broken up with a coffee blender to obtain small crumbs (E). MS-DE granules were air dried overnight in a laminar-flow cabinet (F).

The resulting filter cake was broken up with a coffee blender applying short pulses until small crumbs were obtained. The resulting crumbs were layered on glass Petri plates of 15 cm diameter and air dried overnight in the air flow within a containment laminar-flow (Figure 4.2). The moisture content of the MS-DE granules was determined with an activity meter (Aqualab Lite V4). The granules were dried until the water content was between 3 – 5 % and then sealed in plastic bags and stored at 4°C.

4.2.5 MS-DE granules viability and conidial production determinations

Microsclerotial viability (hyphal germination) and conidial production were determined for the air-dried MS–DE preparations by sprinkling 25 mg of the MS–DE formulation onto the surface of a water agar plate. Two water agar plates were used for each treatment. After 24 hrs incubation at 28°C in light:dark conditions (12:12 h), 100 MS–DE granules on each plate were examined with a stereo microscope (Olympus SZX 12) for hyphal germination as a measure of viability (Figure 4.3). Conidial production was determined after incubating MS-DE granules for 8 days at 28°C on the water agar

plates in the same light dark conditions. Water agar plates were then flooded with 7 mL of a sterile solution of 0.01% Triton X-100. Plates were then rotary agitated at 60 RPM in a rotary shaker platform for 30 min. at room temperature. After agitation, granules were dislodged from agar with the help of a hockey stick and the resulting suspension containing granules and conidia was transferred to a 15 mL Falcon tube and the recovered volume recorded. The concentration of conidia was determined microscopically using a haemocytometer and the total production of conidia per gram was calculated.

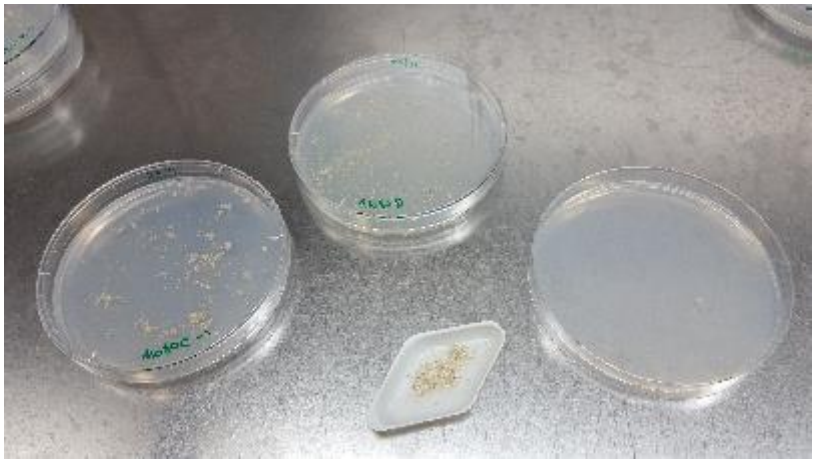

Figure 4.3 **Microsclerotial viability.** Air-dried MS–DE preparations were sprinkled onto the surface of a water agar plate (A). After 24 hr incubation at 28°C on light:dark conditions (12:12 h), 100 MS–DE granules on each plate were examined with a stereo microscope for hyphal germination as a measure of viability (B).

MS-DE were characterized for granules viability and conidial production. Granule evaluation were arranged in a randomized block design with two blocks per trial. In each block, there were the three isolates and samples were processed in duplicates. The microsclerotia characterization was carried out three times. In order to combine data over the three trials, corresponding means were input into an analysis of variance with fermentation runs treated as "blocks" and with three treatments (isolates). Comparison of microsclerotia germination and conidial production between isolates employed an unprotected least significant difference (LSD) procedure (Saville, 2015).

4.2.6 Maize seed coating with microsclerotia

Coating was prepared as detailed in section 3.4.3. The main difference was that the conidial suspension was substituted by an amount in grams of MS-DE which would provide, after MS germination and sporulation, a final conidia concentration per gram of seeds of 1 x 10^8 conidia/mL. The corresponding quantity of MS-DE was added to the polymer, mixed and then used to coat maize seeds as previously (Figure 4.4). Proportions of polymer with MS-DE, bentonite and talc were the same

as indicated before. Control seeds were coated with the polymer and the other ingredients but not with any fungal treatment.

Figure 4.4 **Seed coating with microsclerotia.** Air-dried MS–DE (A) were mixed with the polymer (B - C) until complete dissolution. The resulting biopolymer with MS was added to the seeds and mixed with a spatula (D – E). After uniform coating, bentonite and talc were added (F).

Seeds were coated with MS-DE and CFU were determined. Evaluation were arranged in a randomized block design with one block per trial. In each block, there were two plates of the corresponding dilution of MS coated-seed from each isolate and a coated control (CS) without any fungi. The determination of CFU from MS coated-seeds was carried out three times. In order to combine data over the three trials, corresponding means were input into an analysis of variance which treated trials as “blocks”, with isolates being treatments. Comparison of CFU from MS coated-seeds between isolates employed an unprotected least significant difference (LSD) procedure (Saville, 2015).

4.2.7 Evaluation of maize plant performance in the presence of *Fusarium graminearum* after seeds were coated with microsclerotia of *Metarhizium* spp.

Coated maize seeds with microsclerotia of *M. anisopliae* A1080 & F672, and *M. robertsii* F447 were sown in pots containing 920 g of potting-mix with 0.5% w/w of *F. graminearum*. Controls seeds were grown in presence and absence of *F. graminearum*. *F. graminearum* was obtained as described previously in section 3.4.7.1. Maize plant performance in the presence of *F. graminearum* after coating with MS of *Metarhizium* spp., was evaluated as dry weight as described in section 3.4.8. Coated maize seeds with microsclerotia of *M. anisopliae* A1080 & F672, and *M. robertsii* F447 were sown in pots containing 920 g of potting-mix with 0.5% w/w of *F. graminearum*. Control seeds were grown in presence and absence of *F. graminearum*. *F. graminearum* was obtained as described previously in section 3.4.7.1. Controls included a complete coated seed treatment without fungi grown in absence (CS) and in presence of *F. graminearum* (CS+fg). Each trial consisted of two randomised blocks, with five pots for each treatment in each block. Treatments were one plant per pot from the corresponding seed-coated MS-DE treatment (*M. anisopliae* F672 and *M. robertsii* F447), plus a control plant from coated seeds but without fungi. All these treatments were grown in presence of *F. graminerarum* in the soil (F672+fg; F447+fg and CS+fg). The block was completed with five more pots of control plants grown in absence of *F. graminearum* (CS). Maize plant performance in the presence of *F. graminearum* after coating with MS of *Metarhizium* spp., was evaluated as dry weight as described in section 3.4.8. A logarithm transformation was used for the corresponding length and dry weight of roots and shoots. To combine data over the four trials, treatment means were input into an analysis of variance which treated trials as "blocks" and had isolates and fg (+,-) as treatment factors, followed by an unprotected least significant difference (LSD) procedure (Saville, 2015).

4.2.8 Determination of fungal endophytism by fluorescent and confocal fluorescent microscopy

Samples of plants originated from microsclerotia-coated seeds were analysed for the presence of fungal structures on the surface of the vegetal tissues or internally as endophytes using fluorescent microscopy (Olympus BX51) or confocal microscopy (LSM 510 META - Zeiss, Germany). Wheat germ agglutinin conjugated to Alexa Fluor (WGA-AF488; Molecular Probes, Eugene, OR, USA) was used to stain chitin present in fungal hyphae (Ramonell *et al*. 2005). The hyphal adhesion zone was visualized with the carbohydrate binding lectin concanavalin-A conjugated with Alexa Fluor 633 (ConA-AF633, Molecular Probes, Karlsruhe, Germany). ConA is generally used to visualize glycoproteins since binds to sugar residues like α-mannopyranosyl and α-glucopyranosyl found in glycoproteins and glycolipids (Zuccaro *et al*., 2011). Plant cells were visualized by propidium iodide and Congo red as counter stains (Deshmukh *et al*., 2006).

4.2.8..1 Maize plant inoculation

Maize seeds were coated with microsclerotia as described on section (4.3.5) while controls included coated seeds without fungi. After coating, maize seeds were sown in pots of 1 L of capacity containing 600 g of vermiculite (fine grade 2). Pots were watered with 400 mL of tap water, and transferred to a growth chamber at 25°C with light:dark conditions (12:12). Plants were grown for one month and were watered every 5 days with 400 mL of distilled water.

4.2.8..2 Maize sample preparation

Roots, stems and leaf sheaths were first dehydrated by soaking samples individually in EtOH (96%) contained in Falcon tubes and incubated overnight at 4°C. Subsequently, vegetal tissues were cleared replacing the EtOH with a solution of KOH (10%) and samples were incubated at 96°C for 3 hours. After incubation, KOH was discarded and samples washed once in 1x phosphate-buffered saline (PBS; pH 7.4).

4.2.8..3 Staining of **Metarhizium anisopliae** *and vegetal tissues*

Colonized roots, shoots or leaves were stained by infiltration with WGA-AF488 to visualize fungal structures, CoA-AF633 to visualize the adhesion zone between the hyphae and the plant, and propidium iodide and Congo Red as counter stains for plant cells. Samples were incubated at room temperature for 30 min in staining solution containing 10 µg/mL WGA-AF488, 10 µg/mL ConA-AF633, 20 µg/mL propidium Iodide, 10 µg/mL Congo Red and 0.1% Triton X-100 in 1x PBS (pH 7.4). During incubation, segments were vacuum-infiltrated three times for 2 min at 25 mm Hg. Finally, plant samples were then washed in the PBS buffer for 3 h and then in fresh buffer overnight. Samples were stored in the dark at 4°C until analysis.

4.2.8..4 Fluorescent microscope and confocal laser scanning microscope image acquisition

For microscopic observation vegetal segments of roots, stems and leaves were mounted on glass slides. The visualization of the different fluorophores/chrome and dyes in hyphae and plant cells was achieved using an excitation of 495 nm for WGA-AF488 and detected at 519 nm, ConA-AF633 was excited at 633 nm and detected at 647 nm, while propidium iodide was excited at 530 nm and detected at 615 nm. Confocal fluorescence images were recorded on a multichannel Olympus fluorescent microscope (Olympus, Germany) using the program Cell (Olympus). For microscope observation using a confocal laser microscope the visualization of the different fluorophores and dyes in hyphae and plant cells was achieved using an excitation of 488 nm laser line for WGA-AF488 and detection at 500–540 nm, ConA-AF633 was excited at 633 nm laser line and detected at 650–690 nm, while propidium iodide and Congo Red were excited at 561 nm laser line and detected at 580–660 nm. Confocal fluorescence images were recorded on a multichannel confocal microscope (LSM 510 META - Zeiss, Germany) using the program ZEN 2009.

4.2.9 Statistical analysis

For each individual trial or bioassay, data were analysed by the analysis of variance that was appropriate for the experimental design. For treatment factors with several levels (e.g. isolates), the unrestricted LSD procedure was used to compare means (Saville, 2015). To combine data over several identical trials or bioassays, an analysis of variance was used as above, followed by an unrestricted LSD procedure, with data being treatment means from individual trials, and with "trial" being specified as a blocking factor. All statistical results are given in the Appendix B.

4.3 Results

4.3.1 Microsclerotia production from entomopathogenic fungal isolates in liquid substrate fermentation

4.3.1..1 Primary inoculum evaluation

During production of the primary inoculum, biomass, blastospores and microsclerotia (MS) were quantified at 3 and 4 days post inoculation. Substantial differences occurred among the isolates regarding their ability to produce biomass, blastospores and MS ($p<0.01$).

On average, higher biomass, 85.6 ± 9.7 mg/mL was determined at day 3 than at day 4, when 76.8 ± 7.4 mg/mL was produced ($p<0.01$). On both days, the highest biomass was found in *M. novozealandicum* F99, while the lowest value was determined in *T. harzianum* F327 also in both days (LSD = 0.031). In fact, *T. harzianum* had the lowest biomass production when compared to all isolates of *Metarhizium* spp. (Figure 4.5; LSD = 0.031).

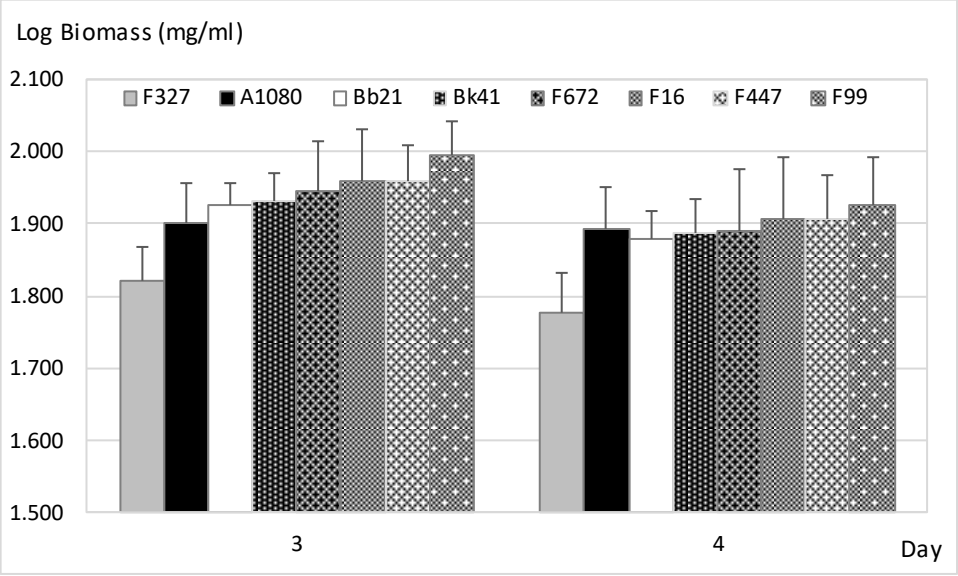

Figure 4.5 Biomass (mg/mL) production during liquid fermentation of entomopathogenic fungi in basal medium casamino acids (C:N ratio 33:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 4 days. Samples were taken at 3 and 4 days for the determination of dry weight. Error bars = standard deviation; $LSD_{5\%}$ = 0.031; $p<0.226$.

While most isolates did not show differences in blastospore production between evaluation days, *M. guizhouense* F16 showed higher blastospore concentration on day 3 than day 4 and for *B. bassiana* Bb21 and *M. robertsii* F447 which produced higher concentrations by day 4 ($LSD_{5\%}$=0.586; Figure 4.6). *T. harzianum* F327 was the only isolate which did not produced blastopores, which is likely because the growing conditions were not suitable for blastopore production for this isolate. Biomass for *T. harzianum* F327 was therefore mycelial.

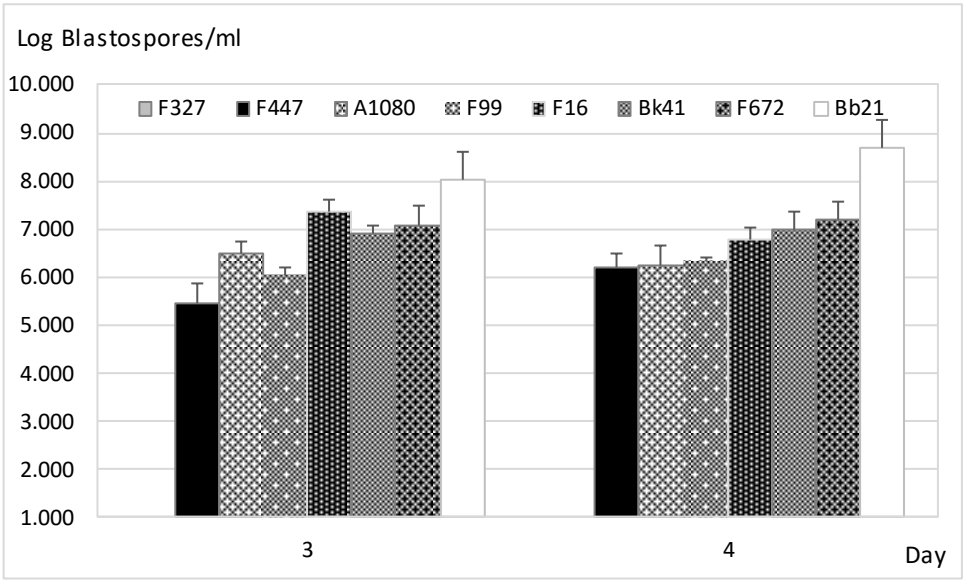

Figure 4.6 **Blastospores (number/mL) during liquid fermentation in basal medium casamino acids (C:N ratio 33:1**). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 4 days. Samples were taken at days 3 and 4 for the determination of blastospore production. Error bars = standard deviation, $p<0.05$, $LSD_{5\%}=0.586$.

The production of MS in the pre-culture medium was general low. Three days from inoculation only *M. guizhouense* Bk41 and F16, and *T. harzianum* F327 were able to produce MS (Figure 4.7).

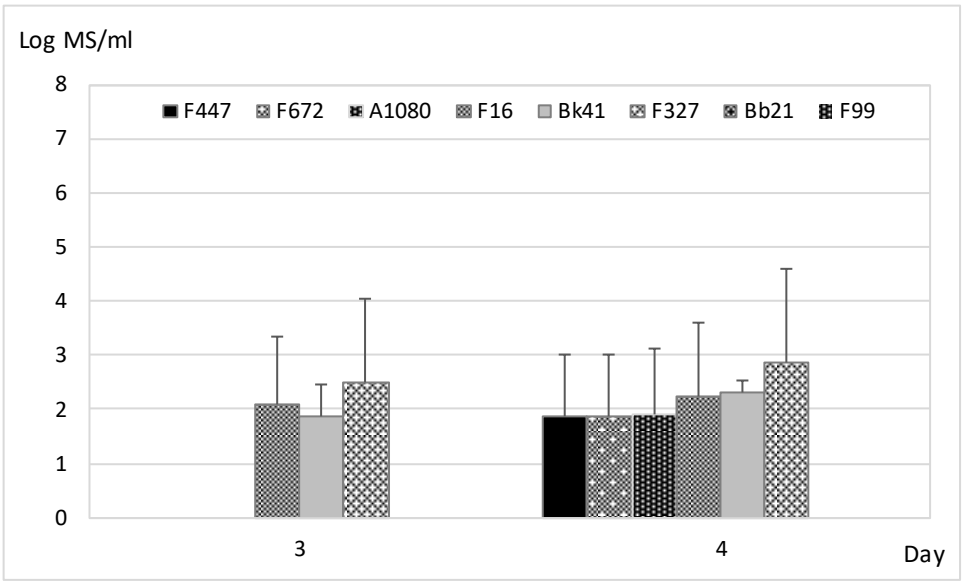

Figure 4.7 **Microsclerotia (number/mL) during liquid fermentation in basal medium casamino acids (C:N ratio 33:1).** Fungi were grown in a rotary shaker at 300 RPM and 28°C for 4 days. Samples were taken at days 3 and 4 for the determination of microsclerotia production. Error bars = standard deviation, $p<0.790$, $LSD_{5\%}=0.864$.

At day 4 all the isolates, except for *B. bassiana* Bb21 and *M. novozealandicum* F99, had MS although still in low concentrations. At this day there were no significant differences among the isolates in MS levels, the highest values on both days were found in *T. harzianum* F327. Pre-cultures of day 4 were used to inoculate a next batch of liquid medium for microsclerotia production (production cultures).

4.3.1..2 Fungal growth evaluation during microsclerotia production in liquid fermentation

Production cultures were evaluated for fungal growth performance, and biomass, blastospore and microsclerotia (MS) densities at 3, 4, 6, 7 and 8 days post inoculation. The isolates show differences in the amount of biomass, blastospores and MS produced ($p<0.01$). On average, during liquid fermentation, biomass was 66.6 mg/mL and ranged from 81.3 to 47.9 mg/mL ($p<0.01$). Greatest biomass density was determined at days 3 and 4 followed by a slow decrease (Figure 4.8; $LSD_{5\%}=0.027$).

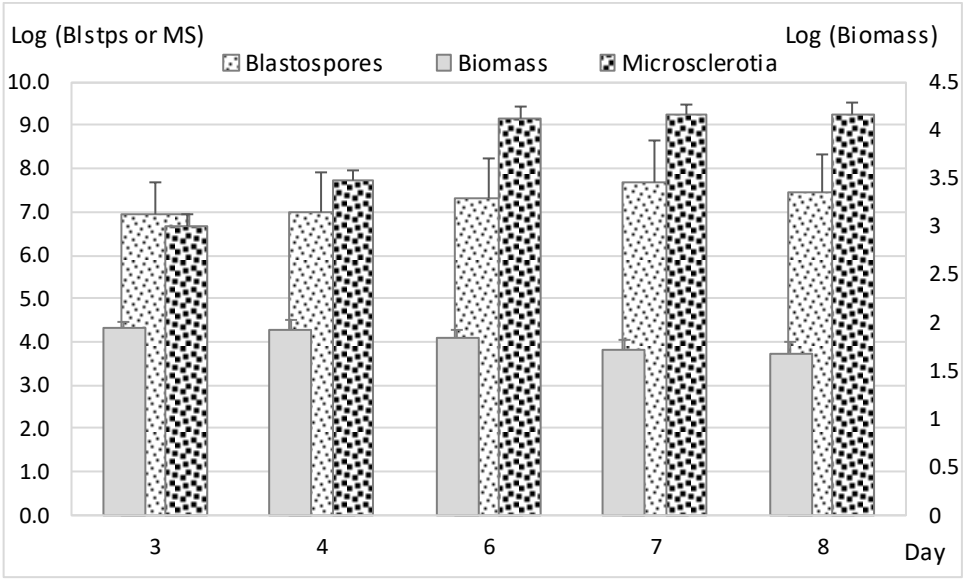

Figure 4.8 Fungal entomopathogenic growth combined for all isolates during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1) for microsclerotia production. Average values determined for biomass (mg/mL), blastospore (number/mL) and microsclerotia (number/mL). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. Error bars = standard deviation; $LSD_{5\%}$ biomass = 0.027; $LSD_{5\%}$ blastospore = 0.197; $LSD_{5\%}$ microsclerotia = 0.176; $p<0.01$.

The average blastospore production was 2.0×10^7 blastospore/mL within a range of 2.5×10^6 to 4.0×10^8 blastospore/mL ($p<0.01$). The highest average value was determined on day 7 ($LSD_{5\%} = 0.197$). A significant increase in the number of MS were found between days 4 and 6 ($LSD_{5\%} = 0.176$). Average MS production was 6.3×10^3 MS/mL within a range of 0.025 to 5.0×10^4 MS/mL ($p<0.01$).

4.3.1..3 Biomass production

All the *Metarhizium* isolates produced their highest biomass by day 3 and 4 after inoculation, while this decreased in subsequent days ($p<0.01$; Figure 4.9). By day 7, *T. harzianum* F327 and *B. bassiana* Bb21 had the lowest biomass among the isolates, while *M. anisopliae* F672 and *M. novozealandicum* F99 had the highest ($LSD_{5\%}=0.075$). At the end of the fermentation process on day 8, only the *T. harzianum* F327 and *B. bassiana* Bb21 isolates had a significant increase in biomass in comparison to day 7 (Figure 4.9, $LSD_{5\%}=0.075$).

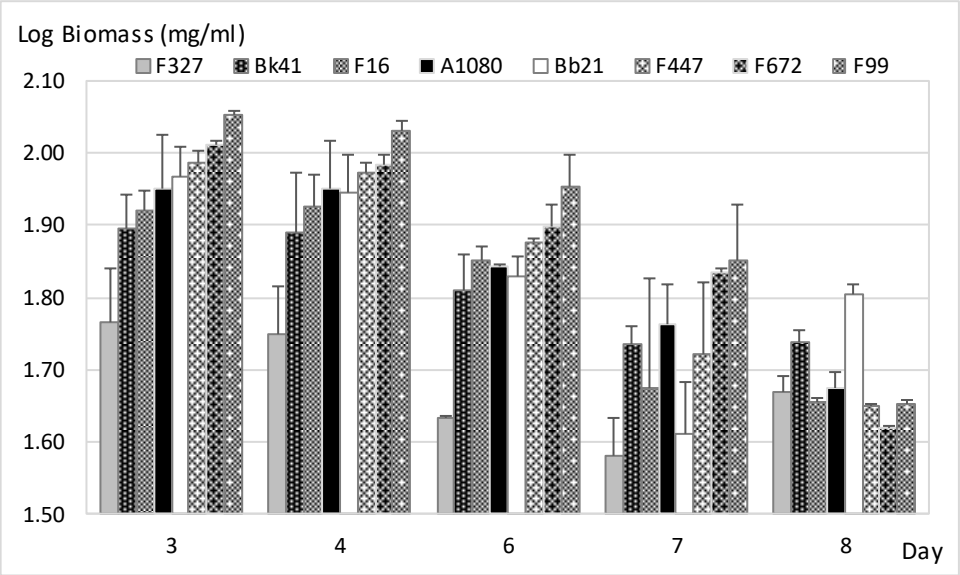

Figure 4.9 Fungal biomass (dry weight Log10mg/mL) during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. Error bars = standard deviation; $LSD_{5\%}$ = 0.075, $p<0.01$.

4.3.1..4 Blastospore production

Contrary to biomass production, blastospore production did not vary significantly during fermentation ($p<0.288$), at least since the beginning of the evaluation on day 3 after inoculation until the end on day 8 (Figure 4.10). The exceptions were the *T. harzianum* F327 and *M. robertsii* F447 isolates, which increased significantly in the number of blastopores per mL at day 6 of the evaluation ($LSD_{5\%}=0.557$). The highest values were determined were in *B. bassiana* Bb21 and *M. guizhouense* Bk41, both with 9.1 x 10^8 blastopores/mL, while the lowest was in *T. harzianum* F327 with 9.6 x 10^6 blastospore/mL (Figure 4.10; $LSD_{5\%}=0.557$).

During the fermentation process, the formation of submerged conidia originating from phialides was observed and also blastospores originating by budding from hyphae. The submerged conidia had a more spherical shape (Figure 4.11 – A), while the blastospores were irregular (Figure 4.11 – B).

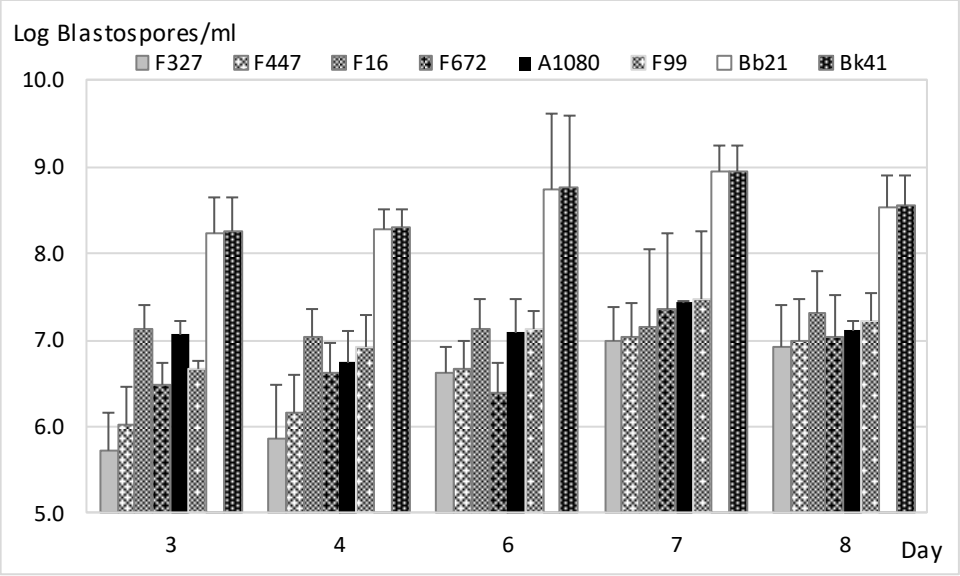

Figure 4.10 Blastospore production (Log10number/mL) during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. Error bars = standard deviation; $LSD_{5\%}$ = 0.557; p<0.288.

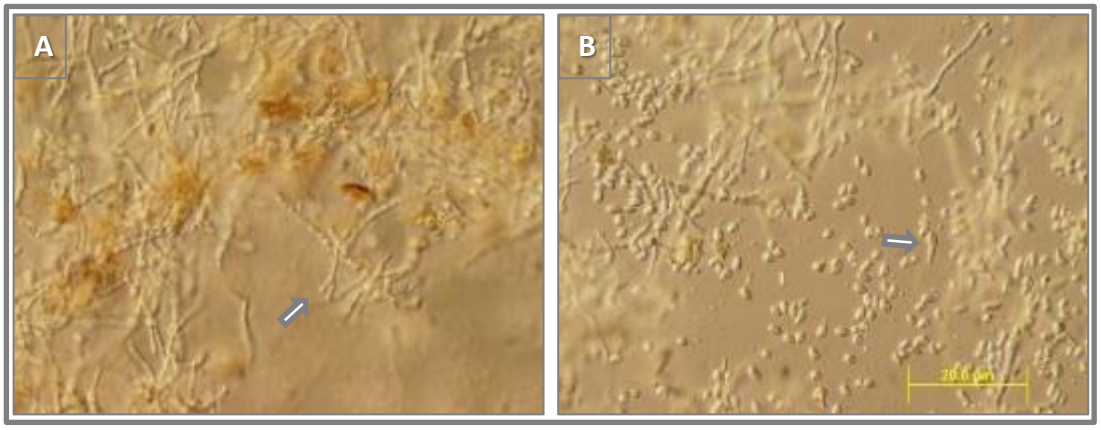

Figure 4.11 Blastospores formation during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. A. *M. guizhouense* Bk41 after 3 days post inoculation. Arrow indicates a phialide in conidiation process. B. *M. guizhouense* Bk41 after 7 days post inoculation. Arrow indicates a blastospore in germination process or the budding of a new blastospore.

4.3.1..5 Microsclerotia production

At the end of the fermentation, it was possible to observe the MS as small melanised heterogenous round structures of differing sizes (Figure 4.12).

Figure 4.12 Microsclerotia production in liquid fermentation in basal salts solution with trace elements and vitamins (C:N ratio 23:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. MS were able to be seen as small round particles which were highly melanised in some cases (circles).

In most fungal isolates, microsclerotia (MS) production increased with duration of fermentation, reaching a maximum value by day 6 after inoculation and remaining steady until day 8 ($p<0.01$). The exception was *M. novozealandicum* F99 which seemed not have any variation in MS during the evaluated period (Figure 4.13; $LSD_{5\%}=0.498$). This isolate, together with *M. guizhouense* Bk41, were able to produce MS in a relatively short time although the number of MS was, on average, lower than production by the other isolates (Figure 4.13).

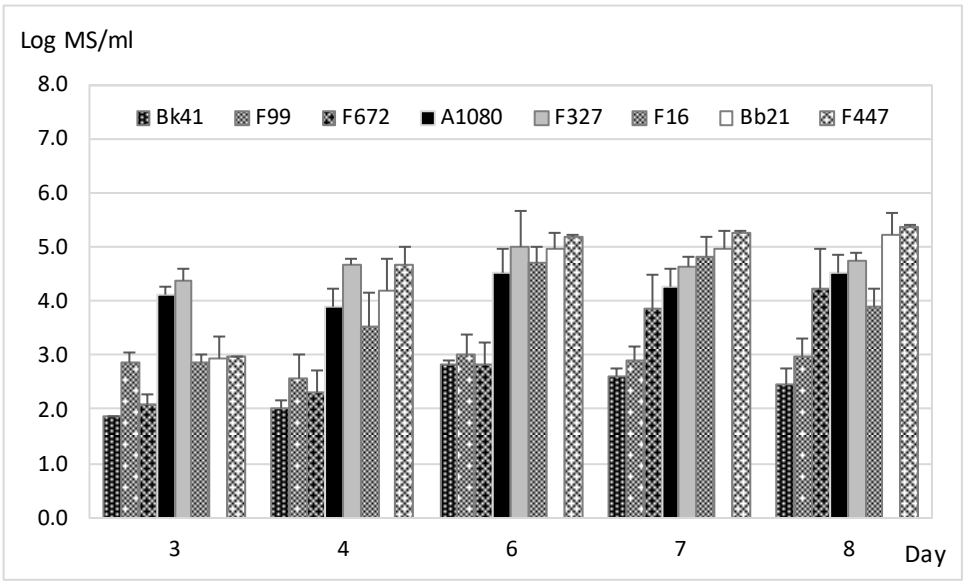

Figure 4.13 Microsclerotia production (Log10number/mL) during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. Error bars = standard deviation; LSD5% = 0.498; $p<0.01$.

The highest production was in *M. robertsii* F447 at day 8 with 2.3 x 10^5 MS/mL, while the lowest value was in the *M. guizhouense* Bk41 with 684 MS/mL at day 6. The *B. bassiana* Bb21 MS production was 1.6 x 10^5 MS/mL at day 8 after inoculation, significantly greater than *M. anisopliae* A1080 (known to produce MS) which highest production was 3.4 x 10^4 MS/mL at day 6 ($LSD_{5\%}$=0.498). The plant growth promotor *T. harzianum* highest production was 9.7 x 10^4 MS/mL also at day 6 after inoculation (Figure 4.13).

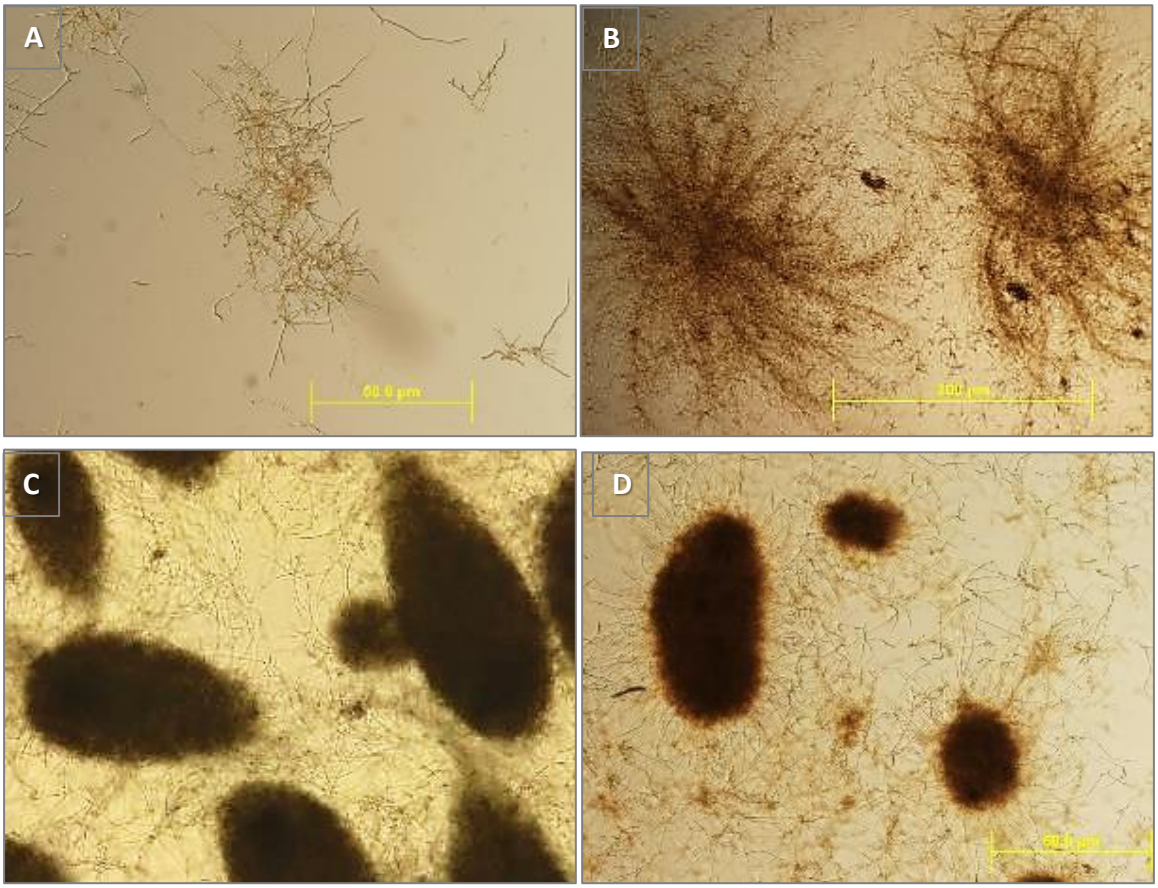

Figure 4.14 Microsclerotia formation during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Fungi were grown in a rotary shaker at 300 RPM and 28°C for 8 days. The initial process for the formation of microsclerotia (MS) started early in the fermentation under liquid conditions with the formation of hyphal aggregates (A and B, 4 days after inoculation). After 7 days of inoculation, the MS were formed as round structures with pseudoparenchymatous cells layers highly melanised (C and D). MS from *M. anisopliae* A1080 4 days post inoculation (A). MS from *M. robertsii* 4 days post inoculation (B). MS from *M. anisopliae* F672 7 days post inoculation (C). MS from *M. novozelandicum* 7 days post inoculation (D).

Even though MS were recorded from the beginning of the fermentation (day 3), it is important to consider that these structures have different development stages. At days 3 and 4 after inoculation, MS still were immature as hyphal aggregates and not completely melanised. Complete melanisation

of MS occurred after several more days of culture (Figure 4.14). The longer the fermentation process (until around 7 days), the greater the melanisation of the microsclerotia. The stages in MS development differed between isolates in rate of development, size and melanisation. For example, MS from *T. harzianum* F327 were relatively small when compared to those from *Metarhizium* isolates. The MS in *M. guizhouense* were melanised faster than those in *M. novozealandicum* F99 (data not shown).

Based on these results obtained from MS production, the isolates *M. anisopliae* A1080, *M. anisopliae* F672 and *M. robertsii* F447 were selected for MS production and coating onto maize seeds.

4.3.2 Microsclerotia production and formulation for seed coating

Primary culture inoculum and production cultures were prepared following the methodology described (section 4.2.3). There were no differences in biomass among the fungal isolates ($p = 0.402$) but the blastospore production was significantly different ($p<0.01$; Table 4.1). *M. anisopliae* F672 had the maximum concentration of blastospores with 1.0×10^7 blastospores/mL, while *M. robertsii* F447 the lowest with 3.0×10^5 blastospores/mL ($p<0.01$).

Table 4.1 Fungal growth in liquid fermentation for primary inoculum production (C:N ratio 33:1). Fungi were grown in basal salts solution with trace metals and vitamins in a rotary shaker at 300 RPM and 28°C. Fungal cultures were evaluated for biomass and blastopore production after four days from inoculation.

Isolate	*Genera*	*Biomass (mg/mL)*	*Blastospores (number/mL)*
A1080	*M. anisopliae*	129.5 ± 44.8 [a]	5.3 ± 0.8 (x 10^5) [b]
F447	*M. robertsii*	100.1 ± 3.8 [a]	3.0 ± 0.1 (x 10^5) [b]
F672	*M. anisopliae*	95.3 ± 9.7 [a]	1.0 ± 0.1 (x 10^7) [a]
LSD		59.6	1.5×10^6
p-value		0.402	<0.01

As both duplicates had similar growth (Figure 4.15), duplicate A was used as inoculum for MS production.

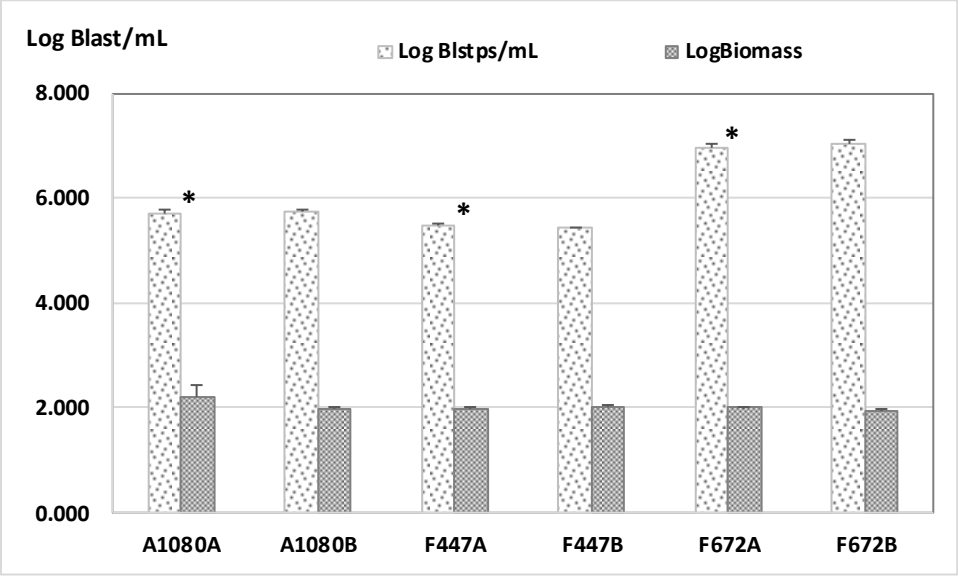

Figure 4.15 Fungal growth during primary inoculum production in basal salts solution with trace metals and vitamins (C:N ratio 33:1). Biomass (mg/mL) and blastospore (N°/mL) production during liquid fermentation of entomopathogenic fungi. Fungi were grown in a rotary shaker at 300 RPM and 28°C for 4 days. After 4 days from inoculation fungal growth was evaluated and cultures A (marked with an asterisk) were used to inoculate production cultures for microsclerotia. Error bars = standard deviation.

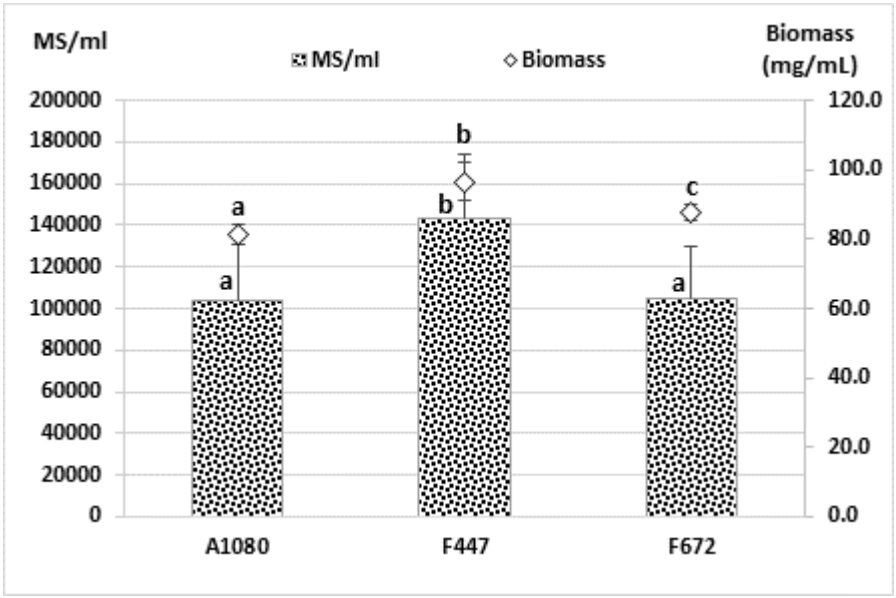

Figure 4.16 Biomass and MS production by entomopathogenic fungi in basal salts solution with trace metals and vitamins (C:N ratio 23:1). After 6 fermentation days in a rotary shaker at 300 RPM and 28°C biomass MS were harvested. Biomass (mg/mL) $LSD_{5\%}$ = 4.9; $p<0.01$ and MS/mL $LSD_{5\%}$ = 32,542.3; $p<0.05$. were determined at this day. Error bars = standard deviation.

On day four, 10 mL of pre-culture broth from each isolate was used to inoculate new cultures with fresh medium with basal salts solution with trace metals and vitamins (C:N ratio 23:1) for the production of MS. After 6 days growing with agitation at 300 RPM and 28°C, samples were taken for the determination of MS. On average, cultures produced around 80 mg/mL of biomass and above 1 x 10^5 MS/mL (Figure 4.16).

All the MS-DE from the three isolates had germination values above 90% (Table 4.2). Although no statistical differences were determined in MS-DE germination between the isolates ($p<0.236$), the production of conidia from the MS-DE was different ($p<0.01$). The highest conidia production per gram of MS-DE was found in *M. anisopliae* F672 while the lowest was in *M. anisopliae* A1080 (Table 4.2).

Table 4.2 **Evaluation of the viability of MS-DE granules.** After harvesting MS from production culture, they were formulated as granules with diatomaceous earth and dried. Viability of MS-DE was determined as granule ability to produce hyphae (germination) and conidia.

Isolate	*Species*	*Germination (%)*	*a_w (%)*	*Conidia production (conidia/g MS-DE)*
A1080	*M. anisopliae*	95.2 ± 3.5 [a]	74.7 ± 15.3 [a]	2.1 ± 0.5 (x 10^9) [a]
F447	*M. robertsii*	91.0 ± 6.9 [a]	70.3 ± 11.4 [a]	4.4 ± 1.2 (x 10^9) [b]
F672	*M. anisopliae*	95.2 ± 2.3 [a]	57.9 ± 1.0 [b]	8.8 ± 1.5 (x 10^9) [c]
LSD		5.7	8.9	1.8 x 10^9
p-value		<0.236	< 0.01	<0.01

Based on the results obtained from the conidia production per gram from MS, the amount of MS necessary was calculated for coating maize seeds that would provide a final conidia production of 1 x 10^8 per gram of seeds. Control seeds (CS) were coated only with the same amount of DE and the rest of the coating ingredients but not fungi. Final loading onto the seeds is shown in Figure 4.17. Seeds coated with MS from *M. anisopliae* F672 had the highest value, 7.8 x 10^6 CFU per gram of maize seeds, while seeds from the coating with *M. robertsii* F447 the lowest, 2 x 10^6 CFU per gram of maize seeds (Figure 4.17). On CS no fungal colonies were obtained.

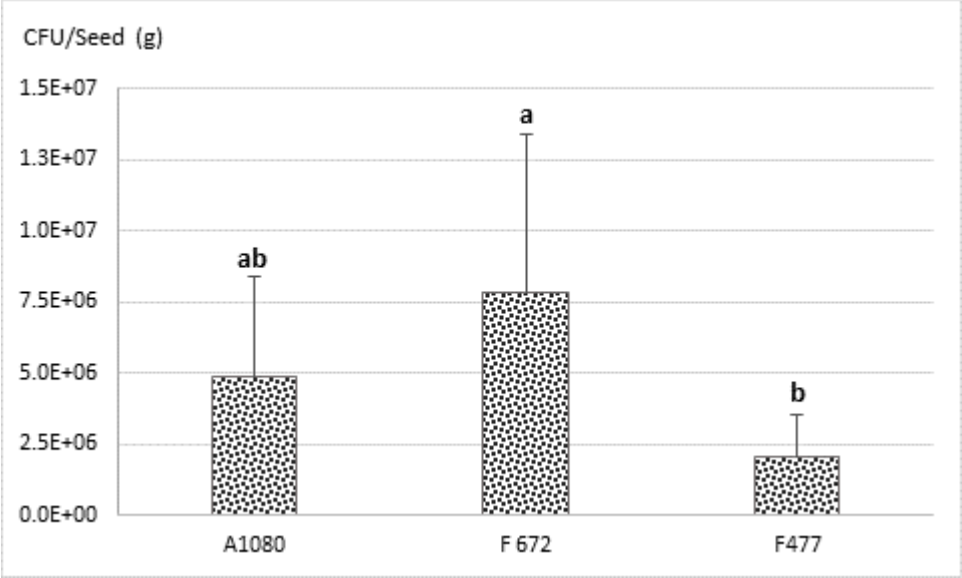

Figure 4.17 Quantification of MS-DE coated to maize seeds. The final amount of the entomopathogenic fungal loading onto maize seeds after seed coating with MS granules was determined as CFU per gram of coated seeds. Error bars = standard deviation; $LSD_{5\%}$ = 4.6 x 10^6; p<0.06.

4.3.3 Evaluation of maize plants after microsclerotia coating and growth in presence of *F. graminearum*

Maize seeds coated with microsclerotia from *M. robertsii* F447 and *M. anisopliae* F672 were sown in potting mix containing *F. graminearum*. After three weeks maize plants were harvested from pots and processed accordingly to determine shoot length and dry weight.

4.3.3..1 Maize plant length

Maize plants grown in the presence of *F. graminearum* had a decrease in total length in comparison to plants grown in absence of the plant pathogen (p<0.01). Control plants in absence of *F. graminearum* (CS) had significantly greater length than plants treated with *M. robertsii* F447 grown in presence of *F. graminearum* (Figure 4.18). On the other hand, length of maize plants with the *M. anisopliae* F672 treatment grown in presence *of F. graminearum* were not significantly different to the CS plants grown in absence of *F. graminearum* (Figure 4.18; $LSD_{5\%}$=0.026).

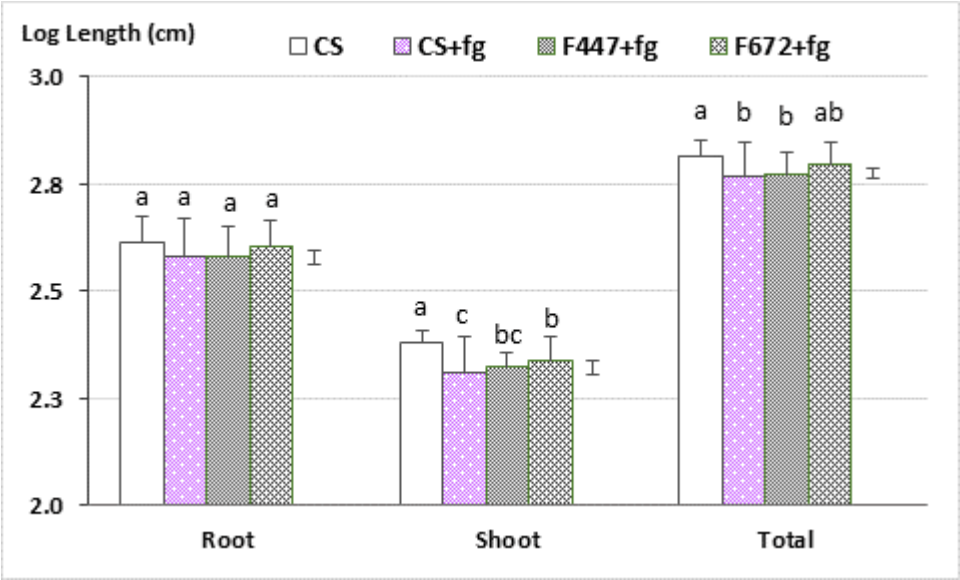

Figure 4.18 Plant maize length after microsclerotia coating. Maize seeds were coated with microsclerotia from *Metarhizium robertsii* F447 and *M. anisopliae* F672 and sown in potting mix containing *Fusarium graminearum*. After three weeks plants were recovered and length of roots and shoots determined. CS: control plants in absence of *F. graminearum*; CS+fg: control plants in presence of *F. graminearum*, F447+fg or F672+fg: coated seeds with microsclerotia from *M. robertsii* or *M. anisopliae*, respectively in presence of *F. graminearum*. Bar = $LSD_{5\%}$ (Roots = 0.031; Shoots = 0.025; Total = 0.026).

The observed variation in plant length in presence of *F. graminearum* was due to the effect on the length of the shoots, rather than roots since these were not affected by the plant pathogen. Maize control plants had the longest shoot length in the absence of *F. graminearum*, while this was reduced approximately 14% when the plant pathogen was present ($p<0.01$, Figure 4.18). However, the *M. anisopliae* F672 treatment a significantly higher shoot length than control plants (CS+fg) in presence of *Fusarium* ($LSD_{5\%}=0.025$).

4.3.3..2 Maize dry weight

Total maize dry weight was significantly different between the treatments ($p<0.01$). Maize growth was the highest in control plants (CS) in absence of *F. graminearum*, while in presence of the pathogen, control plants (CS+fg) showed a 49% decrease in total dry weight (Figure 4.19; $LSD_{5\%}=0.029$). In presence of *F. graminearum*, the *M. anisopliae* F672 coating had significantly greater dry weight than CS+fg, and the *M. robertsii* F447 treatment ($LSD_{5\%}=0.029$). In the presence of *F. graminearum*, plants coated with the *M. anisopliae* isolate had a biomass gain of 32% compared to the maize plants without the coating, while they were almost 26% lower when compared to control plants (CS) in absence of *Fusarium* (Figure 4.19).

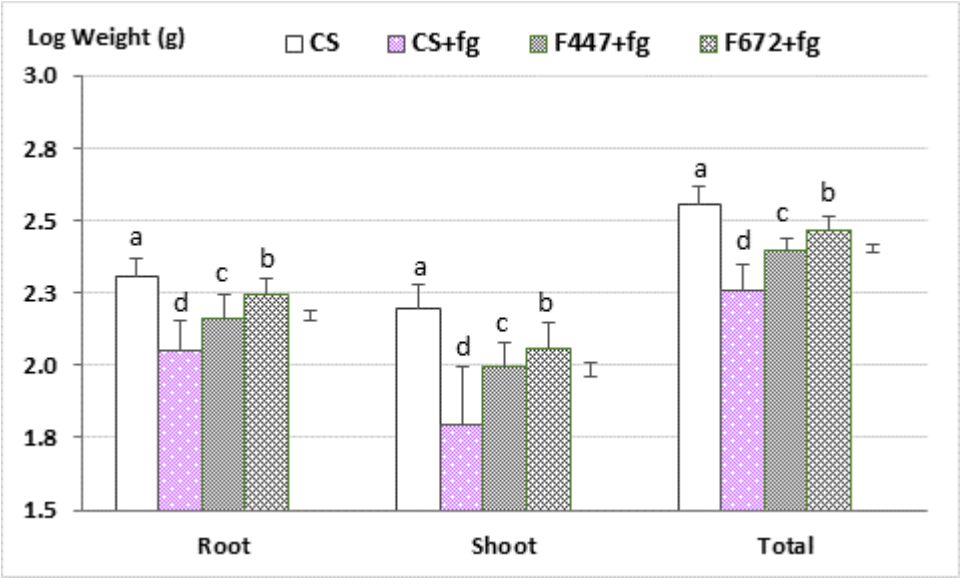

Figure 4.19 Plant maize dry weight after microsclerotia coating. Maize seeds were coated with microsclerotia from *Metarhizium robertsii* F447 and *M. anisopliae* F672 and sown in potting mix containing *Fusarium graminearum*. After three weeks, plants were harvested for the determination of dry weight. Control plants in absence (CS) and in presence *of F. graminearum* (CS+fg); coated seeds with microsclerotia from *M. robertsii* (F447+fg) or *M. anisopliae* (F672+fg) in presence of *F. graminearum*. Bar = $LSD_{5\%}$ (Roots = 0.034; Shoots = 0.055; Total = 0.029), $p<0.01$.

The detrimental effect of *F. graminearum* on maize plants was also shown in shoot weights. Shoot weight in plants grown with the pathogen (CS+fg), was 57% lower than control plants (CS) grown in absence of the pathogen (Figure 4.21, $p<0.01$). The fungal coatings reduced the negative effect caused by *F. graminearum* on shoot development. Compared to control plants in absence of *Fusarium* (CS), shoots from plants treated with *M. anisopliae* F672 had only a 27% weight decrease while plants treated with *M. robertsii* F447 a 37% weight reduction when grown in presence of *F. graminearum* ($LSD_{5\%}$=0.054). The gain in shoot biomass in plants grown from seeds which had been coated with *M. robertsii* and *M. anisopliae* MS treatments represented a 48 and 70% gain, respectively, when compared to maize plants without the fungal entomopathogenic coating in presence of *F. graminearum* (Figure 4.19).

Fusarium graminearum also caused a significant decrease in maize seeding root weight compared to controls ($p<0.01$). The highest root biomass was determined in control plants (CS) in absence of *F. graminearum*, but when this fungal plant pathogen was present it had a significant detrimental effect on maize root weight of control plants which was a 43% lower (Figure 4.19, $LSD_{5\%}$=0.034). In presence of *F. graminearum*, maize plants from seeds coated with the entomopathogenic fungi had a better root development when compared to plants without a fungal treatment ($LSD_{5\%}$=0.034). Compared to control plants (in absence of *Fusarium*) roots in plants from seeds coated with *M. anisopliae* F672 did

not have any difference in dry root weight when *F. graminearum* was present while, root dry weight from plants coated with *M. robertsii* F447 was 28% lower (Figure 4.19). Plants with the *M. anisopliae* treatments, when grown in presence of *F. graminearum*, had 52% more root biomass than CS (CS+fg) grown also in presence of the plant pathogen (Figure 4.19).

4.3.4 Determination of fungal presence by fluorescent and laser confocal microscopy

Maize seeds coated with MS-DE were sown in sterile vermiculite and grown at 22°C. After two weeks and one-month, whole plants were stained with a fluorescent dye. Plants from CS were also processed. For the staining, plants were split in roots, stems and leaves and treated independently and observed with a fluorescent and with laser confocal microscopes.

The presence of hyphae on and in the plant tissues were observed in all the fungal treatments but not in samples from control plants (CS) without a fungal treatment. Fungal hyphae growing on the surface of roots were observed in all MS coated treatments from *M. robertsii* F447 (Figure 4.20), from *M. anisopliae* A1080 (Figure 4.21) and *M. anisopliae* F672 (Figure 4.23). The hyphae were observed growing in major proportions of the distal parts of the roots, and all along the root extension, but not in the root apices.

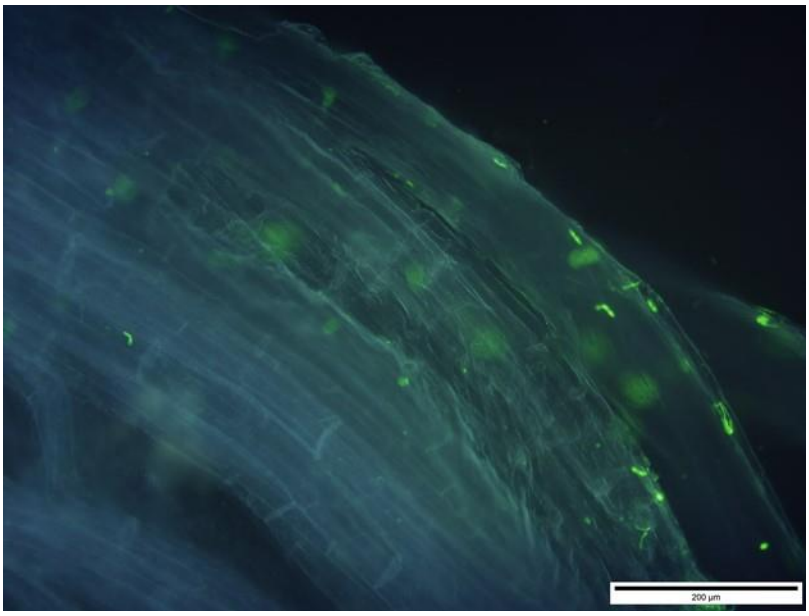

Figure 4.20 *Metarhizium anisopliae* F447 colonizing maize roots cells in two week old plants. Fungal structures were stained with WGA-AF488 (green) and plant cells were stained with propidium iodide (blue). The blurry green images are hyphae growing inside the roots, while the bright green are hyphae in the surface of the root. Images were taken with a fluorescence Olympus microscope with standard settings for WGA- AF488 and propidium iodide. Line bar represents 200 µm.

The capacity of the fungi to colonize the roots and access inside the vegetative tissues was evidenced by the three-dimensional net of hyphae that ran along the cells (Figure 4.21).

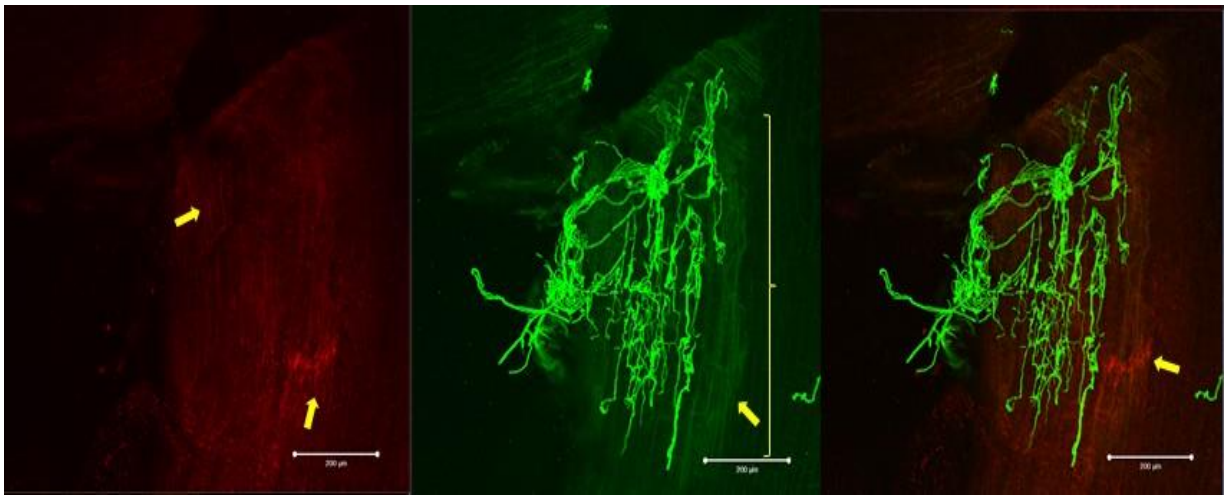

Figure 4.21 ***Metarhizium anisopliae A1080*** **hyphae on roots of two weeks old maize plants.** The left panel shows the presence of glycoproteins, α-mannopyranosyl and/or α- glucopyranosyl residues around hyphal adhesion and penetration sites stained with ConA-AF633 (red, arrows). The tissues were stained with Congo Red (red). In the central panel fungal structures were stained with WGA-AF488 (green). Extensive fungal root colonization was observed on the surface of the root but also in the inner parts of the root (bracket). A three-dimensional net of hyphae that only correspond to growth inside the root. The right panel is an overlay of both pictures. Arrows indicate the signal with CoA -AF633. Image, Z-stack compressed, 12.3 µm, taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. The bar represents 200 µm.

The protrusion zone where secondary roots emerge, seemed to be one of the preferred areas of access to the interior of the root. In these areas extensive colonization was observed (Figure 4.22)

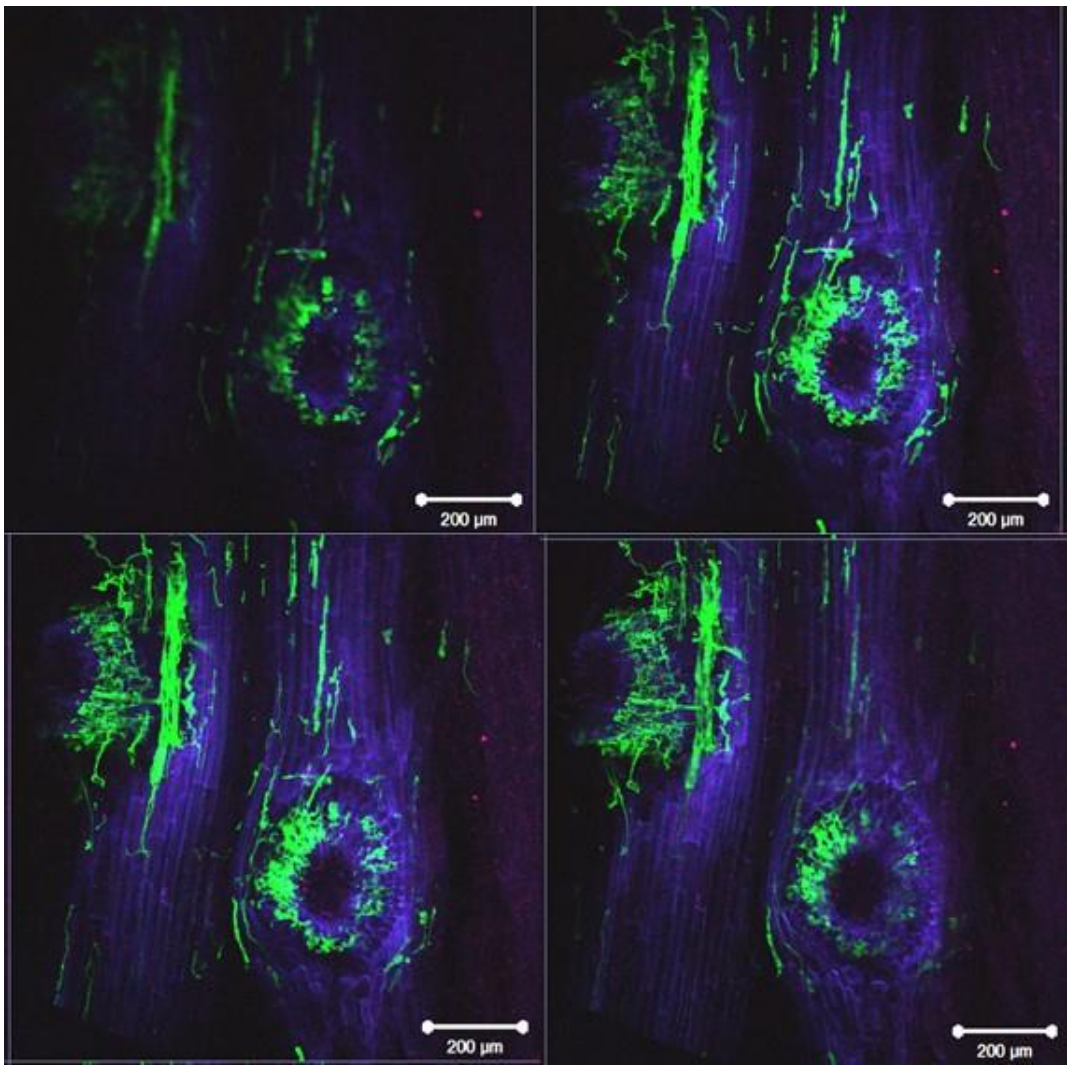

Figure 4.22 ***Metarhizium anisopliae*** **A1080 colonization on roots from two weeks old maize plants**, showing the protrusion zone of secondary root emergence with fungi growing on the surface and inside the tissues. Overlay of images: plant membranes were stained with propidium iodide (blue) and Congo Red (red), while fungal structures were stained with WGA-AF488 (green). Z-Stack image: upper left panel distal focus, bottom right closest focus (51.7 µm). Images were taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. The bar represents 200 µm.

The presence of hyphae growing inside the cortical vegetal cells was observed one-month after sowing (Figure 4.23). The lectin, CoA-AF688, allowed identification of the penetration zone of the hypha through the cell wall (Figure 4.23 - right upper panel), and the constriction zones in the hypha also helped to determine these penetration zones (upper left panel). At this stage, the roots showed extensive fungal colonization on the surface of the root but also endophytic colonization (Figure 4.23 - left upper panel).

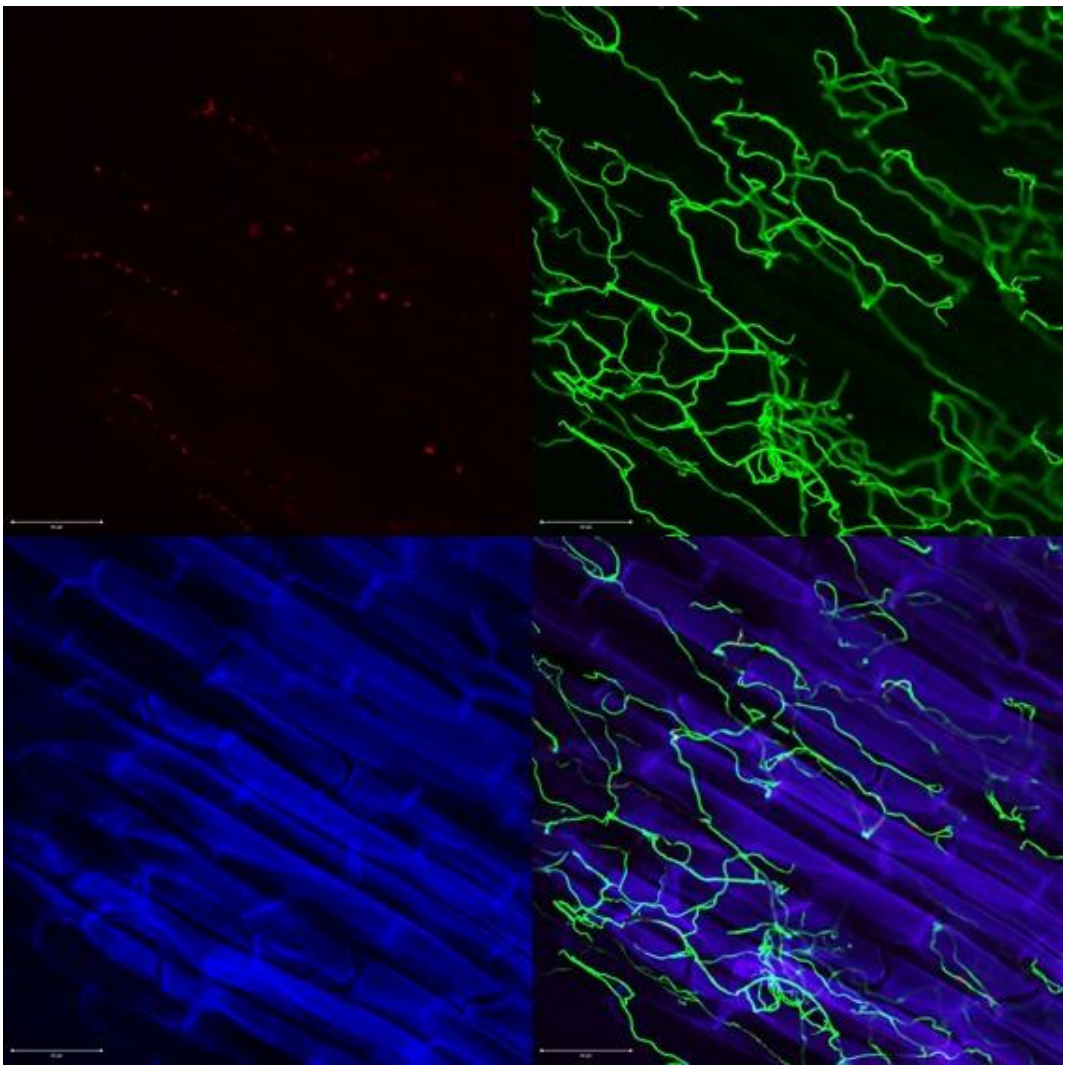

Figure 4.23 ***Metarhizium anisopliae*** **F672 hyphae on 1-month old maize roots.** Root sample was stained with ConA-AF633 (red); WGA-AF488 (green) and propidium iodide (blue). The presence of glycoproteins, α-mannopyranosyl and/or α- glucopyranosyl residues, around hyphal adhesion and penetration sites are visualized in red (left upper panel). The net of hyphae growing around and inside the root cell are stained in green (right upper panel). The root cells are visualized in blue (left bottom panel). Overlay of the three images (right bottom panel). Images were taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. The bar represents 50 µm.

After one month, the seed remnants still attached to the root system were completed colonized by *M. anisopliae* A1080. An extensive net of hyphae was visualized in the interior of the shell of the maize seed, indicating the ability of the fungi to colonize these important reserves for seed development.

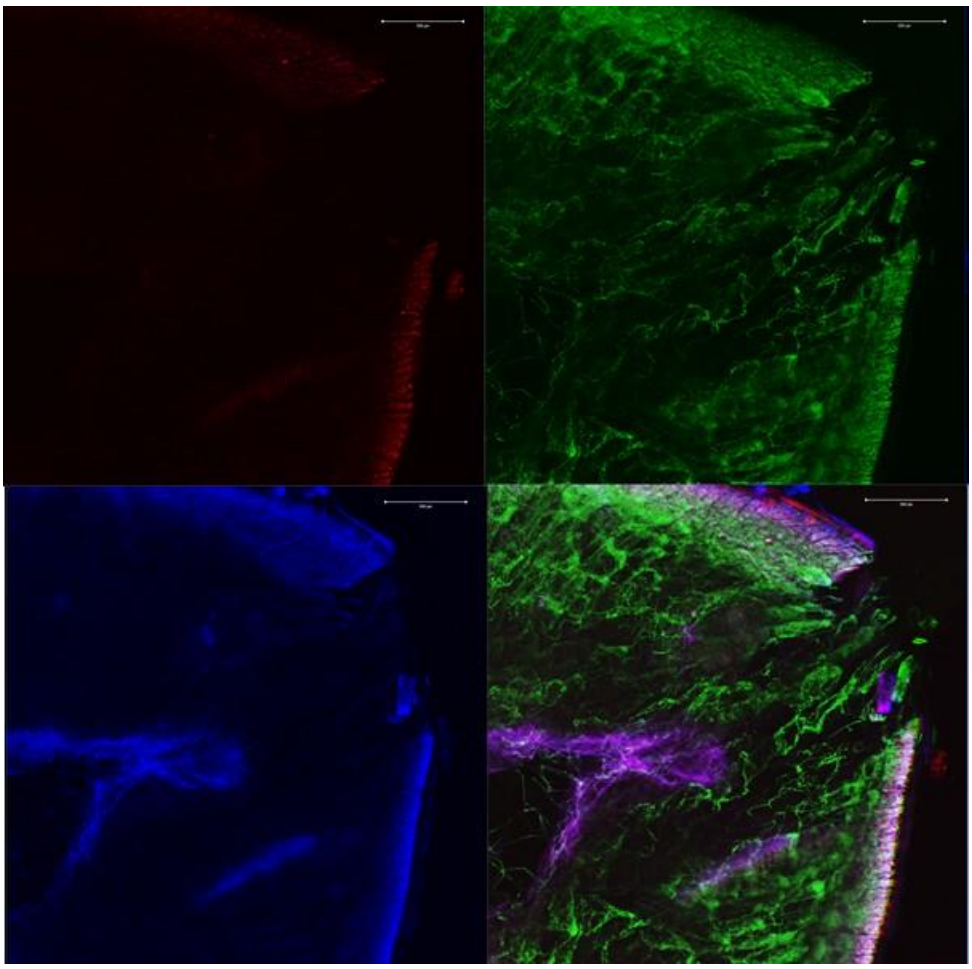

Figure 4.24 ***Metarhizium anisopliae*** **A1080 hyphae inside seed remnants still attached to roots of 1-month old maize plant.** Seed remains attached to root samples was stained with ConA-AF633 (red); WGA-AF488 (green) and propidium iodide (blue). The presence of glycoproteins, α-mannopyranosyl and/or α- glucopyranosyl residues, at the hyphal adhesion sites were visualized in red (left upper panel). The net of hyphae growing inside the seed were stained in green (right upper panel). The remains of the seed were stained in blue (left bottom panel). Overlay of the three images (right bottom panel). Images were taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. Bar represents 200 µm.

Samples of stems and leaves were also analysed for all the treatments by fluorescent and confocal microscopy, but no endophytic colonization was observed. For at least one month after sowing *Metarhizium* seemed to be limited to the colonization of roots.

4.4 Discussion

In this study the production and formulation of microsclerotia (MS) by isolates of *M. anisopliae*, *M. guizhouense*, *M. novozelandicum*, *M. robertsii*, *B. bassiana* and *T. harzianum* was determined. Under the conditions of this study, all isolates produced MS, compact hyphal aggregates that become pigmented with culture age, in addition to more typical blastospores and mycelia. Previous reports have determined the production of these resistant structures in *M. anisopliae*, *M. robertsii*, *M. rileyi*, *M. brunneum* and *T. harzianum* (Jaronski & Jackson, 2008; Jackson & Jaronski, 2009; Behle *et al*., 2014; Mascarin *et al*., 2014; Kobori *et al*., 2015). However, this study also reports on the production of MS in *B. bassiana* and is the first report of MS from isolates of *M. guizhouense* and *M. novozelandicum*. Additionally, when MS were coated to maize seeds and sown in potting mix and challenged with *F. graminearum*, the treated maize plants showed a better performance than control plants. Through fluorescent and confocal microscopy, the ability of the isolates of *Metarhizium* spp. to associate with the roots, not only with the ectorhizosphere but also with the endorhizosphere, was determined.

The biomass obtained by the New Zealand *Metarhizium* spp. isolates, 42 – 55 mg/mL, was higher than the values reported in previous works. Jackson and Jaronski (2009) reported values between 3.8 and 5.0 mg/mL in *Metarhizium* strains, while Mascarin *et al*. (2014) reported between 9.0 and 13.8 mg/mL. Only in research with *M. rileyi*, with a biomass production of 40.7 mg/mL (Song *et al*., 2016), was similar to those values in this study. The highest biomass production was determined in *B. bassiana* Bb21 with 64 mg/mL. Also, this yield was higher than previous works under similar conditions where values were obtained between 15.3 – 20.5 mg/mL (Bidochka *et al*., 1987; Lohse *et al*., 2014). However, the differences in biomass yield might be due to differences in growth conditions and media.

The production of blastospores by the *M. anisopliae* isolates varied between 6.5×10^5 – 1.3×10^7 blastospores/mL and reached these yields after 6 fermentation days. These values were higher than those reported by Jackson & Jaronski (2009), of around $0.9 – 5.8 \times 10^5$ blastospores/mL after 8 fermentation days in identical conditions. The differences in blastospore production might be based on the slightly differences in C:N ratios, but mainly, in the intrinsic properties of the isolates used. *B. bassiana* Bb21 blastospore production after 3 days of fermentation, 2.7×10^8 blastospore/mL was similar than the values reported by Mascarin *et al*. (2015) who reported values between $0.95 – 7.9 \times 10^8$ blastospores/mL in a similar study. However, *B. bassiana* Bb21 maximum production, 4.3×10^9 blastospore/mL, was obtained after 6 fermentation days.

The quantity of MS obtained with both *M. anisopliae* isolates ($3.5 – 4.0 \times 10^7$ MS/L) were close to those reported previously by Jackson & Jaronski (2009) for *M. anisopliae* ($1.8 \times 10^7 – 6.4 \times 10^7$ MS/L) using identical fermentation conditions and similar C:N ratios. Although in their work the highest production

was determined for *M. brunneum* F52 with 1.2 x 10^8 MS/L, in this study the highest MS production was found in the isolate *M. robertsii* F447 with 2.3 x 10^8 MS/L. Mascarin *et al*. (2014) reported yields of MS by strains of *M. anisopliae*, *M. acridum* and *M. robertsii* of around 6.1 – 7.3 × 10^6/L after 3 days growth with a maximum MS yield of 0.7 – 1.1 × 10^7/L after 5 days liquid fermentation. In a different study with *M. rileyi* MS production was reported up to 9.7 x 10^7 MS/L (Behle *et al*., 2013).

Evidently, all *Metarhizium* species tested are capable of producing MS although, as expected, the production of the resting structures varies between species and isolates. In this study, the lowest MS production was found in *M. guizhouense* Bk41 with 3.3 x 10^5 MS/L, while in *M. guizhouense* F16 9.3 x 10^6 MS/mL was achieved. However, *M. guizhouense* Bk41 produced higher amounts of blastospores, 2.6 x 10^7 blastospores/mL than *M. guizhouense* F16, 6.1 x 10^5 blastospores/mL. This fact highlights the importance of a proper isolate characterization to determine the potential use and applicability. Thus, it is apparent that environmental conditions required for formation of MS varies among fungal species and even among isolates of a particular species (Wang *et al*., 2013).

However, MS formation is not restricted to only to *Metarhizium* spp. Other genera are capable of producing these resting structures, under the appropriate conditions including entomopathogenic fungi such as *Isaria fumosorosea* (Mascarin *et al*., 2014) and *Lecanicillum lecanii* (Wang *et al*., 2013). During the production of inoculum for the studies with *F. graminearum* 13083 the formation of MS during liquid fermentation was observed. *T. harzianum* was also reported to be capable to produce MS (Kobori *et al*., 2014). In the current study, isolates *B. bassiana* Bb21 and *M. robertsii* F447 had the highest MS production (2.1 x 10^8 and 2.3 x 10^8 MS/mL, respectively).

An important factor to consider for microsclerotia production is medium optimization especially for industrial production. The use low cost medium would make the production process profitable and with high potential for agronomic uses. Nitrogen is one of the most expensive components of fermentation media (Kobori *et al*., 2015). Previous work has demonstrated that is possible to obtain viable blastospores with glucose as the carbon source and cottonseed flour as the nitrogen source (Mascarin *et al*., 2015) and a low-cost complex nitrogen source based on cottonseed flour effectively supported high MS yields in *T. harzianum* (Kobori *et al*., 2015). Also, reduction in fermentation times would decrease the costs associated with industrial production. Mascarin *et al*. (2015) reported the production of high blastospore yields in only three fermentations days, while Mascarin *et al*. (2014) used a culture medium with low concentration of nutrients to hasten the formation and melanisation of MS. Microsclerotia production and stabilization with low-cost nitrogen sources have been demonstrated for liquid fermentation of *Metarhizium terrestris* and *M. brunneum* with MS showing excellent biocontrol performance in the field (Shearer & Jackson 2006; Behle & Jackson 2014).

Previous results showed that stirred-tank bioreactors can be used to mass produce stable MS of *Metarhizium* and the length of fermentation (4-7 days) for *Metarhizium* cultures had no significant impact on biomass accumulation (Jackson & Jaronski, 2012).

Another factor to consider during MS production is maturation of the resistant structures. Although the production of these resistant structures could be significant early in the fermentation, a complete melanisation of MS did not occur until several days of fermentation had passed. The longer the fermentation process, the higher the melanisation of the microsclerotia occurs not only in entomopathogenic fungi but also in *T. harzianum* (Jackson & Jaronski, 2009; Mascarin *et al*., 2014; Kobori *et al*., 2015). In general, microsclerotia can be observed after 4 fermentation days but this period might be not enough for a complete melanisation. Further studies need to be done to determine the relationship between melanisation and performance of MS. An early harvest of MS and the downstream process during drying and formulation, if they are not completed mature, could result in viability losses. Melanisation has been associated with prolonged persistence in soil and resistance to desiccation (stress tolerance) in various filamentous fungi (Jackson & Jaronski, 2009; Kobori *et al*., 2015).

Kobori *et al*. (2015) found that MS from *T. harzianum* are only formed in high carbon media (36 g/L), while conidiation was supressed in high carbon and high nitrogen media. During preculture fermentation for inoculum production, after 4 days *T. harzianum* F327 was the only isolate that did not produce blastospores. It seems that *T. harzianum* needs specific conditions and nutritional requirements to produce either submerged conidia or blastospores. However, after 7 fermentation days *T. harzianum* F327 produced 3.8 x 10^7 blastospores/mL and 4.4 x 10^7 MS/L. Kobori *et al*. (2015) reported in similar conditions up to 3.4 x 10^8 conidia/mL and 1.7 x 10^7 MS/L after 7 fermentation days. The MS obtained were formulated as granules with DE and were able to reduce damping-off in melon seedlings caused by *Rhizoctonia solani*.

The rehydration and incubation of air-dried MS granules from the isolates *M. anisopliae* A1080 and F672, and *M. robertsii* F447, on water agar plates resulted in hyphal development in sporogenic structures to produce high numbers of conidia. This was also reported in previous works for *M. brunneum* and *M. anisopliae* (Jackson & Jaronski, 2009; Mascarin *et al*., 2014), *Lecanicillium lecanii* (Wang *et al*., 2013), *T. harzianum* (Kobori *et al*., 2015), *M. robertsii* and *M. acridum* (Mascarin *et al*., 2014). However, the levels reached in conidia/g of dried MS-DE were one order higher than those reported by Jackson & Jaronski (2009), and similar to those reported by Mascarin *et al*. (2014).

This was the first work to use MS coated to seeds that proved that after coating MS were viable as determined as CFU/g seed. The coating of maize seeds with MS proved to be a feasible method for delivery of entomopathogenic fungi providing protection of maize plants against *F. graminearum*. As stated by Partida-Martínez & Heil (2011) the potential benefits of MS coating on plant maize growth was observed in presence of the challenge, such as plant disease. Control plants grown in absence of *F. graminearum* (CS) had significantly greater length and weight than plants treated with *M. robertsii* F447 while no differences were observed with the *M. anisopliae* F672 treatment. This indicates that the coating with *M. anisopliae* F672 counteracted the negative effect on plant growth caused by *Fusarium*. In presence of the plant pathogen, the shoot biomass in plants where seeds had been coated with MS from *M. robertsii* and *M. anisopliae* increased by 48 and 70%, respectively, when compared to maize plants without the fungal entomopathogenic coating. These results suggest that the entomopathogenic fungus *M. anisopliae* F672 could also be utilized as a biocontrol agent against certain plant root pathogens and is an area that warrants further research.

Previous work had also found antagonism between an endophytic *M. robertsii* in bean plants and *Fusarium solani*. Sasan & Bidochka (2013) found that beans plants colonized by *M. robertsii*, then exposed to *F. solani*, produced greater plant growth and lower disease indices compared with plants uncolonized by *M. robertsii*. The mechanisms used by *Metarhizium* to counteract the negative effects of the presence of the plant pathogen could be due to multiple factors such as the competence for colonization of the rhizosphere, promotion of the induced response in the plant or production of secondary metabolites that inhibit *Fusarium* growth. Ravindran et al. (2014) found that secondary metabolites extracted from *M. anisopliae* (TK09) showed inhibitory activity against *F. oxysporum* in agar disc diffusion methods. In studies *in vitro*, cell-free culture filtrates of *M. robertsii* inhibited the germination of *F. solani* conidia by 83% and the inhibitory metabolite was heat stable. Dual cultures in Petri dishes showed antagonism of *M. robertsii* against *F. solani* with a relative inhibition of c. 60% of *F. solani* growth (Sasan & Bidochka, 2013). Thus, the overall results suggest that species of *Metarhizium* may be used as a control for plant pathogens as well as insects pest.

Previous works have used the incorporation MS formulated as granules to soil for the control of soil dwelling insects. Jackson & Jaronski (2009) found that seven days after sugar beet root maggot, *T. myopaeformis* larvae, were introduced into soil treated with granules of MS-DE, the mean cumulative mortality ranged from 23.3 to 53.3 %. Bioassays using soil-incorporated MS resulted in significant infection and mortality of western flower thrips, *Frankliniella occidentalis* (Wang *et al*., 2012). MS granules of *M. brunneum* were able to produce viable conidia and effectively infect and kill Asian long horned beetle, *Anoplophora glabripennis* when applied in hydromulch formulations (Goble *et al*., 2016). Behle *et al*. (2013) reported a 74% mortality in ticks, *Ixodes scapularis*, using potting mix

containing granules of MS-DE from *M. brunneum*. Efficacy studies revealed that the incorporation of 0.4 g dried MS granules of *T. harzianum* into potting soil enhanced plant emergence and reduced (>90 % suppression) damping-off disease incited by *R. solani* by increasing survival of melon plants (Kobori *et al*., 2015). The rapid sequence of MS granule germination and conidiation enhanced the ability of *T. harzianum* to quickly colonize plant tissues and rhizosphere to the detriment of plant pathogens.

This study was the first to demonstrate that after MS-coated seeds were sown in potting mix, the environmental conditions were appropriate for MS germination of three different isolates belonging to *M. anisopliae* and *M. robertsii* and, by using fluorescent and laser confocal microscopy, showed that the resulting hyphae were closely associated with the ecto and endorhizosphere of maize roots. The ability of *M. robertsii* ARSEF 2575-GFP to superficially associate with roots of *Phaseolus vulgaris* (haricot bean) and *Panicum virgatum* (switchgrass) was also determined after submerging the seeds in a conidial suspension (Wyrebek *et al*., 2011). Sasan & Bidochka (2012) found that after submerging seeds in a conidial suspensions of *M. robertsii* – GFP not only did they observe fungal rhizospheric colonization in switchgrass roots and but also the endophytic colonization of cortical cells in been roots. In the present work, two isolates, *M. anisopliae* F672 and *M. robertsii* F447, were obtained from plant material, *Pinus radiata* and *Actinidia deliciosa*, respectively, while *M. anisopliae* A1080, from a lepidopteran larva *Trichoplusia ni*. The ability of produce MS as well as the capability to associate with maize roots, seemed to be well conserved in *Metarhizium* independently of origin. This reinforces the idea of *Metarhizium* as a soil dwelling fungus with the ability to form resting structures to survive unsuitable conditions, and the ability to associate with roots which guarantees long term survival in soil.

Vega *et al.* (2009) stated "the ability of *Metarhizium anisopliae* to form sclerotia may be important for rhizosphere competence following a pattern seen in phytopathogenic fungi". Whether the association pattern between hyphae of entomopathogenic fungi and roots is a similar process to that of phytopathogenic fungi or is an exclusive process for entomopathogenic fungi needs to be further studied. However, *M. anisopliae* A1080 and F672 and *M. robertsii* F447 widely colonized the differentiation and the root hair zones, inter- and intracellularly, while it was infrequently detectable in the elongation and meristematic zones. This colonization pattern was also observed in the plant pathogen *Piriformospora indica* (Zuccaro *et al*., 2011). However, the ability of *Metarhizium* to be endophytic and to colonize cortical cell roots, set this genus closer to ecto- and arbuscular mycorrhizal fungi, which either grow intercellularly or colonize predominantly the deeper cortex layers of younger parts of the root (Zuccaro *et al*., 2011). This may indicate that entomopathogenic fungi follow a pattern similar to that observed in *P. indica* and mycorrhiza. In *P. inidica*, the failure of WGA-AF488 to stain the hyphae inside living cells strongly suggested that the fungus remained enveloped in an intact plant-

derived membrane throughout intracellular growth (Zuccaro *et al*., 2011). *M. anisopliae* and *M. robertsii* were possible to observe inside the cells stained with the WGA-AF488 that may indicate that entomopathogenic fungi growing inside the vegetal cell were not enveloped by an endomembrane as in *P. indica*.

The results obtained provide insights into liquid culture production of MS from different species of *Metarhizium* and the potential use of these structures when coated onto seeds for the biocontrol of pest and plant pathogens.

Chapter 5

Fungal transformation with the mcherry and GFP proteins

5.1 Introduction

The transformation of fungal entomopathogens has been used with different purposes: (1) identified and characterized genes involved in colonization of plants or insect´s infection (Wang & St. Leger, 2007; Fang & St. Leger, 2010; Liao *et al*., 2013; Song *et al*., 2016); (2) manipulated the genes of the pathogen to improve biocontrol performance (St Leger *et al*., 1996; Fang *et al*., 2011); and (3) to localize entomopathogens in living organisms or soil (Hu & St. Leger, 2002; Wyrebek *et al*., 2011; Sasan & Bidochka, 2012).

Genetic manipulation of fungal biocontrol agents, such as *Metarhizium*, *Beauveria* and *Isaria*, have been founded in the needs to improve fungal biocontrol agents´ virulence or efficacy against adverse conditions (Ying & Feng, 2006). For example, a strain of *M. anisopliae* was modified to express additional copies of the gene encoding a cuticle-degrading protease to increase the speed of kill, which at the end was 25% faster than the wild-type strains (St. Leger *et al*., 1996). Also, a single-chain antibody fragment that blocks transmission of malaria was expressed in a *Metarhizium* transformed strain (Fang *et al*., 2011). However, the use of such genetically engineered strains have been polemic because of their possible persistence in the environment (St. Leger, 2008; St Leger, Wang & Fang, 2011; Moonjely, Barelli & Bidochka, 2016).

Additionally, genetic modification of entomopathogenic fungi, *Metarhizium* spp., and *Beauveria* spp., with genes that encode for fluorescent proteins have been a useful tool to determine their persistence in soil, observe the type of interaction with plants (rhizospheric or non-rhizospheric), or to localize the fungal transformants inside plant tissues (St. Leger, 2008; Wyrebek *et al*., 2011; Sasan & Bidochka, 2012). For example, a green fluorescent protein (GFP) transformed strain of *M. anisopliae* was used to determine the fungal persistence in soils after application to cabbages, allowing the confirmation of rhizosphere competence of *M. anisopliae* (Hu & St. Leger, 2002). Wyrebeck *et al*. (2011) used also a *M. robertsii* strain expressing the GFP protein to demonstrate the close association of the fungus with the roots of *Phaseolus vulgaris* and *Panicum virgatum*. Sasan and Bidochka (2012) used a genetically modified *M. robertsii* strain expressing the GFP protein and z-stack images taken with a confocal microscopy to demonstrate the endophytic association of the *M. robertsii* and *P. vulgaris*.

The most common method for fungal transformation were based upon the isolation of protoplasts, which is time consuming, requires optimisation for each species, can lead to more than one nucleus per cell, and the transformation frequencies achieved are usually low with entomopathogenic fungi (dos Reis *et al*., 2004; Ying & Feng, 2006). For instance, a conventional method for *B. bassiana* transformation employing polyethylene glycol (protoplast-PEG method) rendered four transformants per µg plasmid DNA per 10^7 protoplasts, while using electroporation to transform *B. bassiana* protoplasts to benomyl resistance was obtained a transformation efficiency of 0.2 to 0.6 transformants per µg plasmid DNA per 10^7 protoplasts (Daboussi *et al*., 1989; Pfeifer & Khachatourians, 1992).

An alternative transformation method for the filamentous fungi was developed with *Agrobacterium tumefaciens* based on the bacterial ability during tumour induction (Ti) to transfers part of its Ti plasmid, T-DNA, to the plant genome (de Groot *et al*., 1998). The ease use, efficiency of transformation and the precision of T-DNA integration has led to widespread use of this microorganism for gene transfer to fungal protoplast of *Aspergillus niger*, *Fusarium venenatum*, *Tirchoderma reesei*, *Colletotrichum gloeosporioides*, *Neurospora crassa* and *Agaricus bisporus* (de Groot *et al*., 1998).

The *Agrobacterium tumefaciens*-mediated transformation methodology was later modified to transfer DNA to conidia instead of protoplasts (Fang *et al*., 2003; dos Reis *et al*., 2004). Conidia of *B. bassiana* were transformed to either hygromycin B or phosphinothricin resistance using the *hph* or *bar* genes, respectively. The efficiency of transformation obtained was higher than with protoplasts with up to 28 and 96 transformants per 10^4 and 10^5 target conidia, respectively. In certain conditions up to 350 transformants were obtained per 10^5 conidia (Fang *et al*., 2003). The stability of the transformants, between 80 to 100%, was demonstrated after five successive transfers on a nonselective medium, while putative transformants were analysed for the presence of the *hph* gene by PCR and Southern analysis (dos Reis *et al*., 2004). However, the *Agrobacterium*-mediated transformation needs 48 h co-cultivation of both fungal and bacterial strains and is not suitable for gene replacement (Ying & Feng, 2006).

A novel methodology based on the transformation of blastospores with PEG-LiAc and shock temperatures have been developed for the integration of the resistance gene to phosphinothricin and the *gfp* into *B. bassiana* (Ying & Feng, 2006). This method has been shown to be advantageous because the blastospores are more resistant to osmotic changes than protoplasts and can be easily stored in LiAc or glycerol at -80°C for sequential use. Blastospore transformation was efficient with 24 transformants per µg of DNA and the transformed blastospores were genetically stable after consecutive cultures in no selective medium (Ying & Feng, 2006).

5.1.1 Objective of this chapter

The aim of this study was the determination of the endophytism capability of entomopathogenic fungal isolates. The main goal was to confirm that seed coating with entomopathogenic fungi worked as a delivery system for either, association with roots or become endophytic in maize plant tissues. To achieve this goal, *Metarhizium* expressing fluorescent markers was required. Therefore, the main activities reported include the transformation of entomopathogenic fungal isolates to express a fluorescent protein, either GFP or mcherry, and then, maize seed coating with conidia from the transformants and observation with laser confocal microscopy.

5.2 Material and methods

5.2.1 Fungal cultures

The isolates selected for the transformation with the fluorescent proteins were the *Metarhizium anisopliae* A1080 and F672 (Table 2.1). Growth inhibition studies of different isolates of *Metarhizium* spp. was tested by inocula of conidia on PDA supplemented with different concentrations, i.e., 0, 2, 5, & 10 µg/mL of benomyl. Complete inhibition of conidia germination was observed in all the isolates at 5 µg/mL (minimal inhibitory concentration). Thus, this concentration was considered suitable for the selection of resistant colonies in transformation studies.

5.2.2 Preparation of the plasmid p-mcherry with resistance to β-tubulin (p-mcherryβTubr)

The β-tubulin gene (insert) which confers resistance to benomyl was cloned into the vector plasmid p-mcherry. The plasmid p-mcherry (4.9 Kb), contained the *mcherry* gene (position 4219 – 4926) flanked by the glyceraldehyde 3-phosphate dehydrogenase promoter (pgpdh) from *Trichoderma harzianum*, and the terminator transcription sequence (Tnos) also from *T. harzianum* also contained an ampicillin resistance gene (Figure 5.1-A). The linearization of the plasmid p-mcherry was attained by digestion with *Not*I (Figure 5.1-B). The β-tubulin gene in the plasmid pCR2.1-tubr was excised by digestion with *Not*I (Figure 5.1-A). A total of 0.5 µg of DNA from p-mcherry (419 ng/µL) and pCR2.1-βtub (168 ng/µL) were digested with 1 µL of *Not*I in a total volume of 25 µL. Reaction was carried out overnight at 37°C in agitation at 460 RPM. The products of reaction were identified and separated in gel electrophoresis in 0.5% agarose. The fragments, 4.9 Kb (p-mcherry) and 3.1 Kb (pCR2.1-βtub) were cut from the gel and purified with the NucleoSpin® purification Kit. Finally, the linearized and purified fragments were ligated. After this last step, two plasmids were possibly obtained (Figure 5.1-C). Products were visualized by gel electrophoresis in agarose 1% or purified by gel electrophoresis in agarose 0.5%. Correct assembly of the vector and insert in the plasmid, and insert orientation were also determined by enzyme digestion and gel electrophoresis in agarose 1%.

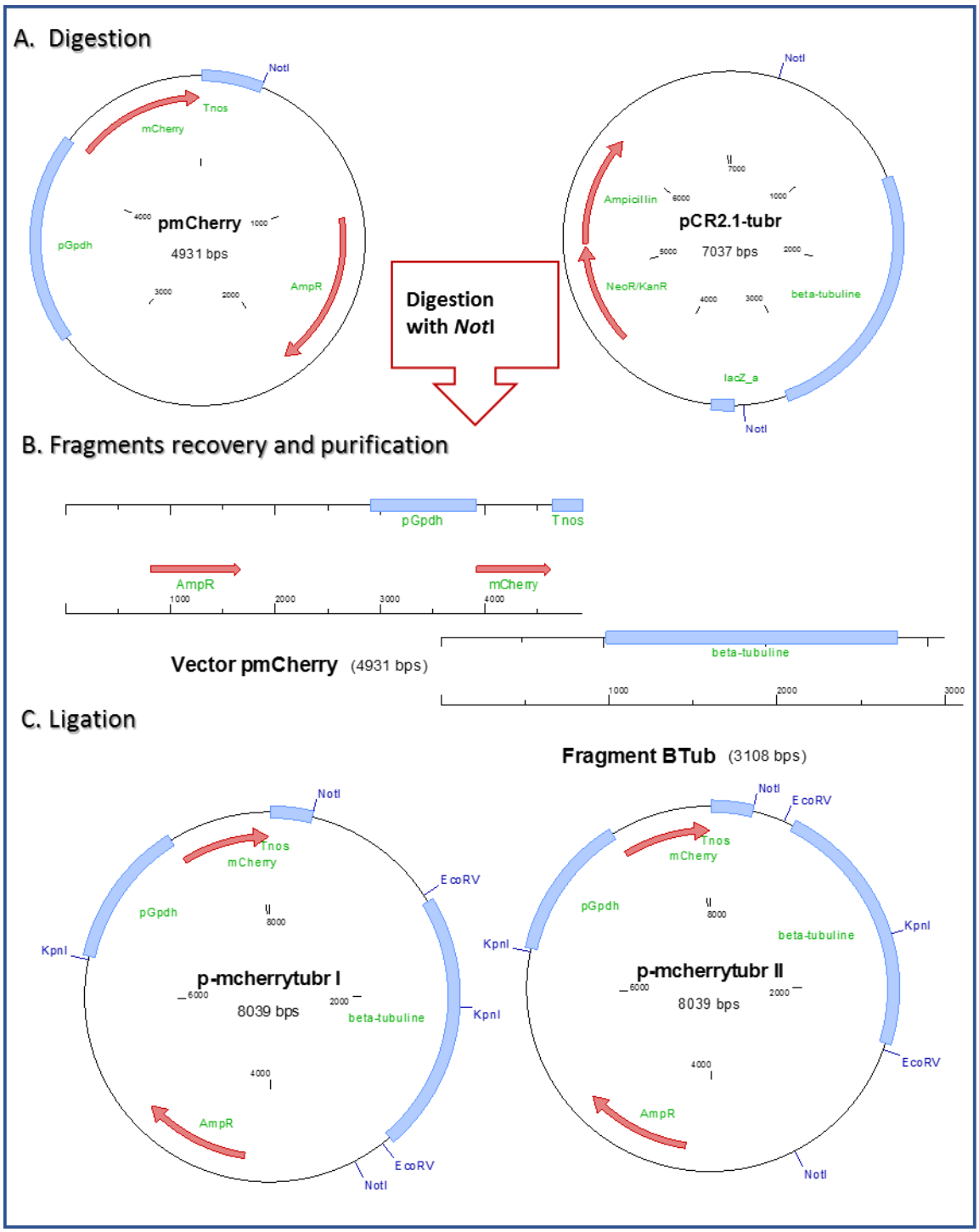

Figure 5.1 **Assembly of the p-mcherryβTubr plasmid.** A. The plasmid p-mcherry, contained the *mcherry* gene flanked by the pGpdh promoter and the Tnos terminator, and the selection marker *Amp*R with resistance to ampicillin. The plasmid pCR2.1-tubR contained the gene of interest with resistance to the fungicide benomyl. B. The digestion of both plasmids with enzyme *Not*I produced the linearization of the p-mcherry (vector), and released the β-tubulin gene (insert). C. Ligation of the vector and the insert rendered the final plasmid p-mcherryβTubr. The β-tubulin gene could be inserted in either direction and for this reason two ligation products were obtained.

5.2.3 Preparation of the plasmid triple GFP with resistance to β-tubulin (p-3xGFPβTubr)

The plasmid p-mcherry-hph was originally designed for the expression of the protein mcherry and with selection markers resistance to ampicillin and hygromycin. Following the strategy schematized in Figure 5.2, this plasmid was used as a vector, through addition of inserts of 3 x GFP and β -tubulin, by substitution of the mcherry and hygromycin resistance genes, respectively, becoming the plasmid p-3xGFPβTubr. The plasmid p-mcherry-hph (6.4 Kb) was digested with the enzymes *Asc*I and *Xho*I excising the fragment that contained the gene encoding the mcherry protein but keeping intact rest of the vector for the 3xGFP and β-tubulin genes (Figure 5.2-A). The inserted 3xGFP was obtained from the plasmid pUC57-3xGFP using a set of 5 restrictions enzymes: *Asc*I, *Xho*I, *Kpn*I, *Hind*III, and *Xmn*I. The insert β-tubulin gene (3108 bps) was obtained from the plasmid pCR2.1-tubr using the enzymes *Not*I and *Xmn*I (Figure 5.2-A). Restriction reaction were carried in a total reaction volume of 50 µL, with 5.0 µg of DNA of each plasmid, and 1 µL of restriction enzymes for the double restriction and 0.5 µL enzyme in the quintuple restriction. Reactions were conducted overnight at 37°C in agitation at 460 RPM. Fragments were identified and separated by gel electrophoresis in 0.5% agarose. The first intermediate plasmid was obtained by cloning (ligation I) the 3xGFP into the vector, rendering an intermediate plasmid p-3xGFP-hph (Figure 5.2-C). The last step was to substitute the sequence of the *hph* for that of the β-tubulin gene. The hph gene was eliminated with the use of the enzyme *Not*I (Figure 5.2-D) which cut the intermediate plasmid in two fragments, the vector named p-3xGFP (6.4 Kb) and a fragment with the hph gene (1.4 Kb). Restriction reaction were carried in a total reaction volume of 50 µL, with 5.0 µg of DNA of each plasmid, and 1 µL of each enzyme. Reactions were carried on overnight at 37°C in agitation at 460 RPM followed by a step at 80° for 30 min to inactivate the enzyme. Products of the restriction were visualized by gel electrophoresis in 0.5% agarose. The final step was to ligate the vector p-3xGFP (6.4 Kb) with the insert β-tubulin (3.1 Kb) to obtain the final plasmid p-3xGFPβTubr of 9.5 Kb (ligation II). Following this strategy two final plasmids were obtained (Figure 5.2-F). Restriction reactions were carried for 1.5 h at 37°C, products were purified by gel electrophoresis in 0.5% agarose. The band of interest was cut off from the gel and purified with the NucleoSpin® Gel and PCR Clean-Up Kit (Macherey-Nagel) purification Kit. After purification the vector and insert fragments were quantified by spectrophotometry and quality confirmed by gel electrophoresis in 1% agarose.

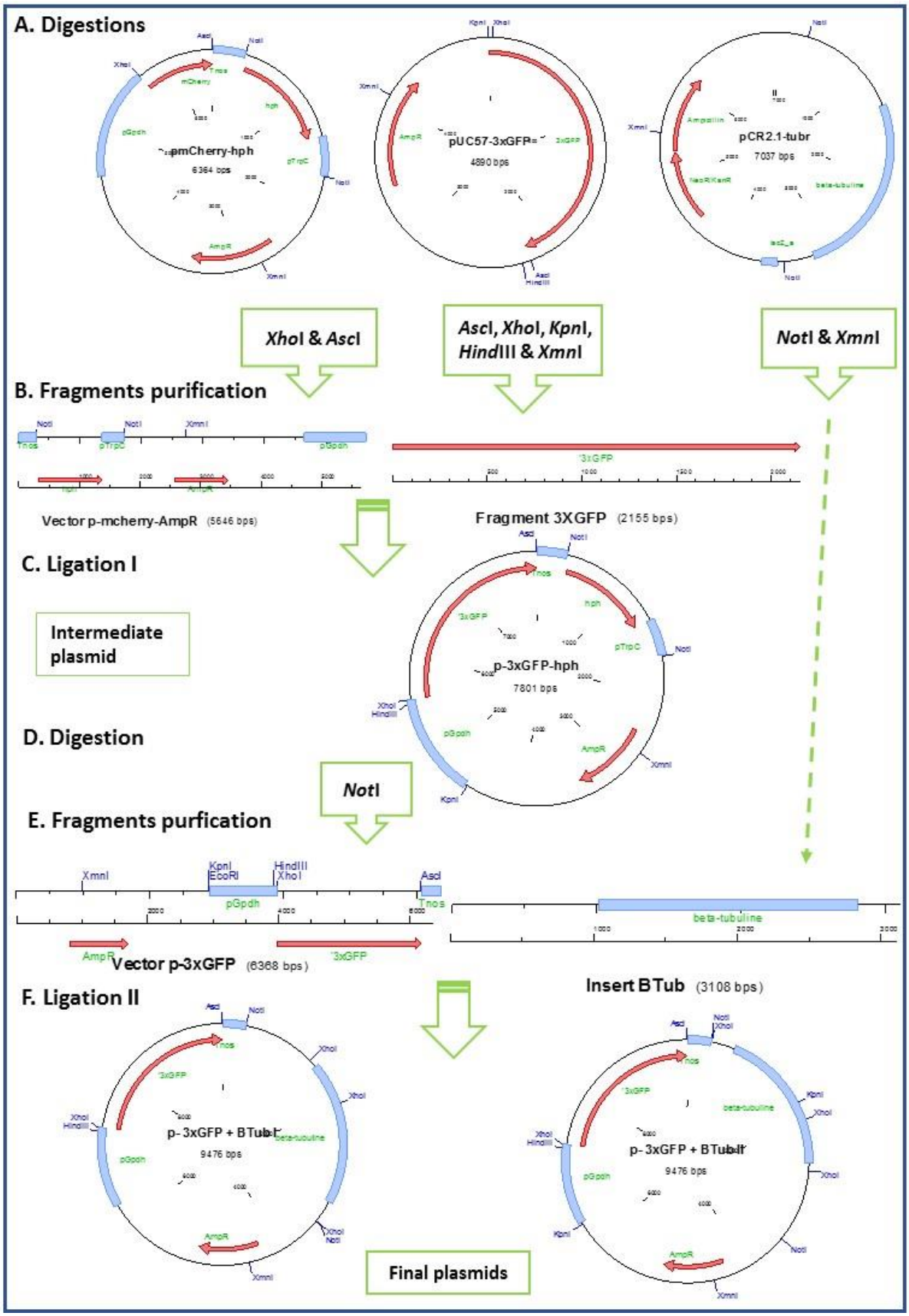

Figure 5.2 **Assembly of the plasmid p-3xGFPβTubr.** A. The mcherry sequence was excised from the p-mcherry-hph plasmid with the enzymes *Xho*I and *Asc*I. Using 5 different restriction enzymes the insert 3xGFP was obtained from the plasmid pUC57-3xGFP. The β-tubulin gene was obtained from the pCR2.1-βtub plasmid with *Not*I and *Xmn*I. The ligation of the vector and 3xGFP generated the intermediate plasmid p-3xGFP-hph (C). Through restriction of this plasmid with *Not*I (D) and ligation with β-tubulin (E) the plasmid p-3xGFPβTubr was obtained (F).

5.2.4 Plasmid assembly: ligation of vector and insert

Final plasmids were obtained after ligation vector and insert with the T4 DNA ligase. Corresponding ligation reactions were done using vector:insert ratios 1:1 and 1:3 in 10 and 20 µL respectively, using the recommended equation (Ec.1).

$$Insert\ (ng) = Vector\ (ng)\ \times \frac{Insert\ \#}{Vector\ \#} \times \frac{Insert\ length\ (bps)}{Vector\ Length\ (bps)} \quad (Ec.1)$$

where, # indicates the corresponding ratio vector:insert

Ligation was carried on in a thermocycler (SuperCycler Trinity, Kyratec) at 22°C for 2.5 hrs and reaction inactivated at 65°C for 30 min. After ligation, plasmid quantity was increased by incorporation to *E. coli* competent cells (bacterial transformation) and cultured in Luria-Bertani Broth (LBB).

5.2.5 *E. coli* competent cells TOP10 transformation

Fifty µL of TOP10 cells (1 x 10^6) were transferred to a 1.5 mL Eppendorf tube and 10 µL of the plasmid from the 1:1 vector: insert mix or 20 µL of 1:3 was added. The mixture was incubated for 30 min on ice, then 45 seconds at 42°C and again 2 min on ice. Subsequently 1 mL of LBB (room temperature) was added to the mixture of TOP10 cells and plasmid, and incubated at 37°C in agitation at 200 RPM for one hour. Finally, 50 and 100 µL from each ligation ratio (1:1 and 1:3) and controls were used to inoculate Luria-Bertani agar (LBA) plates containing ampicillin (100 µg/mL) per duplicate and incubated at 37°C overnight. Controls were TOP10 competent cells without added plasmid inoculated onto LBA plates with and without the antibiotic selection marker. Colonies of *E. coli* TOP10 competent cells grown overnight in LBA with ampicillin were transferred individually to a 24 plate well containing 100 µL of LBB with ampicillin (100 µg/mL) using a loop stick of approximately 1 µL. The plate was incubated at 37°C in agitation at 220 RPM for 4 h. After this period 2 µL of the culture broth was used for a PCR screening to confirm the transformation of the *E. coli* TOP10 competent cells with the corresponding plasmid.

5.2.5..1 E. coli competent cells TOP10 plasmid transformation assessment by PCR

E. coli competent cells TOP10 were screened for the presence of the corresponding final plasmids targeting the sequence of the β-tubulin resistance gene. The primers TnosF (5'-ACATGTAATGCATGACGTTAT) and M13F (5'- GTAAAACGACGGCCAGT) were used to check for the p-mcherryβTubr with an amplification product of approximately 3364 bps. The plasmid p-3xGFPβTubr was screened using the primers BTubF (5´- TCTCCTTCTCTGCCTTTCTC) and Ben-F (5´-CTCGAGATCGACGAGGACAG) with an amplicon of 633 bps. The intermediate plasmid p-3xGFP-hph was screened using GFPScF (5´- TTCCCATCCTGGTCGAATTG) and GFPScR (5´- AAGGCGCTCTGAGTAGAAAG) which targeted the GFP gene producing an amplicon of 586 bps. PCR amplifications were performed in a total volume of 25 µl, which included 5 µl of 5X PCR buffer (10mM Tris/HCl pH 8.0, 50 mM KCl, 1.5

– 2.0 mM $MgCl_2$, dNTP mix 10 mM each dATP, dCTP, dGTP, and dTTP), 0.75 µl of each primer (10 µM), 0.25 µl Taq polymerase (MyTaq™, Bioline, Roche) and 2 µL of LBB culture broth from *E. coli* TOP10 competent cells. PCR reaction volume was completed with ddH_2O. PCR amplifications were initiated with a 4 min initial denaturation at 95°C, followed by 35 cycles of amplification cycles which each consisted of 15 s denaturation at 95°C, a 30 s annealing step at 56°C and a final extension at 72°C for 180 s. After completion of the amplification cycles an additional final extension step for 120 s at 72°C was performed. PCR products were visualized by gel electrophoresis in agarose (1%) to confirm *E. coli* TOP10 competent cells that carried the plasmid.

5.2.5..2 Plasmid recovery from E. coli Top10 competent cells - Miniprep

E. coli Top10 competent cells transformant colonies confirmed by PCR were selected for miniprep. From the culture broth of the corresponding PCR positive transformant, 2 µL was transferred to 50 mL Falcon tubes, containing 10 mL of LBB with ampicillin and incubated overnight with agitation at 300 RPM at 37°C. Plasmid was recovered from culture broth using the Presto™ Mini Plasmid Kit (Geneaid) following the manufacturer's instructions. Minipreps used approximately 1.5 – 7.0 mL of the culture broth.

5.2.5..3 Plasmid assembly confirmation

Plasmid correct conformation and insert orientation was confirmed by enzymatic restriction and sequencing. Plasmid concentration was determined with a nano-drop spectrophotometer (NanoDrop 1000 Thermo-Fisher Scientific).

5.2.6 Preparation and harvest of blastospores for *Metarhizium* transformation

The blastospore preparation and harvest was developed following a procedure described by Ying & Feng (2006) with modifications. Flasks containing 100 mL of Sabouraud dextrose broth (SDB) were inoculated with a conidia suspension of *M. anisopliae* A1080 or F672 to a final conidia concentration of 1 x 10^7 conidia/mL per flask. Inoculated cultures were shaken by 300 RPM at 28°C and after 4 days a 5-mL aliquot of the SDB culture was transferred into 50 mL GM medium (w/v, 4% glucose, 0.4% NH_4NO_3, 0.3% KH_2PO_4, and 0.3% $MgSO_4$) and kept in the same incubation conditions for another 4 days. The resultant blastospores were collected by filtering through a single-layer of Myracloth in a 50 mL Falcon tube. The filtered blastospores were concentrated at 2400×g centrifugation at 4°C for 20 min and supernatant discarded. The harvested blastospores were washed twice with 800 µL of sterile dd-H_2O by 2400×g centrifugation at 4°C for 10 min and supernatant discarded. Harvested blastospores were resuspended in 0.5 mL 0.1 M LiAc and kept on ice for 30 min. Conidia concentration was determined by haemocytometer count and adjusted, if necessary, to 1 x 10^8 conidia/mL. Subsequently, 100 µL sterile glycerol was added to each tube, mixed thoroughly by shaking, and the

resultant suspension was standardized to 1×10^8 blastospores per millilitre, then stored in 50-µL aliquots at −80°C for later use.

5.2.6..1 Blastospore-based transformation

The following blastospore-based transformation system was developed using a blastospore protocol developed for *B. bassiana* (Ying & Feng 2006). One tube of the blastospore suspension was thawed on ice, followed by 4,720×g centrifugation at 4°C for 10 min. The supernatant was removed by pipetting out the glycerol-LiAc solution leaving a small white pellet of the precipitated blastospores. The following agents were added in order: 240 µL 50 % PEG 4000, 36 µL l M LiAc, 25 µL denatured salmon sperm DNA (4 mg/mL), 10 - 20 µL linearized-plasmid (0.1 µg μL^{-1}), and 35 µL 1 M dithiothreitol. Suspension was mixed after each reagent was added by inversion and gently tapping tube with a finger. After thorough mixing, the suspension was incubated on ice for 45 min and then subjected to heat shock at 42°C for 20 min. The blastospores were subsequently collected by 4,720×g centrifugation at 4°C for 10 min and resuspended in 0.5 mL sterile dd-H_2O. Aliquots of 100 µL suspension were plated on Czapek's medium containing benomyl (5 µg/mL) and incubated at 25°C. Positive and negative controls were non-transformed blastospores where PEG 4000 50% was used instead plasmid and plated onto Czapek´s with and without benomyl. After 4-6 days of incubation colonies were visible. Emerging transformant colonies were transferred to fresh Czapek´s agar medium containing 5 µg/mL of benomyl and then sub-cultured three consecutive rounds in non-selective Czapek´s agar, followed by a final round on selective Czapek´s agar but with 10 µg/mL of benomyl. The blastospore concentration for the transformation with the m-cheery protein was 1 x 10^8 blastospores/mL, while for the GFP protein were used $1x10^6$ blastospores/mL. Blastospore concentration was determined using a Neubauer chamber.

5.2.7 Plasmid PCR amplification

The sequence of the p-mcherryβTubr was obtained using two pair of primers that amplified the plasmid in two regions named, Mc1 (6.2 Kb) and Mc2 (3.7 Kb). The region, Mc1, was amplified with the primers BtubF (5´- TCTCCTTCTCTGCCTTTCTC) and 5730pmChR (5´- CGCTGCCTTCCATGTGAAC), while the Mc2 region with M13R (5´- GGAAACAGCTATGACCATG) and BenF (5´- CTCGAGATCGACGAGGACAG). The sequence of the p-3xGFPβTubr was obtained also with using primers which divided the plasmid in GFP1 (5.1 Kb) and GFP2 (6.1 Kb). Part of the region GFP1 was amplified with the pair M13R/BenF, and the GFP2 with the BenR (5´- CTGTCCTCGTCGATCTCGAG) and GFPScR (5´- AAGGCGCTCTGAGTAGAAAG). PCR amplification were performed in a total volume of 50 µl, which included 10 µl of 5X PCR buffer (10mM Tris/HCl pH 8.0, 50 mM KCl, 1.5 – 2.0 mM $MgCl_2$), 1 µl of dNTP mix (10 mM each dATP, dCTP, dGTP, and dTTP), 2.5 µl each of the opposing amplification primers (10 µM), 0.5 µl Taq polymerase (Q5 Hot Start High Fidelity, New England BioLabs) and 1 pg - 1 ng of the plasmid DNA. PCR for all primers combination PCR amplifications were initiated with a 1 min

initial denaturation at 98°C, followed by 40 cycles of amplification cycles which each consisted of 15 s denaturation at 98°C, a 35 s at the annealing temperature appropriated for each pair plasmids (see below) and a final extension at 72°C for 240 s. After completion of the amplification cycles an additional final extension step for 240 s at 72°C was performed. The annealing temperatures for the p-mcherryβTubr plasmid were 53°C for the primers BTubF/5730pmCh and 52°C for the M13R/BenF pair. The annealing temperatures for the p-3xGFPβTubr were 60°C for the pair M13/BenR and 63°C for the BenR/GFPScR primers. PCR products were visualized by gel electrophoresis in agarose (1%) to confirm the presence of the genes in the fungal DNA.

5.2.8 PCR product purification and sequencing

Amplicons were purified using HighPrep™ PCR purification kit based on paramagnetic beads technology. Sequencing of the DNA was performed using an ABI Prism 3130xl Genetic Analyser with a 16 capillary 50 cm array installed and using Performance Optimized Polymer 7.

Table 5.1 Primers using for sequencing of plasmids p-mcherryβTubr and p-3xGFPβTubr.

Region	Forward	Sequence 5´- 3´	Reverse	Sequence 5´- 3´	Amplicon (bps)
Mc1 a	BenR	CTGTCCTCGTCGATCTCGAG	M13-F	GTAAAACGACGGCCAGT	1650
Mc1 b	BenR	CTGTCCTCGTCGATCTCGAG	5730pmChR*	CGCTGCCTTCCATGTGAAC	5565
Mc1 c	M13R	GGAAACAGCTATGACCATG	5730pmChR	CGCTGCCTTCCATGTGAAC	1181
Mc2 a	M13R*	GGAAACAGCTATGACCATG	5730pmChR	CGCTGCCTTCCATGTGAAC	1181
Mc2 b	M13R	GGAAACAGCTATGACCATG	Top10ScR	TTTGAATGGAGGCGACGC	3131
Mc2 c	6217pmCh F	AGAAGCCCGTCCAACTTCC	Top10ScR	TTTGAATGGAGGCGACGC	1464
Mc2 d	Top10ScF	CAGTGACATGCCTTTGCCG	BenF	CTCGAGATCGACGAGGACAG	1109
GFP1 a	M13R	GGAAACAGCTATGACCATG	GFPScR	AAGGCGCTCTGAGTAGAAAG	1725
GFP1 b	GFPScF	TTCCCATCCTGGTCGAATTG	Top10ScR	TTTGAATGGAGGCGACGC	3429
GFP1 c	Top10ScF	CAGTGACATGCCTTTGCCG	BenF	CTCGAGATCGACGAGGACAG	1109
GFP2 a	BenR	CTGTCCTCGTCGATCTCGAG	M13F*	GTAAAACGACGGCCAGT	1650
GFP2 b	BenR	CTGTCCTCGTCGATCTCGAG	GFPScR	AAGGCGCTCTGAGTAGAAAG	6109
GFP2 c	M13R*	GGAAACAGCTATGACCATG	GFPScR	AAGGCGCTCTGAGTAGAAAG	1725

***; indicates that no sequence was obtained with the respective primer.**

5.2.9 Fungal transformants molecular characterization

5.2.9..1 Fungal biomass for DNA extraction

Fungal transformant colonies were grown for 10 days in Czapek´s agar with benomyl (5 µg/µL) at 25°C, light:dark conditions (12:12 h). After colonies sporulated, a conidia suspension in agar (0.2%) was prepared and 10 µL of the suspension was used to inoculate a 24-plate well with each well containing 1.5 ml of malt extract broth (MEB) with benomyl 5 (µg/ml). Inoculated plates with MEB+ben were incubated at 22°C at 220 RPM. After 5 days, biomass was harvested by filtration through two layers of Myracloth and washed with sterile distilled water. Fresh biomass weight was determined.

5.2.9..2 Fungal DNA extraction

Genomic DNA was extracted from grinded frozen mycelia with liquid N_2, and using the Gentra Puregene Tissue Kit (Qiagen) according to the manufacturer's instructions and recommendations.

5.2.9..3 PCR screening

The *Metarhizium* transformants were screened by PCR to determine the presence of the corresponding genes, *mcherry* or *gfp*. The primers 6217pmChF and BenF were used for the amplification of 2008 bps from the p-mcherryβTubr plasmid. The primers GFPScF and GFPScR were used for the amplification of 586 bp from the p-3xGFPβTubr plasmid. PCR amplifications were performed in 25 µl, which included 5 µl of 5X PCR buffer (10mM Tris/HCl pH 8.0, 50 mM KCl, 1.5 – 2.0 mM $MgCl_2$), 0.5 µL of dNTP mix 10 mM each (dATP, dCTP, dGTP, and dTTP), 0.5 µl each primer (10 µM), 0.25 µl Taq polymerase (Q5 Hot Start High Fidelity, New England BioLabs) and 2 µL of genomic DNA (1 ng – 1 µg). PCR reaction volume was completed with ddH_2O. PCR amplifications were performed with a 1 min initial denaturation at 98°C, followed by 40 cycles of amplification cycles which each consisted of 18 s denaturation at 98 °C, a 35 s annealing step determined for each gene (see below) and a final extension at 72 °C for 120 s. After completion of the amplification cycles an additional final extension step for 420 s at 72°C was performed. The annealing temperature used for the mcherry sequence was 61°C while for the GFP sequence was 58°C. PCR products were visualized by gel electrophoresis in agarose (1%) to confirm the presence of the genes in the genomic fungal DNA.

5.2.10 Determination of fungal endophytism by confocal fluorescent microscopy

Samples of roots, stems and leaves of maize plants from coated-seeds with the fungal transformants expressing GFP or mcherry, were analysed for the presence of fungal structures on the organ´s plant surfaces or internally as endophytes using confocal microscopy (LSM 510 META - Zeiss, Germany). Additionally, the hyphal adhesion zone to the plant cell wall was visualized with the carbohydrate binding lectin concanavalin-A conjugated with Alexa Fluor 633 (ConA-AF633, Molecular Probes, Karls-ruhe, Germany). ConA selectively binds to α-mannopyranosyl and α-glucopyranosyl residues found in glycoproteins and glycolipids (Zuccaro *et al*., 2011). Plant cells were visualized with propidium iodide as a counter stain (Deshmukh *et al*., 2006).

5.2.10..1 Maize plant inoculation

Maize seeds were coated as described in section (3.5.3) but using a 1 x 10^7 conidia/mL conidial suspension of transformant fungi. After coating, maize seeds were sown on pots of 1 L of capacity containing 600 g of vermiculite (fine grade 2). Pots were watered with 400 mL of tap water, and transferred to a growth chamber at 25°C with light:dark conditions (12:12 h). Plants were grown for two weeks and were watered every five days with 400 mL of distilled water. Controls included coated seeds without fungi.

5.2.10..2 Maize sample preparation

Roots, stems and leaf sheaths were cleared with a solution of KOH (10%) and incubated at 96°C for 3 hours. After incubation, KOH was discarded and samples washed once in 1x phosphate-buffered saline (PBS; pH 7.4).

5.2.10..3 Staining of vegetal tissues

Colonized roots, shoots or leaves were stained by infiltration with CoA-AF633 to visualize the adhesion zone between the hyphae and the plant, and propidium iodide as counter stain for plant cells. Samples were incubated at room temperature for 30 min in staining solution containing 10 µg/ml ConA-AF633 and 20 µg/ml propidium Iodide and 0.1% Triton X-100 in 1x PBS (pH 7.4). During incubation, segments were vacuum-infiltrated three times for 2 min at 25 mmHg. Finally, plant samples were then washed in the PBS buffer for 3 hrs and then in fresh buffer overnight. Samples were stored in the dark at 4°C until analysis.

5.2.10..4 Confocal laser scanning microscope image acquisition

For microscopic observation, vegetal segments of roots, stems and leaves were mounted on glass slides. The visualization of the different fluorophores and dyes in hyphae and plant cells used an excitation of 488 nm laser line for the GFP protein and detection at 500–540 nm. Mcherry protein was exited at 588 nm laser line and detected at 610 nm, ConA-AF633 was exited at 633 nm laser line and detected at 650–690 nm, while propidium iodide was excited at 561 nm laser line and detected at 580–660 nm. Confocal fluorescence images were recorded on a multichannel confocal microscope (LSM 510 META - Zeiss, Germany) using the program ZEN 2009.

5.3 Results

5.3.1 Preparation of the plasmid p-mcherry with resistance to β-tubulin (p-mcherryβTubr)

After restriction of the p-mcherry (4.9 Kb) with *Not*I the vector p-mcherry-AmpR (4.9 Kb) and the insert β-tubulin resistant gene (3.1 Kb) were cut from gels and cleaned up for ligation (Figure 5.3).

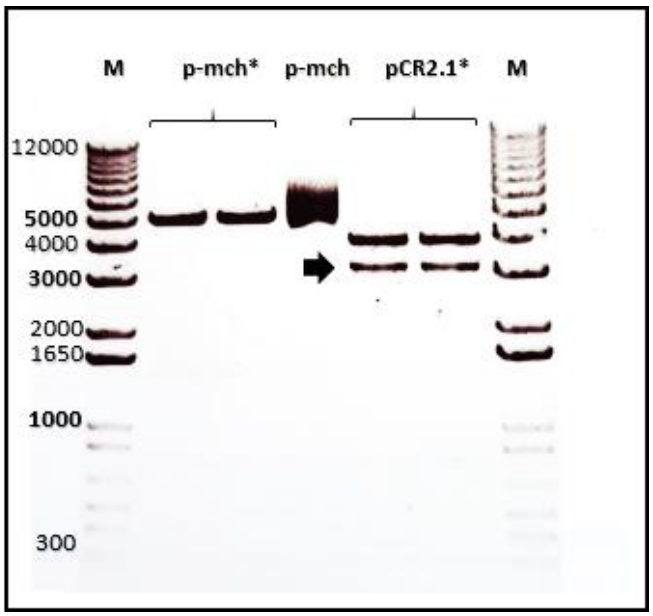

Figure 5.3 Enzymatic digestion of plasmids p-mcherry and pCR2.1-βtub with the enzyme *Not*I. The fragments, 4.9 Kb (p-mcherry) and 3.1 Kb (pCR2.1-βtub) were cut from the gel and recovered for ligation. M, molecular marker; p-mch*, product of digestion p-mcherry with *Not*I; p-mch, p-mcherry without enzyme; pCR2.1*, plasmid pCR2.1-βtub digested with *Not*I. Arrow, the fragment of interest from pCR2.1-βtub.

The final concentration obtained of both fragments after the purification step was approximately of 10 ng/µL (Figure 5.4), determined with the High mass molecular marker (High DNA Mass Ladder, Invitrogen).

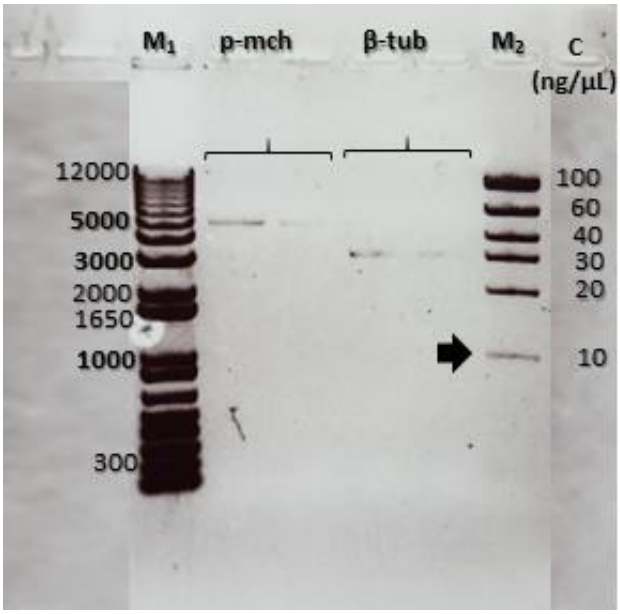

Figure 5.4 Determination of purity and concentration of vector p-mcherry-AmpR and insert β-tub. The fragments obtained from digestion with *Not*I of 4.9 Kb (from p-mcherry) and 3.1 Kb (from pCR2.1-βtub) were purified with the NucleoSpin® purification Kit. After gel band recovery, purity and concentration of vector p-mcherry-AmpR and insert B-tub, were determined by gel electrophoresis. M_1, molecular marker 1 Kb Plus DNA Ladder; p-mch, vector p-mcherry-AmpR; β-tub, insert β-tubulin; M_2, molecular marker High DNA Mass Ladder; C, equivalent concentration of intensity of fragments in ng/µL. Arrow indicates the fragment concentration in comparison with the molecular marker.

The products of ligation from both ratios (ratio 1:1 and ration 1:3) were used for the transformation of *E. coli* competent cells TOP10. In total were obtained 18 colonies, 7 from ratio 1:1 and 11 from ratio 1:3. A total of 10 *E. coli* transformant colonies were selected for screening of the plasmid of interest by PCR with the pair of primers BtubF/BtubR. The expected amplicon of 3.4 Kb was confirmed only in the colonies identified as 6 and 9 (Figure 5.5).

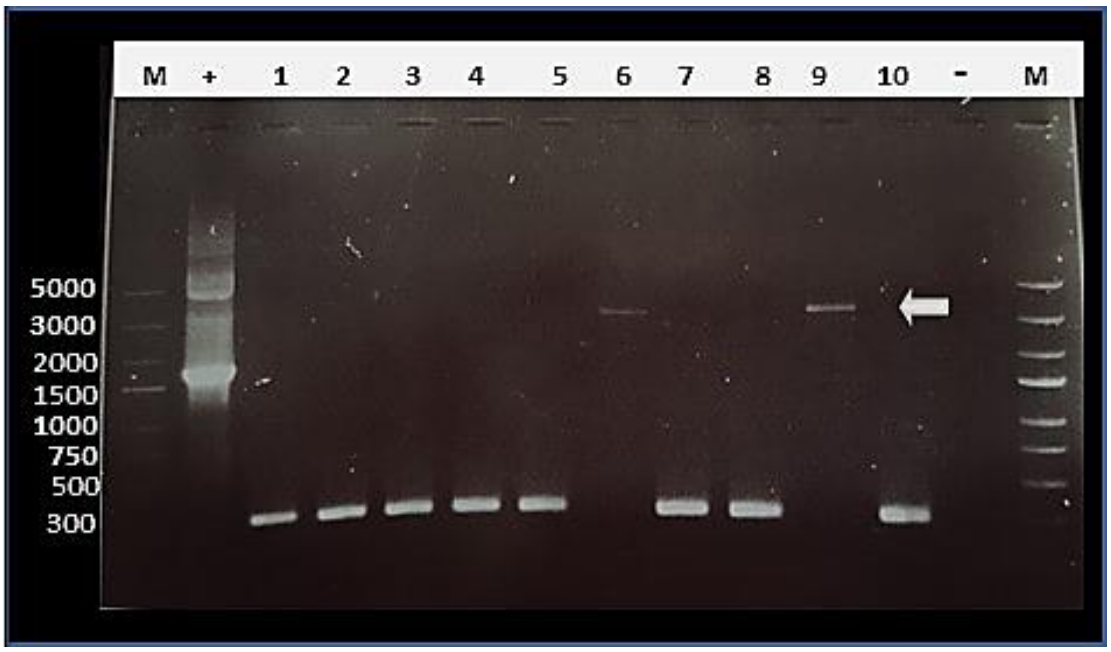

Figure 5.5 Screening for the presence of the plasmid p-mcherryβTubr in *E. coli* TOP10 cells. The products of ligation from both ratios were used for the transformation of *E. coli* competent cells TOP10. A total of 10 *E. coli* transformant colonies were selected for screening of the plasmid of interest by PCR with the pair of primers BtubF/BtubR. The expected amplicon of 3.4 Kb was confirmed only in the colonies identified as 6 and 9 (arrow). M, molecular marker (O´Gene Ruler Express DNA Ladder, Thermo Scientific), +; p-mcherry; 1-10, transformants from *E. coli* TOP10; -; negative control.

Plasmid concentrations after recovered and purified from colonies 6 and 9 were, 338.9 and 312.5 ng/µL, respectively. According to the possible orientation of the insert into the vector two different plasmids of 8.0 Kb each were possible to be obtained, p-mcherrytubr I and p-mcherrytubr II (Figure 5.1). Determination of the final p-mcherryβTubr (8.0 Kb) plasmid assembly was done by restriction with the enzymes *EcoR*V and *Kpn*I in two different reactions. The plasmid was confirmed only in colony 9 assembled as p-mcherryβtubr I type (Figure 5.6).

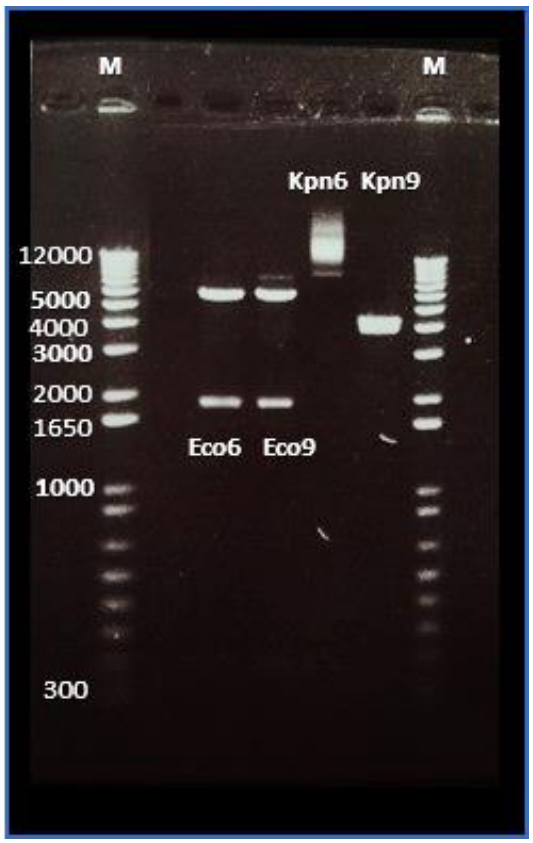

Figure 5.6 Determination of the final p-mcherryβTubr (8.0 Kb) plasmid assembly by restriction with the enzymes *EcoR*V and *Kpn*I. The expected products of this reactions were two fragments of 1.9 and 6.1 Kb with *EcoR*V from both plasmids, p-mcherryβtubr I and p-mcherryβtubr II. The restriction of plasmid p-mcherryβtubr I with *Kpn*I would result in two fragments of 3.8 and 4.2 Kb, while the restriction of plasmid p-mcherrytubr II with the same enzyme, two fragments of 3.4 and 4.7 Kb. The correct orientation was only confirmed in colony 9 assembled as p-mcherryβtubr I type. M, molecular marker (1 Kb Plus DNA Ladder, Life Technologies); Eco, *EcoR*V; Kpn, *Kpn*I; 6 and 9, refers to the corresponding *E. coli* TOP10 colonies 6 and 9, respectively.

5.3.2 Preparation of the plasmid triple GFP with resistance to β-tubulin (p-3xGFPβTubr)

The first stage was to obtain the intermediate plasmid vector p- 3xGFP-hph (7.8 Kb). The restriction of the p-mcherry-hph plasmid (0.554 ng/µL) with enzymes *Xho*I and *Asc*I produced two fragments, the fragment of interest of 5.7 Kb and the exscinded mcherry gene of 718 bps (Figure 5.7)

Theoretically, only the enzymes *Asc*I and *Xho*I would have been enough to exscind the insert 3xGFP from the remaining plasmid pUC57 and then, separate both fragments in an agarose gel (0.5%). However, in practice it was found that both fragments tended to re-ligate because of cross contamination between both given the closer size of the fragments, 2155 and 2735 bps (Figure 5.7).

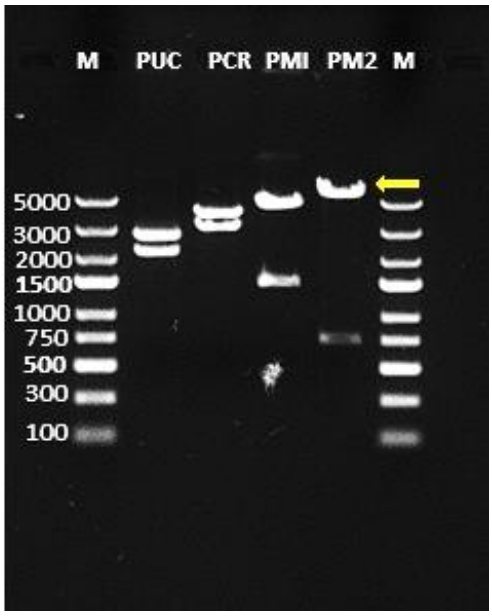

Figure 5.7 Digestion of plasmid p-mcherry-hph (6.4 Kb). The plasmid was digested with the enzymes *Xho*I and *Asc*I. The expected products of this reactions were the vector-hph (5.7 Kb) and the cherry gene (0.7 Kb). The arrow indicates the fragment of interest from the p-mcherry-hph. This gel corresponds also to a first attempt to prepare the plasmids pUC57-3xGFP and pCR2.1-βtub for ligation with *Not*I. However, the fragments obtained were re-ligated later in the original plasmid and it was necessary to use additional enzymes. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); PUC, pUC57-3xGFP; PCR, pCR2.1-βtub; PMI, p-mcherry-hph; PM2, p-mcherry-hph.

Using a pool of 5 enzymes, the non-interest fragments were reduced to smaller sizes which allowed for the separation from the 3xGFP fragment. The restriction of pUC57-3xGFP plasmid (508.2 ng/µL) produced the insert of 2.2 Kb with *Asc*I and *Xho*I, a fragment of 800 bps with *Xmn*I, two fragments of 1851 and 58 bps with *Hind*III, and a small fragment of 26 bps with *Kpn*I (Figure 5.8).

The final concentration obtained after gel band clean-up was 25.33 ng/µL for the p-mcherry-AmpR and 28.13 ng/µL for the 3xGFP fragments. These fragments were respectively the vector and insert for ligation I. The product of this reaction was the intermediate plasmid p-3xGFP-hph (7.8 Kb).

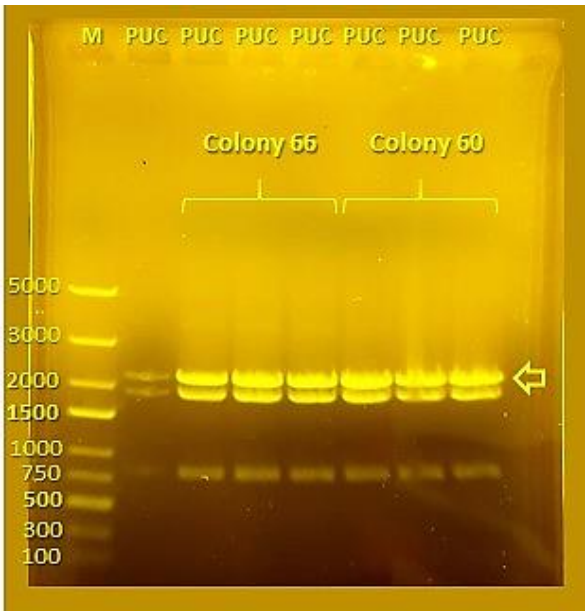

Figure 5.8 **Digestion of plasmid pUC57-3xGFP (4.9 Kb).** The plasmid (508.2 ng/µL) was digested with *Asc*I, *Xho*I, *Xmn*I, *Hind*III and *Kpn*I. Product of the digestion was obtained the insert of 2.2 Kb (arrow) and also other fragments of 1851 Kb, 0.8 Kb, 58 bps and 26 bps. The plasmid pUC57-3xGFP was obtained from two different back up colonies 60 and 66. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); PUC, pUC57-3xGFP.

After fragment ligation and transformation of *E. coli* competent cells, 26 colonies were obtained, 5 from ratio 1:1 and 21 from ratio 1:3. A total of 24 *E. coli* transformant colonies were screened through PCR, allowing to confirm the plasmid in at least 18 bacterial colonies (Figure 5.9).

The colonies identified as 7, 10, 21, 22, 23, 24 and 25 were selected for miniprep or plasmid recovery. The proper assembly of the plasmid was confirmed using the enzymes *Asc*I and *Xho*I which products of restriction represents the original fragments used for the ligation: the vector p-mcherry-hph (5.7 Kb) and the insert 3xGFP (2.2 Kb). Using this strategy, the correct plasmid was identified in *E. coli* colonies 7, 10, 21, 22 and 23 (Figure 5.10). The next stage was to replace in the later intermediate plasmid, p-3xGFP-hph (7.8 Kb), the gene for resistance to hygromycin for the gene of resistance to benomyl as selection marker for *Metarhizium* fungal transformants.

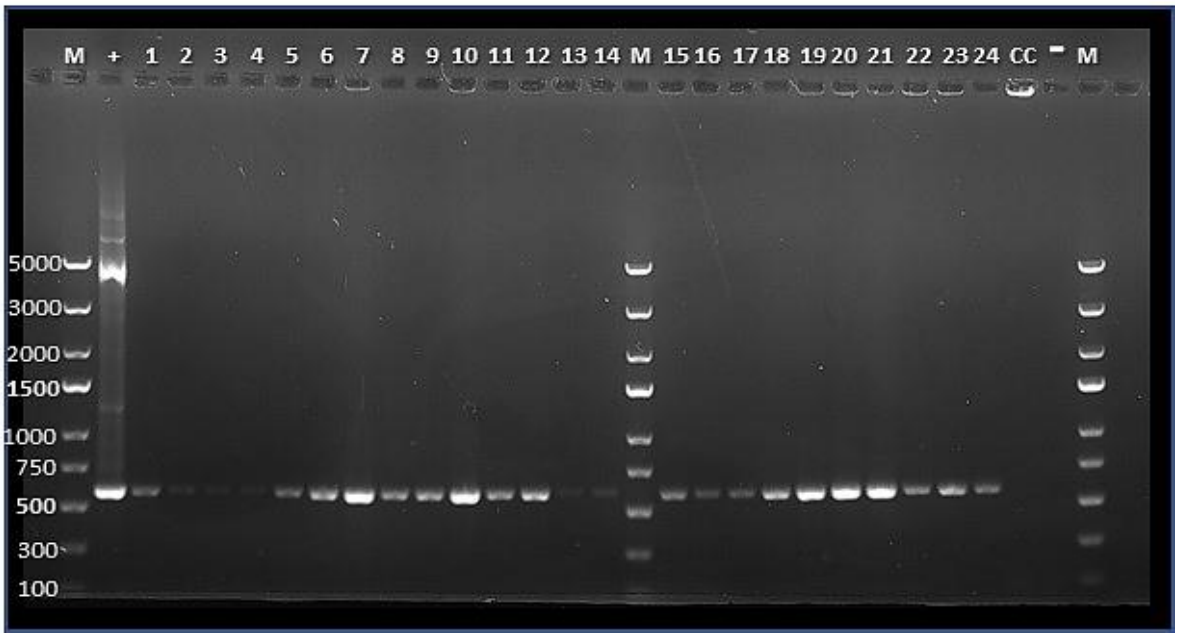

Figure 5.9 **Screening in *E. coli* TOP10 cells for plasmid p-3xGFP-hph.** The intermediate plasmid obtained in ligation I was screened through PCR in 24 *E. coli* transformant colonies using the pair of primers GFPScF and GFPScR. This pair of primers targeted a fragment of 586 bps from the GFP gene. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); +, pUC57-3xGFP; 1 – 24, *E. coli* TOP10 transformants; CC, *E. coli* TOP10 competent cells; -, negative control.

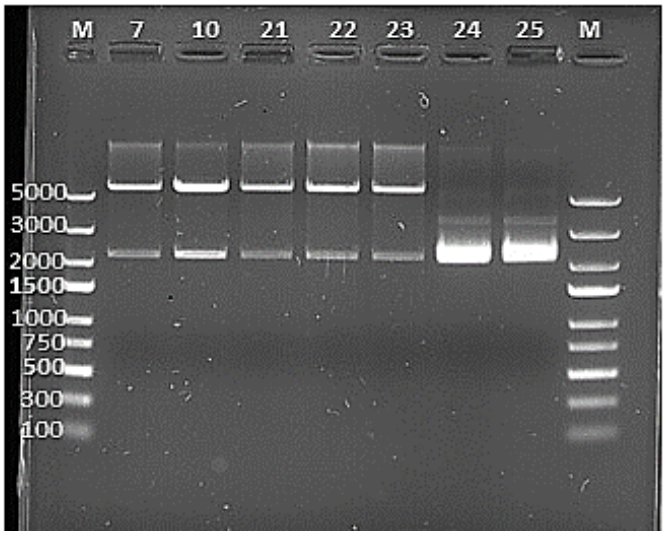

Figure 5.10 **Restriction digestion of putative intermediate plasmid p-3xGFP-hph.** The assembly of the plasmid was confirmed using the enzymes *Asc*I and *Xho*I with expected products of 5.7 Kb and2.2 Kb. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); *E. coli* TOP10 transformants; 7, 10, 21, 22, 23 24, 25.

Restriction of the plasmid pCR2.1-βtubr (0.187 µg/µL) with the enzymes *Not*I and *Xmn*I released the β-tubulin gene. This fragment would have been also obtainable using only the enzyme *Not*I, but both fragments were of similar size: 3.1 and 3.9 Kb (Figure 5.7). The use of an additional enzyme, *Xmn*I, allowed further separation of the β-tubulin of 3.1 Kb, from the two other fragments of 1.9 and 2.0 Kb (Figure 5.11).

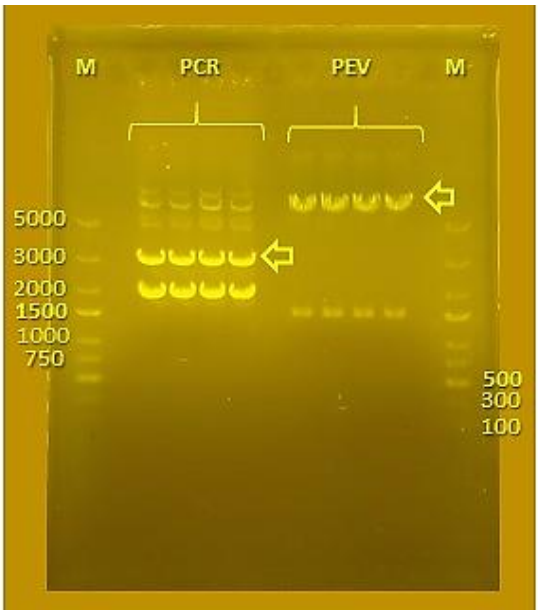

Figure 5.11 Fragment purification after enzymatic restriction of the plasmid pCR2.1-βtubr (PCR). The enzymes *Not*I and *Xmn*I released the β-tubulin gene (arrow). The intermediate plasmid p-3xGFP-hph (PEV) obtained from the E. coli TOP10 transformant colony 10 was restricted also with the enzyme *Not*I to linearize the plasmid for the next ligation and also to eliminate the hph gene sequence.

The intermediate plasmid p-3xGFP-hph (0.364 µg/µL) obtained from the *E. coli* TOP10 transformant colony 10 was restricted also with the enzyme *Not*I to linearize the plasmid for the next ligation and also substitution of the hph gene by the β-tubulin gene (Figure 5.11). The final concentration obtained after gel band clean-up were 127.4 ng/µL for the fragment p-3xGFP-AmpR (6.4 Kb) and 50.4 ng/µL for the β-tubulin fragment (3.1 Kb), these would be respectively the vector and insert in the next ligation step (ligation II). The transformation of *E. coli* competent cells with the product of the ligation rendered only 10 colonies, 6 from ratio 1:1, and 4 from ratio 1:3. The screening of the colonies though PCR was confirmed in all the colonies (Figure 5.12).

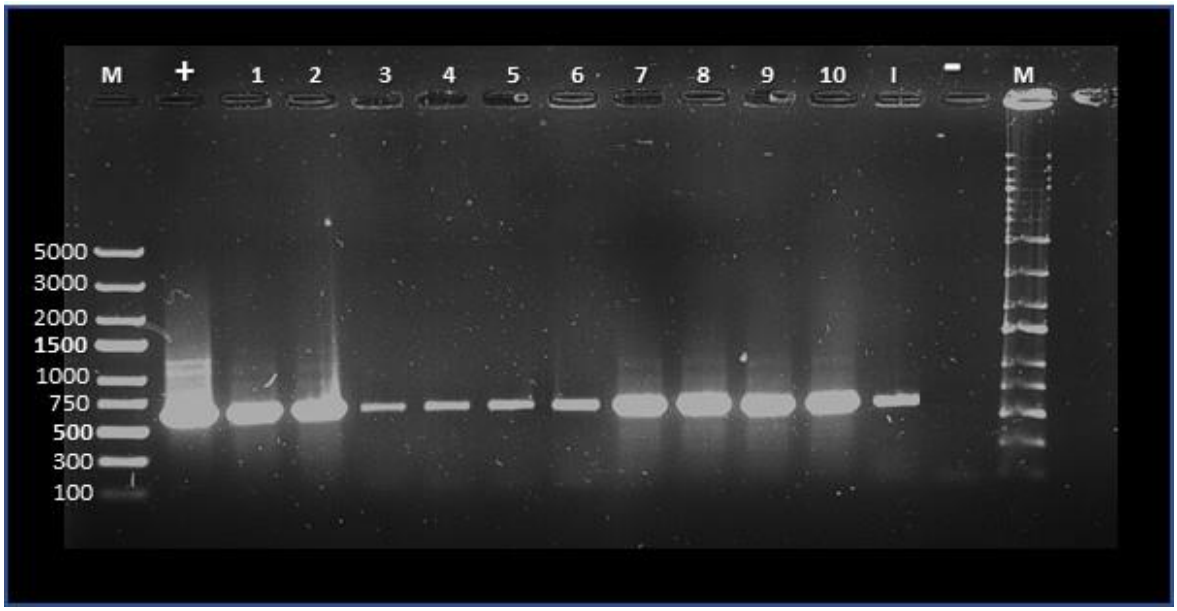

Figure 5.12 Screening in *E. coli* TOP10 cells for the β-tubulin gene. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); +, plasmid pCR2.1-βtub; 1 – 10, E. coli TOP10 transformants; I, insert Btub; **-,** negative control.

Confirmation of the correct assembly of the fragments into the final p-3xGFPβTubr (9.5 Kb) plasmid was done by restriction with the enzyme *Not*I. The expected products of this reaction corresponded to the original intermediate vector (6.4 Kb) and the insert (3.1 Kb). Only the colonies 1, 2, 9 and 10 had the expected restriction pattern, colonies 7 and 8 had the same pattern as the original plasmid pCR2.1-βtub while colonies 3 – 6 had a small fragment of 2 Kb (Figure 5.13-A). A second restriction reaction with the enzyme *Xmn*I was used on colonies 1, 2, 5, 7, 9 and 10 in order to confirm the proper size of the plasmid p-3xGFPβTubr. This reaction allowed the identification of the plasmid in colonies 1, 2 and 9 (Figure 5.13-B). The *E. coli* transformant identified with the number 9 was selected for plasmid extraction which was used for the fungal transformation of *M. anisopliae*.

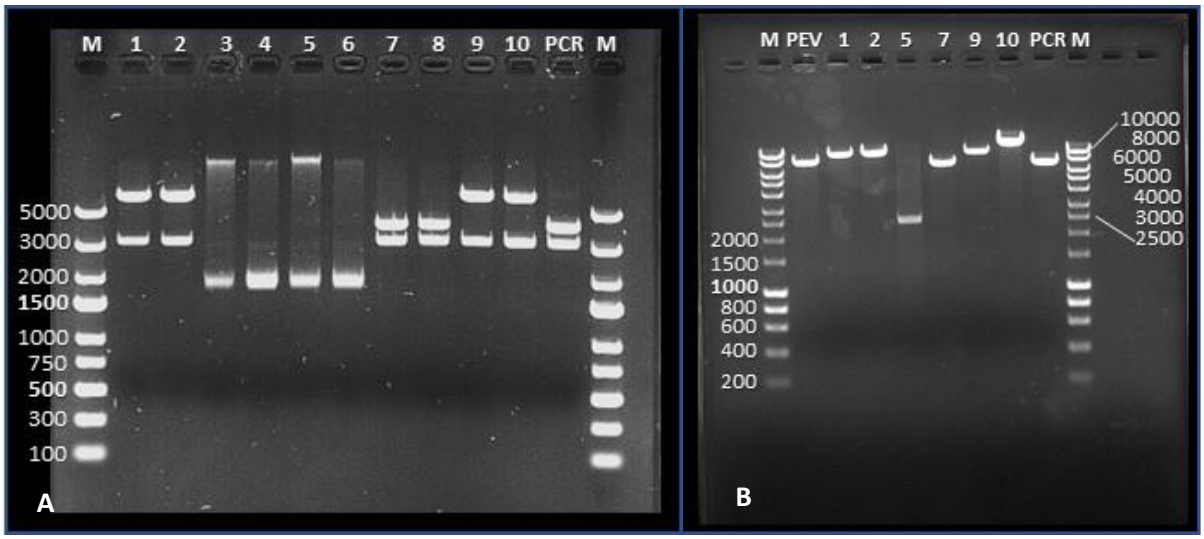

Figure 5.13 Digestion of the plasmid p-3xGFPβTubr. The products of restriction with *Not*I represents the original fragments used for the ligation: p-3xGFP-AmpR (6.4 Kb) and β-tubulin insert (3.1 Kb). Using this strategy, the plasmid properly assembled was identified in *E. coli* TOP10 colonies 1, 2, 9, and 10 (A). A second Restriction with *Xmn*I confirmed that p-3xGFPβTubr was present in colonies 1, 2 and 9, while colony 10 probably have more than one copy of the insert (B). M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); *E. coli* TOP10 transformants; 1 – 10; PCR, plasmid pCR2.1-βtub (7.0 Kb); PEV, plasmid p-3xGFP-AmpR (6.4 Kb).

After culturing the bacterial isolate 9 by triplicate (A, B and C) in 5 mL of LBB with ampicillin the plasmid was recovered. Final concentration of the plasmid from using the 5 mL of each culture were 636.4 ng/µL (9A), 606.6 ng/µL (9B) and 609.1 ng/µL (9C). Plasmid were linearized with the enzyme *Xmn*I for the fungal transformation of *M. anisopliae* A1080 and F672 isolates.

5.3.3 Plasmid sequence aligment

In Figure 5.14, a graphic representation of the alignment of the sequences from the regions Mc1 and Mc2 with the p-mcherryβTubr plasmid is shown. The alignment of the sequences with the original plasmid, determined that the p-mcherryβTubr was correctly assembled during the different cloning stages.

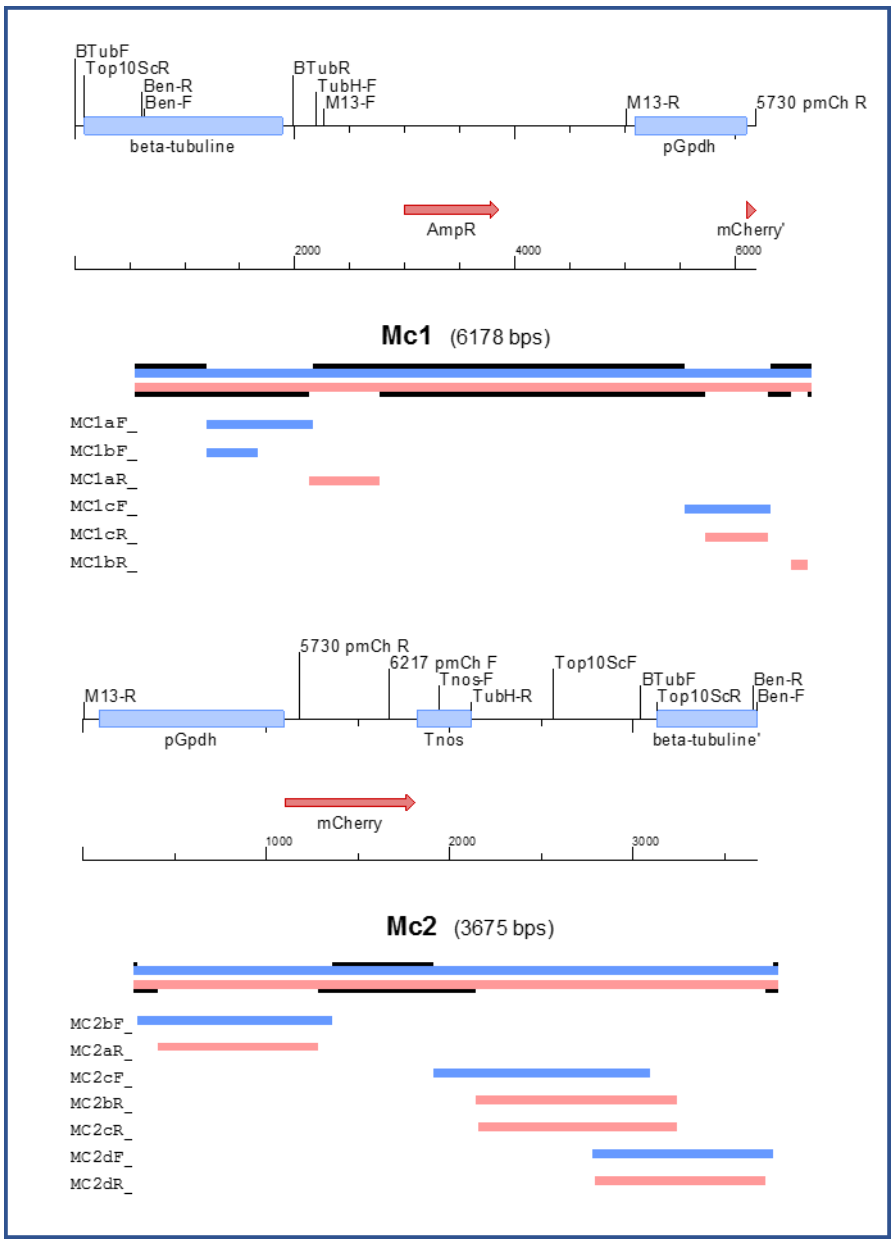

Figure 5.14 Sequence assemby with the p-mcherryβTubr plasmid (8.0 Kb). Primers are indicated at the top. Two main regions, Mc1 and Mc2, were first amplified and then sequenced with the primers indicated in the figure to obtain different fragments (in blue and pink). The fragments obtained were aligned with the original sequence to determine the correct assembly of the plasmid. Regions in black indicates were no amplification was obtained. Mc1, region Mc1 (6.2 Kb); Mc2, region Mc2 (3.7 Kb); a, b, c and d = sub regions amplified from each Mc1 or Mc2; f/r = forward or reverse primers.

In Figure 5.15, a graphic representation of the alignment of the sequences from the regions GFP1 and GFP2 with the p-3xGFPβTubr plasmid is shown. The alignment of the sequences with the original plasmid, determined that the p-3xGFPβTubr showed correct assemble during the different cloning stages. Although no sequences were obtained in certain regions of both, GFP1 and GFP2, the principal sections for the β-tubulin, promotor, terminator and GFP protein sequence were in the expected order.

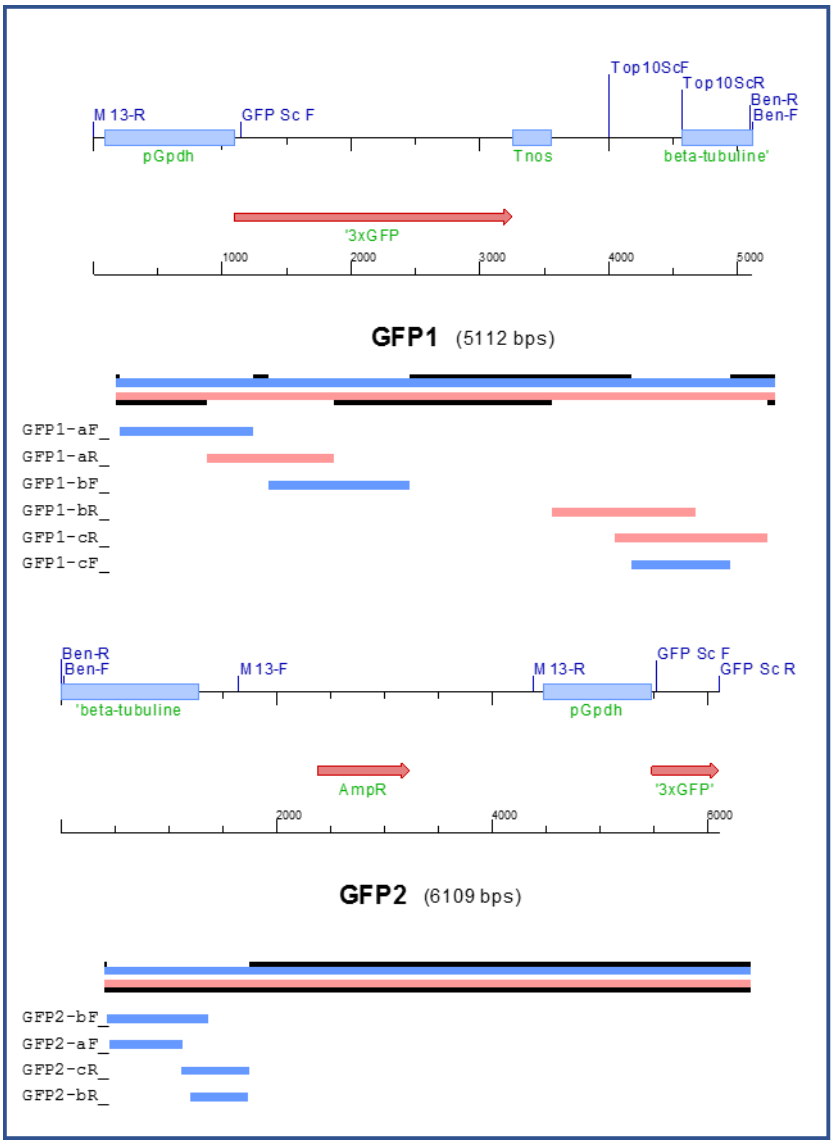

Figure 5.15 Sequence assemby with the p-3xGFPβTubr (9.5 Kb). Primers are indicated at the top. Two main regions, GFP1 and GFP2, were first amplified and then sequenced with the primers indicated in the figure to obtain different fragments (blue and pink). The fragments obtained were aligned with the original sequence to determine the correct assembly of the plasmid. Regions in black indicates were no amplification was obtained. GFP1, region GFP1 (5.1 Kb); GFP2, region GFP2 (6.1 Kb); a, b and c = sub regions amplified; f/r =forward or reverse primers.

This assembly confirmed that both plasmids were properly assembled during the different stages used (Figures 5.14 and 5.15).

5.3.4 Transformation of *Metarhizium anisopliae* with the plasmids p-mcherryβTubr and p-3xGFPβTubr

Only *M. anisopliae* A1080 was successfully transformed with p-mcherryβTubr plasmid (Figure 5.16). After transformation 16 fungal colonies were obtained. After the final round on selective Czapek's agar with benomyl (10 µg/ml), 4 colonies were selected (A1080-A; A1080-B; A1080-C & A1080-D).

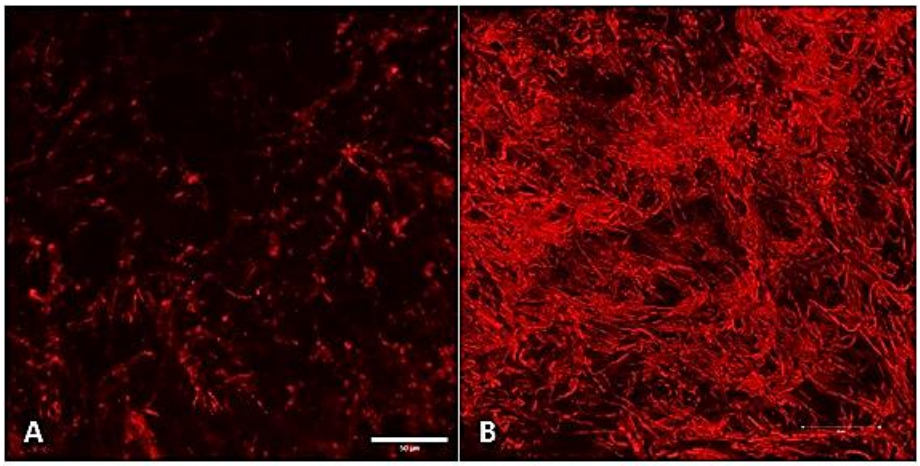

Figure 5.16 Confocal scanning electron micrographs of mcherry *M. anisopliae* A1080 transformants grown in liquid cultures. (A) *M. anisopliae* A1080 - C (B) *M. anisopliae* A1080 - D. Bar = 50 µ m.

Metarhizium anisopliae A1080 and F672 were both transformed with the p-3xGFPβTubr plasmid. In total 22 colonies were obtained from *M. anisopliae* A1080 and 40 colonies from *M. anisopliae* F672. However, some of these colonies during the consecutive rounds in non-selective Czapek's agar showed slow growth or non-sporulating colonies and for these reasons were discarded as unviable fungal transformants or abortive transformants. At the last rounds on selective Czapek's agar, from the original 42 fungal transformants only two colonies were selected from *M. anisopliae* A1080 and 23 colonies from *M. anisopliae* F672 (Figure 5.17). This last round in selective Czapek´s agar selected for mitotic stable transformants.

The transformation efficiency for *M. anisopliae* A1080 with the p-mcherryβTubr plasmid was 8.0 ± 2.8 transformants colonies each 1 $x10^8$ blastospores. The transformation efficiency with the p-3xGFPβTubr plasmid, it was 11.0 ± 1.2 transformants colonies for *M. anisopliae* A1080 and 20.0 ± 11.3 transformants colonies for the *M. anisopliae* F672 isolate for every 1 x 10^6 blastospores.

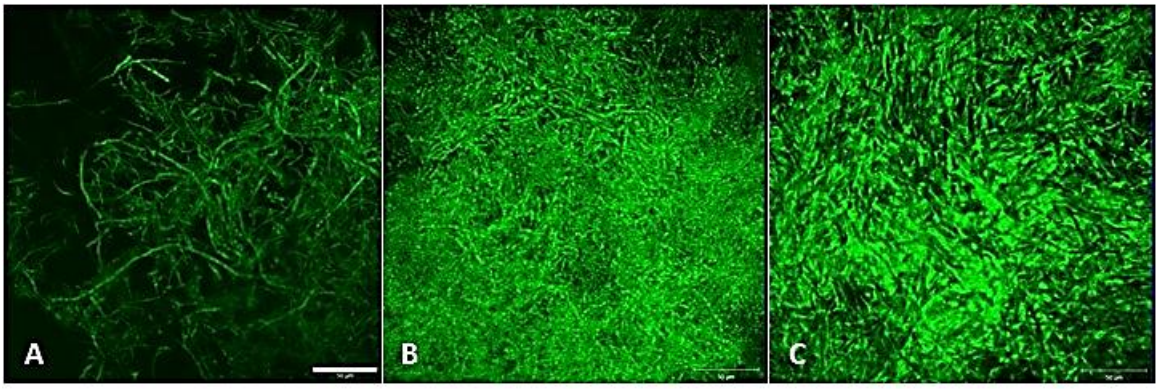

Figure 5.17 Confocal scanning electron micrographs of GFP *M. anisopliae* F672 transformants grown in liquid cultures. (A*) M. anisopliae* F672 - 30 (B) *M. anisopliae* F672 – 34 (C) *M. anisopliae* F672 – 37. Bar = 50 µm.

The fungal transformants were subjected to a PCR to confirm the presence of fluorescent protein genes in the fungal genome. All the transformants, except *M. anisopliae* A1080-A, had the expected amplicon of 2.0 Kb corresponding to the *mcherry* gene, but also an identical pattern among the transformants corresponding to unspecific amplifications (Figure 5.18). The pattern was also observed in the wild type isolate.

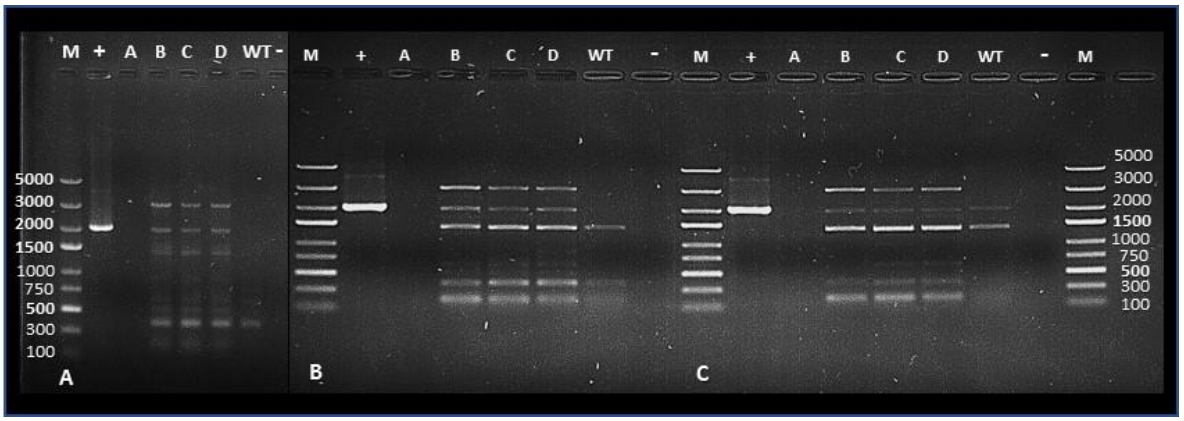

Figure 5.18 Confirmation of the presence of the *mcherry fluorescent* gene in *M. anisopliae* A1080 transformants through PCR with the primers 6217pmChF and BenF which amplify an amplicon of 2008 bps. A. Annealing temperature, 58°C. B. Annealing temperature 61°C. C. Annealing temperature 63°C. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); +, plasmid p-mcherryβTubr; A – D, *M. anisopliae* A1080 transformants; WT, *M. anisopliae* A1080 *wild type*; -, negative control.

All the *M. anisopliae* transformants for the *gfp* gene, had an identical expected PCR amplification of 586 bps, except for the transformant *M. anisopliae* A1080-1 (Figure 5.19). A similar pattern of unspecific amplifications was observed with these gfp transformants, however this was resolve

increasing the annealing temperature from 54 to 58°C. Neither of the *wild type* isolates corresponding to the *M. anisopliae* A1080 or F672 transformants amplified for the targeted fragment.

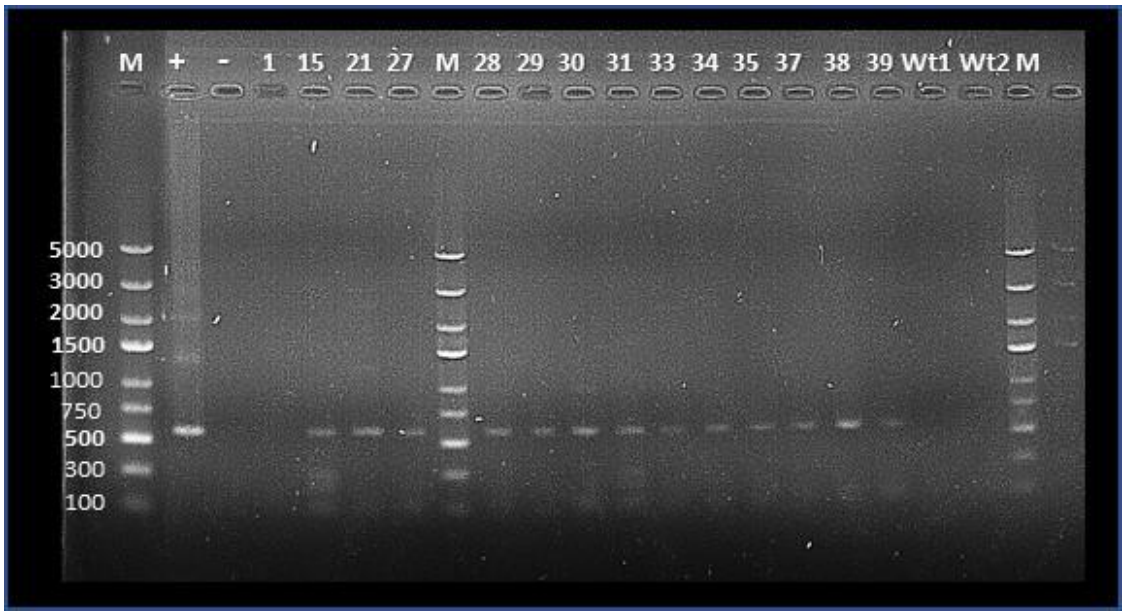

Figure 5.19 Confirmation of the presence of the *gfp fluorescent* gene in *M. anisopliae* A1080 and F672 transformants by PCR with primers GFPScF and GFPScR which amplified an amplicon of 586 bps. M, molecular marker (O´GeneRuler Express DNA Ladder, Thermo Scientific); +, plasmid p-3xGFPβTubr; -, negative control; 1-15, *M. anisopliae* A1080 transformants; 21-39, *M. anisopliae* F672 transformants; Wt1*, M. anisopliae* A1080 *wild type*; Wt2, *M. anisopliae* F672 *wild type*.

5.3.5 Determination of fungal endophytism by confocal fluorescent microscopy

Based on the molecular characterization and observation through confocal microscopy the mcherry transformants *M. anisopliae* A1080-C and A1080-D, and the GFP transformants *M. anisopliae* F672-30, F672-34 and F672-37 were selected for maize seed coating.

The presence of fungi on plant tissues was observed only in the *M. anisopliae* A1080 mcherry transformants – D (Figure 5.20), and in the *M. anisopliae* F672 GFP transformants 30 and 34 (Figure 5.21 and 5.22). Fluorescence corresponding to the mcherry or the GFP proteins was not observed in control plants treatments (CS). The presence of hyphae of the *Metarhizium* transformants were only observed in root samples, what might indicate that *Metarhizium* is able to establish associations exclusively with roots and does not translocate to the upper parts of the plants, at least not in two weeks old maize plants.

Hypha with the mcherry protein was only observed with the *M. anisopliae* - D transformant, in the surface and internally in the root. The presence of glycoproteins, α-mannopyranosyl and/or α-glucopyranosyl residues, around hyphal adhesion and penetration sites were visualized with ConA-AF633 (Figure 5.20).

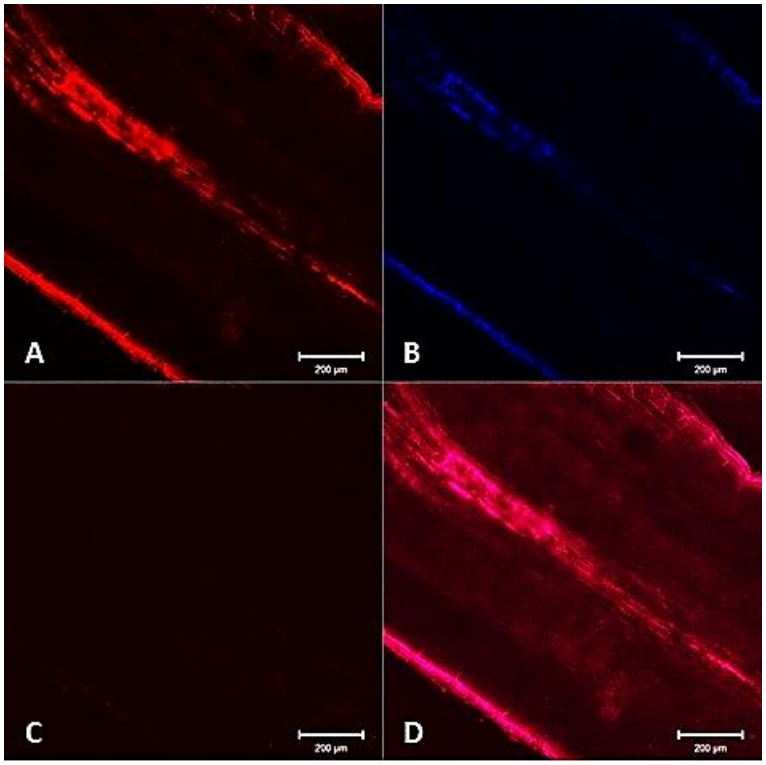

Figure 5.20 Confocal laser scanning micrographs obtained after 2 weeks of interaction between maize roots and mcherry transformant *M. anisopliae* A1080. (A) A section of a whole maize root treated with *M. anisopliae* A1080 - D showing associated mcherry-expressing hyphae. (B) Same root section stained with propidium iodide to visualize vegetal tissues (blue). (C) The presence of glycoproteins, α-mannopyranosyl and/or α- glucopyranosyl residues, around hyphal adhesion and penetration sites are visualized with ConA-AF633 (red). (D) Overlay of the three previous images. Images were taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. The bar represents 200 µm.

The presence of hyphae marked with the GFP protein from the *M. anisopliae* F672 transformants– 30 was observed in the surface of maize roots although the colonization was not extensive along the roots. The hyphal adhesion and penetration zones were also determined with the fluorescent dye ConA-AF633 (Figure 5.21).

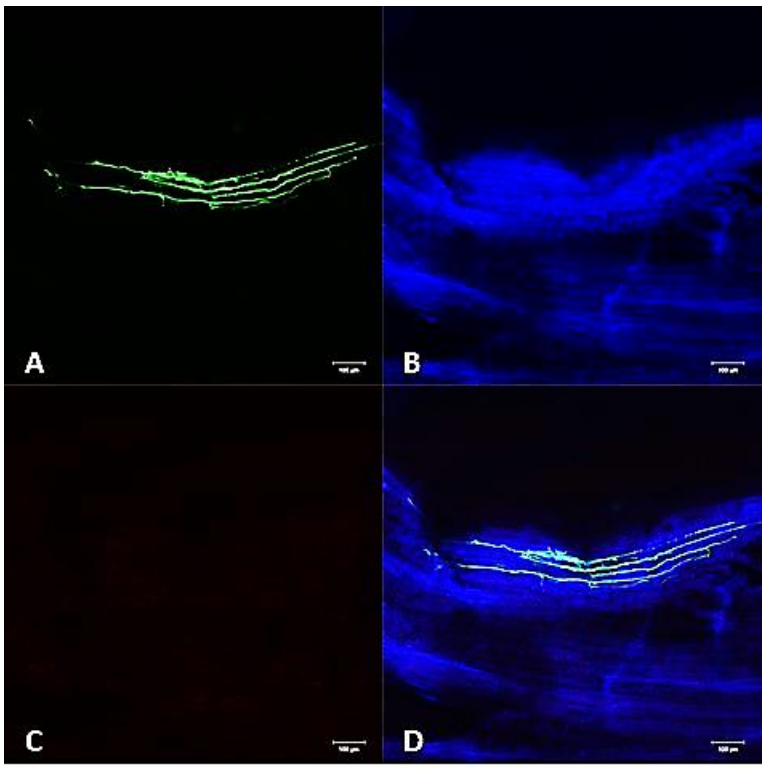

Figure 5.21 Confocal laser scanning micrographs obtained after 2 weeks of interaction between maize roots and *M. anisopliae* F672 - GFP. (A) Maize roots showing GFP transformant *M. anisopliae* F672 - 30 hyphae colonizing the surface of a maize root (green). (B) Same root section stained with propidium iodide to visualize vegetal tissues (blue). (C) The presence of glycoproteins, α-mannopyranosyl and/or α- glucopyranosyl residues, around hyphal adhesion and penetration sites are visualized with ConA-AF633 (red). (D) Overlay of the three previous images. Images were taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. The bar represents 100 µm.

The transformant *M. anisopliae* F672 - 34 was observed on the surface of maize roots and internally as endophyte, along with the penetration and hyphal adhesion sites marked with the fluorescent dye ConA-AF633 (Figure 5.22). The colonization by this mutant was higher than the *M. anisopliae* F672-30.

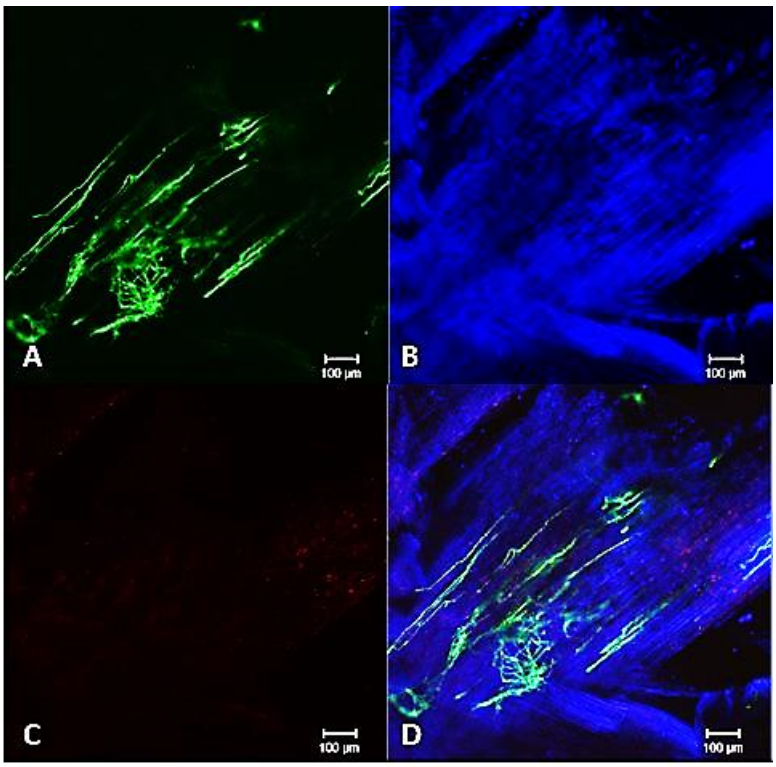

Figure 5.22 Confocal laser scanning micrographs obtained after 2 weeks of interaction between maize roots and *M. anisopliae* F672 - GFP. (A) Maize roots showing GFP transformant *M. anisopliae* F672 - 34 hyphae colonizing the surface, and the internal tissues of a maize root (green). (B) Same root section stained with propidium iodide to visualize vegetal tissues (blue). (C) The presence of glycoproteins, α-mannopyranosyl and/or α- glucopyranosyl residues, around hyphal adhesion and penetration sites are visualized with ConA-AF633 (red). (D) Overlay of the three previous images. Images were taken with a confocal microscopy (LSM 510 META - Zeiss) using the program ZEN 2009. The bar represents 100 µm.

The hyphae were observed growing in major proportion of the distal parts of the roots, and all along the root extension, but not in the root apices. Samples of stems and leaves were also analysed for all the treatments by fluorescent and confocal microscopy, but no endophytic colonization was observed.

5.4 Discussion

Two vectors, p-mcherryβTubr and p-3xGFPβTubr, carrying two genes markers, a fluorescent protein and benomyl, were expressed in *M. anisopliae* A1080 and F672. The analysis of the transformants showed that the markers were stably integrated into the genome. Cao *et al*. (2007) highlighted the advantage of genetic transformations with vector carrying two selective markers which avoid the reiterative screening and verification of the transformants in co-transformation experiments.

This is the first report where *M. anisopliae* was transformed using blastospores. Previous research on *Metarhizium* spp. transformations with the GFP gene or to confer resistance to benomyl with the β-tubulin gene used conidia or fungal protoplasts in combination with different techniques involving *Agrobacterium tumefaciens*, biolistic or PEG 8000 transformations (Valadares-Inglis & Inglis, 1997; Furlaneto *et al*., 1999; Inglis *et al*., 2000; Fang *et al*., 2006; Cao *et al*., 2007; Duarte *et al*., 2007). The transformation of *Metarhizium* using blastospores is easier and faster that working with *A. tumefaciens* or fungal protoplasts. Additionally, the transformation efficiency, 11.0 ± 1.2 or 20.0 ± 11.3 transformants per 10^6 blastospores/mL, obtained with *M. anisopliae* A1080 or F672, respectively, was similar to the efficiency determined using *A. tumefaciens* where were obtained 17.0 ± 1.4 *M. anisopliae* transformants per 10^6 conidia/mL (Fang *et al*., 2006). Inglis *et al*. (2000) using the micro-projectile bombardment reported up to 50 *M. acridum* transformants per 10^9 conidia, while Duarte *et al*. (2007) using an agro-transformation of *M. acridum* reported up to 53 transformants per 10^5 conidia.

Ying & Feng (2006), used this methodology for the transformation of *B. bassiana* and found a transformation efficiency of 24.0 ± 4.6 transformants per 10^8 blastospores. However, in transformations of conidia from *B. bassiana* with *A. tumefaciens* it was determined an increase in transformation efficiency from 1.7- to 4.6-fold when 1 x 10^5 conidia were used instead of 1 x 10^4 (dos Reis *et al*., 2004). Fang *et al*. (2006) also found variations in the transformation efficiency when working with different conidia concentrations of *M. anisopliae*. The transformation of *Metarhizium* using blastospores is still not optimized and needs further studies to determine the maximum efficiency values achievable, i.e. the optimal number of blastospores/mL or incubation times at different temperatures when transformation the blastospores.

So far, the transformation of *M. anisopliae* using blastospores has proved to be a successful methodology for the integration of two foreign genes, and obtained mitotically stable transformants as it was determined after culturing *M. anisopliae* clones in non-selective medium (without benomyl) and a last round in selective medium with 10 µg/ml of benomyl. This method offers considerable advantages over traditional protoplast-mediated transformations or agro-transformations. These include avoiding the production and use of osmotically sensitive, often multinucleate protoplast, or

the supplementary cultivation and quantification of *A. tumefaciens* cultures and additional co-cultivation times. Additionally, the blastospores can be stably stored in LiAc and glycerol at -80°C.

The GFP from jellyfish *Aequorea victoria* has been used as a reporter gene in *Metarhizium* and *B. bassiana* to determine the rhizosphere survival or localization in vegetal tissues (Hu & St Leger, 2002; Ying & Feng, 2006; Sasan & Bidochka, 2012; Wyrebek *et al*., 2011).

The intensity of GFP fluorescence varied among the transformants in this study. Such results were also previously reported (Inglis *et al*. 2000; Cao *et al*., 2007), showing that individual transformants differ in gene expression. Furthermore, the fluorescence was much stronger in mycelia than in conidia. A possible reason may be that the *T. harzianum* promoter used is more active during hyphal growth than sporulation.

Further studies should be done to determine if the *mcherry* or *gfp* gene markers and resistance to benomyl are maintained after the plant colonization process. Previous studies with a transformant strain of *M. anisopliae* with the GFP protein and resistance to benomyl determined that the traits were kept during the whole infection process in insects (Cao *et al*., 2007).

Different species of *Metarhizium* have been shown to have bifunctional lifestyles as insect pathogens as well as plant endophytes (Sasan & Bidochka, 2012). Many fungi from other genera, traditionally known as insect pathogens, have also been found to be endophytes, including species of *Acremonium*, *Beauveria*, *Cladosporium*, *Clonostachys*, *Lecanicillium* and *Isaria* (Vega, 2008; Ownely *et al*., 2010; Sasan & Bidochka, 2012). It has been proposed that *Metarhizium* may have evolved refined ecological adaptations to insect parasitism in the soil shifting from a plant-root associate fungi (Spatafora *et al*., 2007; Vega *et al*., 2009). On the other hand, Sasan & Bidochka (2012) have proposed that the ancestral lifestyle of *Metarhizium* might have started as an insect pathogen followed by adaptations to rhizosphere colonization. The fact that *Metarhizium* shares a phylogenetic origin with fungal grass-endosymbionts like *Claviceps* and *Epichloë* suggests that the ancestral lifestyle was originally as a plant associate (Spatafora *et al*., 2007; Sasan & Biodocka, 2012).

This study confirms, that *Metarhizium anisopliae* conidia coated to maize seeds worked as a suitable delivery system which granted fungal maize root colonization.

Chapter 6

General Discussion

6.1 Summary of main results

This study contributes to the goal of developing improved biological control agents from selected insect pathogenic, rhizosphere competent, entomopathogenic fungi delivered efficiently through seed coating. This PhD study used molecular and phenotypic characterisation, microbial formulation studies, laboratory and glasshouse experiments, and molecular transformations to establish a base of knowledge for development of improved biological controls.

The molecular characterization involved the identification of different isolates of *Metarhizium* obtained from different samples and locations around New Zealand. The phylogenetic study determined that most of the isolates corresponded to *M. novozealandicum*, while the remaining isolates were distributed among *M. robertsii*, *M. brunneum*, *M. guizhouense*, *M. anisopliae* and *M. frigidum*. This is the first report of the presence of recently described new species of *Metarhizium* spp. in New Zealand. A further molecular characterization based on the presence of the genes *mad1*, *mad2* and *mrt,* reported as involved in the colonisation of plants and insects, was then carried out. One of the main findings from this work was the high frequency of the *mrt* gene among the different *Metarhizium* isolates, while presence of *mad1* and *mad2* genes depended on the species or even the isolate.

The phenotypic characterization involved the spectrophotometric determination of fungal growth of the entomopathogenic isolate in root exudates. Determination of the kinetic growth parameters allowed discrimination of the isolates according to their growth in roots exudates (RE) which reflected their individual abilities to metabolize nutrients present in the rhizosphere and could predict their ability to establish in the rhizosphere. This is the first time that entomopathogenic fungal growth in RE has been evaluated as a potential indicator of rhizocompatibility.

The mortality among larvae of *C. giveni* was, as expected, dependent on soil moisture, with increasing mortality correlated with higher water content, demonstrating the importance of water for conidia germination and fungal infection.

One of the main activities of this study was to examine the effect of entomopathogenic fungal seed coatings on maize plant development and performance against biotic factors. Additionally, the effects in the maize plant induced response due to the seed coating with *M. anisopliae* was evaluated. In general, when maize plants were grown in the absence of the challengers, there were no significant

differences between the plants with the fungal treatments and the control plants. In the presence of *C. giveni* and *F. graminearum*, some entomopathogenic fungal treatments produced improved growth performance of the seedlings. It was further demonstrated that seed coating with entomopathogenic fungi could provide a delivery system for fungal biocontrols and improve the opportunity for close association with plant roots after conidia germination and hyphal growth. The principal plant – fungal association was in the rhizosphere but also some fungi become endophytic. The *M. anisopliae* isolates A1080 and F672 and the *M. robertsii* isolate F447 had significantly higher colonization of the rhizosphere than the other isolates. The establishment in the rhizosphere of the maize roots would assist the persistence of the fungi.

It was also demonstrated that colonisation of the rhizosphere by *M. anisopliae* A1080 and F672 produced changes in the levels of salicylic acid (SA) and jasmonic acid (JA) when compared to CS. The *Metarhizium* treatments increased the level of JA in roots and shoots but SA only in the roots. These effects were reduced through feeding of *C. giveni* larvae. Increased levels of JA and SA in the plant have been shown to provide resistance to plant pathogens and herbivore insects. At least one isolate induced both, the *systemic acquired resistance* (SAR) (measured as increased SA) and *the induced systemic resistance* (ISR) (measured as increased JA) in the plant. This is the first report where the effect of *M. anisopliae* on the content of the phytohormones SA and JA was evaluated.

The seed coating was further developed by replacing fungal conidia with resistant structures known as microsclerotia (MS). Production of MS by isolates of *M. anisopliae*, *M. guizhouense*, *M. novozelandicum*, *M. robertsii*, *B. bassiana* and *T. harzianum* was evaluated. This is the first report of the production of MS in *M. guizhouense* and *M. novozelandicum*. Under the conditions of this study, all isolates produced MS in levels comparable to previous works using similar fermentation conditions and C:N ratios. The highest production of MS was in *B. bassiana* Bb21 and *M. robertsii* F447. MS were formulated in diatomaceous earth (MS-DE) and coated onto maize seeds. Maize plants treated with the MS-DE treatment had better performance than control plants when grown in presence of *F. graminearum*. Thus, the overall results suggest that *Metarhizium* can be used for the control of plant pathogens and insect pests. This is the first report where maize plant performance was evaluated after seeds were coated with MS.

This study was the first to use fluorescent and laser confocal microscopy to observe rhizosphere and endophytic fungal association with maize roots after MS-DE from *Metarhizium* spp. were coated onto maize seeds. Soil conditions were appropriate for MS germination and hyphae developed along the maize roots, associated with the ecto and endorhizosphere. These studies were also confirmed using maize seeds coated with conidia of isolates of *Metarhizium* transformed with the fluorescent proteins mcherry and GFP. This is the first report of a blastospore-based transformation of *M. anisopliae*.

6.1.1 Molecular characterization – specific gene study

Metarhizium isolates, from different locations and samples from around New Zealand, were identified to species level using the complete sequence of the EF-1α. The resulting phylogenetic tree placed most isolates in the *M. novozealandicum* clade highlighting the importance of this taxon in New Zealand. Other isolates were identified as belonging to *M. robertsii*, *M. brunneum* and *M. guizhouense*, which have been reported as having agriculture habitats (Kabaluk & Ericsson, 2007; Vega *et al*., 2009; Behle *et al*., 2013; Keyser *et al*., 2015; Moonjely, Barelli & Bidochka, 2016). Only one isolate was grouped in *M. anisopliae* and none of the isolates were found in the *M. flavoviride* taxon a worldwide common specie of *Metarhizium*. Finally, two isolates were identified as *M. frigidum*. This is the first report on the presence of recently described new species of *Metarhizium* spp. in New Zealand.

The presence of the genes *mad1*, *mad2* and *mrt,* previously reported as involved in colonisation of plants and insects, was then used to further characterise the isolates. The *mad1* gene is mainly related with the ability of *Metarhizium* to infect insects, therefore, a desirable attribute for control insect populations. The genes *mad2* and *mrt* play an important role in the ability of *Metarhizium* to survive in the rhizosphere of plants.

One of the main results was the high frequency of the *mrt* gene among the different *Metarhizium* isolates used in study. This fact supports the theory about the ancestral origin of *Metarhizium* as a plant associated fungus which later evolved into an insect pathogen (Spatafora *et al*., 2006; Vega *et al*., 2009; Moonjely, Barelli & Bidochka, 2016). The genes *mad1*, *mad2* and the *mrt* seem to be uniformly distributed among the isolates in the PARB and MGT clades. The taxon *M. frigidum* seemed not to have the *mad2* gene and neither did some isolates in the *M. novozealandicum* clade. This is in agreement with previous works where it was found that plant adhesins (*mad2*) had diverged among *Metarhizium* lineages, contributing to clade-and species-specific variation, while insect adhesins (*mad1*) had been largely conserved (Wyrebek & Bidocka, 2013).

These results suggested that certain species of *Metarhizium* not only play a role in the regulation of insect populations but also have the capability to associate with plants, and therefore have specific genetic adaptations that support these ecological roles. This variability in the composition of *Metarhizium* genome has been noticed in previous molecular and ecological studies, which explains the versatility of certain species of *Metarhizium* to act as insect pathogens or plant endophytes (St. Leger, Wang & Fang, 2011; Keyser *et al*., 2015).

6.1.2 Spectrophotometric determination of fungal growth in roots exudates as an indicator of rhizosphere competence

The molecular characterization mirrored the results observed when entomopathogenic fungal isolates were grown in the presence of roots exudates (RE). In general, entomopathogenic fungal species belonging to the clades PARB or MGT grown in RE had kinetic parameters values corresponding to a high capability to establish and survive on the rhizosphere. Concordantly, in these group of isolates it was found that the genes *mad2* and *mrt* were present among the species in the clades. On the other hand, *M. novozealandicum* and *M. frigidum* were two species with the lowest performance in RE, and probably with poor rhizo-compatibility. In these species in particular, the *mad2* gene was present only in some of the *M. novozealandicum* and it was not found in either *M. frigidum* isolate, which may explain, in part, the poor performance in RE and probably poor rhizo-compatibility.

The spectrophotometric determination of entomopathogenic fungal growth in RE as a measure of rhizosphere-compatibility has not been carried out previously. Using this methodology and estimating kinetic parameters it was possible to characterize the isolates according to their growth in RE. The final results were in agreement between the isolates which have a significantly greater growth in RE and those species of *Metarhizium* reported as rhizosphere-competent (Hu & Leger, 2002; Sasan & Bidochka, 2012; Liao *et al*., 2013; Liao *et al*., 2014; Keyser *et al*., 2014; Krell *et al*., 2017). For example, when fungal growth was evaluated as quantity (change, slope or maximum value) the species *M. anisopliae*, *M. robertsii*, *M. brunneum* and *M. guizohuense* produced more biomass than *M. novozealandicum* or *M. frigidum*. When fungal growth was evaluated as speed (average rate), *M. anisopliae*, *M. brunneum* and *M. robertsii* were faster than *M. novozealandicum* and *M. frigidum*. Finally, when growth was evaluated as time-based, *M. anisopliae*, *M. guizohuense* and *M. brunneum* started to grow before than *M. novozealandicum* or *M. frigidum*, while *M. anisopliae*, *M. guizhouense* and *M. robertsii* reached a maximum rate faster than *M. novozealandicum* and *M. frigidum*. It would be interesting to evaluate these parameters at lower temperatures and determine if colder temperatures would be more favourable for cold adapted species such as *M. frigidum,* where it may have an advantage over the other *Metarhizium* species.

Finally, the determined kinetic values allowed ranking of the isolates accordingly to their growth in RE where those isolates with the best performance were obtained from plant organs as endophytes: *M. robertsii* F447, *M. anisopliae* F672 and *M. guizhouense* Bk41. In this group were also those with proven plant promotion capacities, like *M. anisopliae* A1080 (Hu & St. Leger, 2002) and *T. harzianum* F327 (Chirino-Valle *et al*., 2016).

In summary, the determination of the kinetic parameters allowed discrimination of the isolates according to their growth in RE which reflected their individual abilities to metabolize nutrients present

in the rhizosphere and so had a high probability to establish on the rhizosphere. However, given the complexity of the nutrients present in the exudates from roots which varies between plant species, and the fact that *Metarhizium* also is compatible with certain plant species, it would be necessary to evaluate each situation in particular when selecting entomopathogenic isolates with rhizo-compatibility for a certain type of plant.

6.1.3 Grass grub pathogenicity

The mortality in *C. giveni* due to entomopathogenic fungi was, as expected, dependent on soil moisture. Increasing mortality correlated with higher water content demonstrating the importance of water for conidia germination and fungal infection (Bruck, 2005). On average, mycosis was higher at 10^7 than at 10^9 conidia/ml, except for *M. guizhouense* F16. There are reports indicating that high conidia densities can negatively affect germination by increasing the concentration of fungal volatile compounds, like 1-octen-3-ol, which is produced by numerous species (Nassr, 2013; Wyatt *et al*., 2013; Roeland *et al*., 2013). *M. guizhouense* F16, in the three conidial suspensions applied, was the first to cause fungal infection on larvae of *C. giveni* at a soil-humidity content of 20%. This fact might indicate that this isolate is able to cope with drier conditions than the remaing isolates. The increase in moisture helped to accelerate the infection process for all the conidia concentrations tested and improved the infection performance of the isolates in general.

6.1.4 Seed coating with conidia and maize plant performance

The isolates used in this study when applied as a coating to maize seeds at 10^5 and 10^8 conidia/mL did not have any detrimental effect on seed viability or plant development, since maize plant performance was comparable to that obtained with control seeds. However, higher conidial concentrations of 10^9 conidia/seed have been shown to reduce germination and also root growth (Kabaluk & Ericcson; 2007).

In general, when maize plants were grown in the absence of *C. giveni* or *F. graminearum*, there were no significant differences between the plants from the fungal entomopathogenic coated seeds and the control treatments. While the fungal-seed coating did not have any detrimental effect, neither was there an effect on maize plant growth promotion. Only maize plants coated with the *B. bassiana* Bb21 and *M. novozealandicum* F99 treatments had smaller shoot or root dry weight, respectively, than controls and the other fungal coatings. One explanation could be that these isolates might be less cooperative with the plant, and take nutrients from roots. Plants can perceive this negative effect and use more resources to limit fungal growth, what in the end would affect plant growth (Vega *et al*., 2009; Ownley *et al*., 2010; Pangesti *et al*., 2016).

On the other hand, effects on maize plant growth because of the fungal coating were determined when plants were grown in the presence of *C. giveni* and *F. graminearum*. In general, control plants (CS),

and also plants treated with the *T. harzianum* F327 had significantly lower growth in the presence of the plant pathogen or the insect pest. Conversely, plants treated with the entomopathogenic fungal coating were not significantly different from each other when grown in presence or absence of *C. giveni* and *F. graminearum*. This suggests a plant benefits from associated microorganisms would only be noticed in the presence of detrimental abiotic or biotic factors (Partida-Martínez, 2011).

Plants with the *M. anisopliae* F672 treatment had no variations in root or shoot dry weight in presence of the challengers, which might indicate the ability of this isolate to promote maize growth and at the same time, reduce the possibly negative effect on growth associated with the presence of the biotic factors. The mechanisms by which the entomopathogenic fungal coating attenuated the negative effects on maize growth in the presence of *C. giveni* or *F. graminearum* are still to be determined. However, it may be the pathogenic capacity of the isolates to infect *C. giveni* or direct competition with *F. graminearum* for the colonization of the rhizosphere. All *Metarhizium* isolates coated to maize seeds were pathogenic to *C. giveni* in a range between 33 and 67%. Bruck (2005) also described that colonization of the rhizosphere of *Picea abies* by a rhizosphere competent isolate of *M. brunneum* provided nearly 80% control of black vine weevil larvae within two weeks of exposure to inoculated roots. In presence of *F. graminearum*, symptoms of *Fusarium* root rot were higher on maize plants from the CS treatment, than on plants with some of the fungal treatments. This result highlights the potential of some isolates to either, reduce the ability of *F. graminearum* to cause disease or to increase the tolerance in the plant. Another important factor to consider in the interaction between entomopathogenic fungi and *Fusarium* species is the production of mycotoxins by *Fusarium*. Some species of *Fusarium* produces higher amounts of fumomonisins when growth is inhibited by another fungus in competition for resources in plant (Marín *et al*., 2004).

When the *Metarhizium* isolates where coated on seeds and planted, all were able to not only grow in the rhizosphere of the maize plants, but also to colonize endophytically the plant tissues. In particular, *M. anisopliae* A1080 and F672, and *M. robertsii* F447 were the species with major rhizosphere colonization capacity. Previous reports also indicated these species with high ability to colonize the plant rhizosphere (Hu and St. Leger, 2002; Sasan & Bidochka, 2012; Liao *et al*., 2013; Liao *et al*., 2014; Keyser *et al*., 2014; Kepler *et al*., 2015). In these isolates were also found the genes for adhesion to plant surfaces, *mad2*, and for the utilization of sugars from the root, *mrt*. Furthermore, these isolates had significantly greater performance when grown in RE. Colonization by these isolates was not systemic and remained localized exclusively in roots. However, the short period of time of the experiment would not necessarily have been enough for the fungi to colonize the plant aboveground. Maize plant trials of more of three months would be necessary to determine the colonization extension in the plant by entomopathogenic fungi.

The presence of *C. giveni* was detrimental for rhizosphere colonization in most of the isolates. The decrease in rhizosphere colonization when *C. giveni* was present is most likely associated with root biomass loss because of the feeding behaviour by the larvae, while in the case of *Fusarium* may be competence for root colonization in addition to the systemic response in the plant. Previous works also reported a reduction in rhizosphere colonization in the presence of root feeding larva (Cosme *et al*., 2016; Zitapolca-Hernández *et al*., 2017). Another explanation for the decrease in rhizosphere colonization might be linked to changes in roots exudates composition. Plants are able to modify the microbiota present on the rhizosphere by altering the root exudates composition as a strategy utilized by the plant as consequence of insect attack (Partida-Martínez & Heil, 2011; Pangesti *et al*., 2013).

The decrease in rhizosphere colonization observed in *M. anisopliae* A1080 and *M. robertsii* F447 in the presence of *C. giveni* supports the bodyguard hypothesis (Elliot *et al*., 2000). If the plant response to root-feeding larvae is a change in roots exudate composition which reduces the ability of the entomopathogenic fungi to survive in the rhizosphere, this could be sensed by the fungus as nutrient deprivation. Nutrient deprivation will force fungi to initiate the conidiation process releasing infective conidia in the proximity of roots where the larvae are feeding. In particular, species of *Metarhizium* and *Beauveria*, in unfavourable conditions, can initiate the formation of conidia directly from the tips or sides of hyphae, in a process known as microcycle conidiation where the development of conidiophores is arrested (Jung *et al*., 2014). Further studies are necessary in order to confirm this theory. If this mechanism is valid, it would explain how entomopathogenic fungi as endophytes can still be pathogenic to insect, and why there was an observed reduction in fungal endophytism in presence of *C. giveni*.

In the presence of *F. graminearum,* rhizosphere colonization in *M. robertsii* F447, *M. anisopliae* A1080 and F672 was decreased, while in *T. harzianum* F327, *M. guizhouense* F16 and *M. novozealandicum* F99 was increased. The decrease in the ability of the entomopathogenic fungi to colonize the rhizosphere may also be a consequence of changes in root exudates composition. This observation also fits with the bodyguard hypothesis where the maize plant in the presence of *F. graminearum* responded by increasing the rhizosphere competence of beneficial fungal species, like *T. harzianum*, which could compete with the plant pathogen. This increase in rhizosphere competence may explain why there was an increase in the endophytic colonization of the fungal treatments in presence of *F. graminearum*. Entomopathogenic fungal isolates may have different roles in and on the plant, with some being able to better compete with *F. graminearum* than others (e.g. *B. bassiana* Bb21, *M. anisopliae* A1080, *M. guizhouense* F16 and *M. novozealandicum* F99) while other fungal isolates would be more suitable as insect pathogens (e.g. *M. robertsii* F447 and *M. anisopliae* A1080). This is supported by the fact that root infection symptoms were lower in treatments where there was an increase in rhizosphere colonization such as with *M. guizhouense* F16 and *M. novozealandicum* F99,

while *F. graminearum* symptom incidence was similar to CS in *M. anisopliae* F672 and *M. robertsii* F447 where a decrease in rhizosphere colonization was determined. It would still be necessary to determine which changes occurred in root exudates composition, if this is the reason, or if the changes in rhizosphere colonization was the result of plant hormonal regulation.

6.1.5 Effect of the maize seed coating with conidia from *Metarhizium anisopliae* in the content of salicylic acid and jasmonic acid of plants

There have been no previous reports on the effects of entomopathogenic fungi on the plant content of salicylic acid (SA) and jasmonic acid (JA). In this study, maize plants, after coating with *M. anisopliae* A1080 and F672, had significantly different levels of SA from each other, although, both levels there were not significantly different from control plants. The higher content of SA in plants with the *M. anisopliae* A1080 treatment than in plants with the *M. anisopliae* F672 treatment may indicate that this particular isolate is an elicitor of the SAR or also that this isolate is recognised by the plant as a potential pathogen.

SA is a key hormone, regulating plant defence against biotrophic pathogens and against insect herbivores with a piercing-sucking feeding mode (Pangaesti *et al*., 2013; Fernandez-Conradi *et al*., 2016). Also, SA-dependent signalling is crucial in the interaction of plant roots with non-pathogenic microbes (Pangesti *et al*., 2013). The lower levels of SA in plants from the *M. anisopliae* F672 treatment could indicate that this isolate is not recognized by maize plants as an invasive microorganism. The selection of entomopathogenic fungal strains that are not recognized by the plant as a potential invader could be another desirable characteristic when plant growth and yield is one of the aims. Whether or not the increase in the levels of SA induced by the *M. anisopliae* A1080 treatment is translated to an increase in the SAR in maize needs to be evaluated. The cost associated with this phytohormone boost could be compensated with the additional control of above ground pests, which in the end may result in plant growth promotion.

The JA profile was also distinctive between the different fungal treatments, but maize plants with the *M. anisopliae* A1080 treatment had higher content of JA in roots and shoots than control plants. This seems to indicate that the isolate *M. anisopliae* A1080 is capable not only to induce SAR in maize but also ISR. JA-signalling has also been described as the main pathway in ISR against aboveground herbivores and is stimulated by root-associated microbes (Pangesti *et al*., 2013).

Plant signalling pathways are also modulated by non-pathogenic microbes that colonize roots without producing disease symptoms in the plant. During biotrophic root colonization it has been observed that JA signalling is required (Cosme *et al*., 2016). However, the activation of the JA-signalling pathway also affects the plant´s interaction with root-colonizing microbes (Pangesti *et al*., 2013; Cosme *et al*.,

2016). In this study, the *M. anisopliae* A1080 treatment had the highest levels of the hormone in roots and leaves. One ecological advantage for this isolate of *Metarhizium* above other microorganisms is that the boost in the phytohormone content in the plant could impair root colonization by other microorganisms, like *F. graminearum*. This hypothesis could also explain why the symptoms of *F. graminearum* observed in plants treated with *M. anisopliae* A1080 were significantly lower than those observed in control plants, in the presence or absence of *C. giveni*.

Another clue for the distinctive hormone profile observed between both isolates might reside in the origin of these fungi. While *M. anisopliae* A1080 was obtained from an insect larva, *M. anisopliae* F672 was isolated as a Pine root endophyte. Further research would need to be done to determine if natural endophytic isolates have evolved a different colonization mechanism which does not induce resistance in the plant.

6.1.6 Microsclerotia production and seed coating

Seed coating with conidia of entomopathogenic fungi has been proposed as a potential strategy for the delivery of these fungal biocontrol agents, because after conidia germination the developing hyphae would survive on the exudates from growing plant roots (Bruck, 2005). However, the application of formulations based only on conidia seems not to be appropriate since most conidia are likely to lose viability rapidly in the environment and only minimal proportions will presumably succeed in infecting new hosts (Meyling & Eilenberg, 2007). Additionally, the production of conidia, and the different stages of harvest, formulation, storage and delivery to the farmers, may compromise the viability of the infective units. In this study it was proposed to investigate coating the seeds with microsclerotia (MS) instead of conidia as a strategy to improve the formulation of the coating and the survival of the biological control agent.

The production and formulation of MS by isolates of *M. anisopliae*, *M. guizhouense*, *M. novozelandicum*, *M. robertsii*, *B. bassiana* and *T. harzianum* was determined. Under the conditions of this study, all isolates produced MS in levels comparable to previous studies using similar fermentation conditions and C:N ratios (Jackson & Jaronski, 2009; Behle *et al*., 2013; Mascarin *et al*., 2014). This is the first report on the production of MS in *M. guizhouense* and *M. novozelandicum*.

However, it is still necessary to further study another important factor for microsclerotia production which is medium optimization, especially for industrial production aims. The use low cost medium would make the production process profitable and with high potential for agronomic uses. A culture medium with low concentration of nutrients can hasten the formation and melanisation of MS that will reduce production costs (Mascarin *et al*., 2014). Microsclerotia production and stabilization with low-cost nitrogen sources have been demonstrated for liquid fermentation of *Metarhizium terrestris*

and *M. brunneum* with MS showing excellent biocontrol performance in the field (Shearer & Jackson 2006; Behle & Jackson 2014). The industrial production of MS is feasible since previous works have shown that stirred-tank bioreactors can be used to mass produce stable MS of *Metarhizium* (Jackson & Jaronski, 2012).

Another factor to consider during MS production is maturation of the resistant structures. Although the production of these resistant structures could be significant early in the fermentation, a complete melanisation of MS did not occur until after several days of fermentation. The longer the fermentation process the higher the melanisation of the microsclerotia occurred not only in entomopathogenic fungi but also in *T. harzianum* (Jackson & Jaronski, 2009; Mascarin *et al*., 2014; Kobori *et al*., 2015). Further studies are needed to determine the relationship between melanisation and performance of MS. An early harvest of MS and the downstream processing during drying and formulation, if they are not completed mature, could result in viability losses. Melanisation has been associated with prolonged persistence in soil and resistance to desiccation (stress tolerance) in various filamentous fungi (Jackson & Jaronski, 2009; Kobori *et al*., 2015).

Additionally, this was the first work to show that MS retained viability after coating as determined by CFU/g seed. The MS coated to maize seed also proved to be effective in promoting plant growth in presence of *F. graminearum* producing better performance than control plants. Furthermore, MS granted protection against a range of different insects present in the soil (Jackson & Jaronski, 2009; Behle *et al*., 2013; Wang *et al*., 2012; Goble *et al*., 2016).

Through fluorescent and confocal microscopy, the ability of the isolates of *Metarhizium* spp. to associate with the roots, not only with ectorhizosphere but also within the endorhizosphere, was demonstrated. The association pattern between hyphae of entomopathogenic fungi and roots still needs to be further studied. However, it was observed that *M. anisopliae* A1080 and F672 and *M. robertsii* F447 widely colonized the differentiation and the root hair zones, inter- and intracellularly, while it was infrequently detectable in the elongation and meristematic zones. This colonization pattern was also observed in the plant pathogen *Piriformospora indica* (Zuccaro *et al*., 2011). However, the ability of *Metarhizium* to be endophytic and to colonize cortical cell roots, set this genus closer to ecto- and arbuscular mycorrhizal fungi. This may suggest that entomopathogenic fungi follow a pattern similar to that observed in *P. indica* and mycorrhiza.

6.1.7 Colonisation of plants by isolates of *Metarhizium* expressing the mcherry or GFP protein

This is the first report where *M. anisopliae* has been transformed using blastospores. This methodology was easier and faster than working with *A. tumefaciens* or fungal protoplasts, and made

it possible to transform *M. anisopliae* with two foreign genes, obtaining mitotically stable transformants. Additionally, the transformation efficiency using blastospores (11.0 ± 1.2 or 20.0 ± 11.3 transformants per 10^6 blastospores/mL), was similar to the efficiency determined using *A. tumefaciens* with 17.0 ± 1.4 *M. anisopliae* transformants per 10^6 conidia/mL (Fang *et al*., 2006). The transformation of *Metarhizium* using blastospores needs to be optimized and needs further studies to determine the maximum efficiency values achievable, i.e. the optimal number of blastospores/mL or incubation times at different temperatures when transforming the blastospores.

A vector carrying two foreign genes, a fluorescent protein and a benomyl selective marker (p-mcherryβTubr and p-3xGFPβTubr) was used in the transformation of blastospores of *M. anisopliae* A1080 and F672. This represent an additional advantage which avoids the reiterative screening and verification of the transformants in co-transformation experiments (Cao *et al*., 2007). Additional studies, like a characterization of the transformants through a southern blot, are necessary to determine the number of copies of the plasmid that had been integrated in the fungal genome (dos Reis *et al*., 2004; Fang *et al*., 2006; Ying & Feng, 2006; Duarte *et al*., 2007). These studies may explain the variations in the intensity of the GFP fluorescence among the transformants.

6.1.8 Conclusions

In summary, this study confirmed, that conidia from entomopathogenic fungi coated to maize seeds worked as a suitable delivery system for fungal maize root colonization. Furthermore, the use of resistant structures known as microsclerotia improved the survival of the entomopathogenic fungi during production, harvest and distribution and at the same time provided fresh infective propagules after the seeds have been sown. *Metarhizium* species belonging to the PARBA or MGT clades, like *M. anisopliae*, *M. robertsii*, *M. guizhouense* and *M. brunneum*, seem to be more appropriate for their use as colonising fungi in agricultural systems, although a specific study for each specific plant-fungus interaction is necessary. A preselection of entomopathogenic fungi based on growth in roots exudates will improve the chances of survival of the selected fungus on the rhizosphere and allow the colonization of roots, rhizosphere and the plant tissues endophytically. *Metarhizium* isolates, coated to maize seeds as microsclerotia, are able to protect plants not only from insect pests present in the soil, like *C. giveni*, but also from soil plant pathogens like *F. graminearum*. In the presence of these pests and pathogens, plant growth performance is improved. *Metarhizium* can also elicit an induced defense response in the plant which can also benefit the plant against insect and disease attack above ground.

Finally, in the selection of entomopathogenic fungi for association with plants, the bidirectional response between the plant and the fungus and the interaction with the challengers which are needed to be controlled must be considered. Specific compatibility between the fungus and plant species concerning trade of nutrients, hormone regulation and biocontrol activity are need to be evaluated to avoid unwanted negative effects.

Appendix A

Media

A.1 Malt extract broth (MB)

	g/L
Glucose	20.0
Malt extract	20.0
Bacto tryptone	1.0

Complete with distilled water till 1 litre. Sterilize 15 min at 121°C and 1.5 atm.

A.2 *Metarhizium* selective medium (MSM)

	g/L
Oat	30.0
CTAB	0.60
Chloranphenicol*	0.50
Cycloheximide*	0.25
Agar	20.0

Complete with distilled water till 1 litre. Sterilize 15 min at 121°C and 1.5 atm.

** Sterilization by filtration and incorporated to the sterilized medium*

CTAB, cetyl trimethyl ammonium bromide

A.3 *Trichoderma harzianum* selective medium (TSHM)

	g/L
$MgSO_4 \cdot 7H_2O$	0.2
K_2HPO_4	0.9
NH_4NO_3	1.0
KCl	0.15
Rose Bengal	0.15
Glucose	3.0
Agar	20

Complete with distilled water till 1 litre. Sterilize 15 min at 121°C and 1.5 atm.

Josie Williams, J., Clarkson, J. M., Mills, P. R. & Cooper, R. M., 2003. A Selective Medium for Quantitative Reisolation of *Trichoderma harzianum* from *Agaricus bisporus* Compost. Appl Environ Microbiol., 69, 4190–4191.

A.4 *Fusarium* selective medium

	g/L
Peptone	15.0
$MgSO_4$	1.0
$KH2PO_4$	0.5
Malachite green oxalate	2.5 mg
Agar	15.0

Complete with distilled water till 1 litre. Sterilize 15 min at 121°C and 1.5 atm.

Thompson, R.S., Aveling, T.A.S., Blanco-Prieto, R. (2013). A new semi-selective medium for *Fusarium graminearum*, *F. proliferatum*, *F. subglutinans* and *F. verticillioides* in maize seed. South African Journal of Botany, 84, 94–101.

A.5 Sabouraud Dextrose Broth (SBD)

	g/L
Dextrose	20.0
Mycological peptone	10.0

Appendix B

Experimental design and statistics results

B.1 Statistical Analysis Chapter 2: Characterization of the entomopathogenic fungi

Section 2.3.4 Fungal radial growth in PDA

Table A.1 Analysis of variance for fungal growth in PDA

Variate: Diameter

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolates	27	62156.98	2302.11	238.91	<.001
Residual	140	1349.0	9.64		
Total	167	63505.98			

Tables of means
Grand mean: 35.35

Isolates	A1080	B14	Bb18	Bb21	BK4-1	C14	F11
	32.67	26.50	35.17	74.33	83.83	28.50	17.17
Isolates	F120	F133	F137	F138	F142	F144	F148
	34.83	31.67	32.33	33.67	25.50	76.33	24.33
Isolates	F16	F178	F264	F387	F401	F628	F672
	37.17	19.67	26.00	31.33	25.33	24.17	50.00
Isolates	F98	F99	FCC327	FCC447	MFI	MW#2	MW#8
	15.50	20.17	81.83	34.67	19.17	23.50	24.33

Least significant differences of fungal diameter means (5% level)

Table	Isolates
rep.	6
d.f.	140
l.s.d.	3.5

Section 2.3.6 – Characterization of fungal growth

Table A.2 Analysis of variance for the kinetic parameter change for fungi grown in roots exudates

Variate: Change

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	0.516950	0.019146	9.62	<.001
Run	2	0.058134	0.029067	14.61	<.001
Residual	54	0.107459	0.001990		
Total	83	0.682542			

Least significant differences of fungal change means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	0.07302	0.02390

Table A.3 Analysis of variance for the kinetic parameter maximum rate for fungi grown in roots exudates

Variate: Maximum rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	1.682E-07	6.229E-09	8.50	<.001
Run	2	1.399E-08	6.993E-09	9.54	<.001
Residual	54	3.958E-08	7.331E-10		
Total	83	2.217E-07			

Least significant differences of fungal maximum rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	4.4E-5	1.5E-5

Table A.4 Analysis of variance for the kinetic parameter slope for fungi grown in roots exudates

Variate: Slope

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	1.476E+10	5.465E+08	32.61	<.001
Run	2	2.973E+08	1.487E+08	8.87	<.001
Residual	54	9.050E+08	1.676E+07		
Total	83	1.596E+10			

Least significant differences of fungal slope means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	6701.5	2193.6

Table A.5 Analysis of variance for the kinetic parameter time to maximum rate for fungi grown in roots exudates

Variate: Time to maximum rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	3.361E+11	1.245E+10	10.15	<.001
Run	2	1.183E+10	5.913E+09	4.82	0.012
Residual	54	6.626E+10	1.227E+09		
Total	83	4.142E+11			

Least significant differences of fungal time to maximum rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	57343.1	18769.9

Table A.6 Analysis of variance for the kinetic parameter maximum value for fungi grown in roots exudates

Variate: Maximum

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	0.615733	0.022805	20.28	<.001
Run	2	0.044702	0.022351	19.88	<.001
Residual	54	0.060720	0.001124		
Total	83	0.721155			

Least significant differences of fungal maximum value means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	0.05489	0.01797

Table A.7 Analysis of variance for the kinetic parameter average rate for fungi grown in roots exudates

Variate: Average rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	1.429E-11	5.293E-13	20.69	<.001
Run	2	7.948E-13	3.974E-13	15.54	<.001
Residual	54	1.381E-12	2.558E-14		
Total	83	1.647E-11			

Least significant differences of fungal average rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	2.6E-07	8.6E-08

Table A.8 Analysis of variance for the kinetic parameter average value for fungi grown in roots exudates

Variate: Average value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	0.2272351	0.0084161	32.68	<.001
Run	2	0.0046418	0.0023209	9.01	<.001
Residual	54	0.0139081	0.0002576		
Total	83	0.2457850			

Least significant differences of fungal average rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	0.026	0.009

Table A.9 Analysis of variance for the kinetic parameter specific growth rate for fungi grown in roots exudates

Variate: growth rate (μ)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	2.63E-4	9.75E-06	2.80	<.001
Run	2	6.75E-05	3.38E-05	9.71	<.001
Residual	54	1.88E-04	3.48E-06		
Total	83	5.19E-04			

Least significant differences of fungal growth rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	3.1E-03	1.00E-03

Table A.10 Analysis of variance for the kinetic parameter lag phase ends for fungi grown in roots exudates

Variate: Lag phase ends ($Tlag_f$)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	9002.88	333.44	21.65	<.001
Run	2	354.89	177.45	11.52	<.001
Residual	54	831.55	15.40		
Total	83	10189.32			

Least significant differences of fungal lag phase ends means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	6.424	2.103

Table A.11 Analysis of variance for the kinetic parameter generation rate for fungi grown in roots exudates

Variate: generation rate (v)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	622.58	23.06	1.98	0.016
Run	2	139.30	69.65	5.98	0.005
Residual	54	629.00	11.65		
Total	83	1390.88			

Least significant differences of fungal generation rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	5.587	1.829

Table A.12 Analysis of variance for the kinetic parameter duplication time for fungi grown in roots exudates

Variate: Duplication Time (Td)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	622.58	23.06	1.98	0.016
Run	2	139.30	69.65	5.98	0.005
Residual	54	629.00	11.65		
Total	83	1390.88			

Least significant differences of fungal duplication time means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	5.6	1.8

Table A.13 Analysis of variance for the kinetic parameter change for fungi grown in PDB

Variate: Change

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	1.4905221	0.0552045	80.25	<.001
Run	2	0.0061520	0.0030760	4.47	0.016
Residual	54	0.0371492	0.0006879		
Total	83	1.5338234			

Least significant differences of fungal change means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	0.04294	0.01405

Table A.14 Analysis of variance for the kinetic parameter maximum rate for fungi grown in PDB

Variate: Maximum rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	4.714E-07	1.746E-08	24.86	<.001
Run	2	1.056E-09	5.278E-10	0.75	0.477
Residual	54	3.792E-08	7.023E-10		
Total	83	5.104E-07			

Least significant differences of fungal maximum rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	4.3E-5	1.4E-05

Table A.15 Analysis of variance for the kinetic parameter slope for fungi grown in PDB

Variate: Slope

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	2.739E+10	1.014E+09	115.77	<.001
Run	2	1.597E+08	7.986E+07	9.11	<.001
Residual	54	4.732E+08	8.762E+06		
Total	83	2.802E+10			

Least significant differences of fungal slope means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	4845.7	1586.1

Table A.16 Analysis of variance for the kinetic parameter time to maximum rate for fungi grown in PDB

Variate: Time to maximum rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	2.794E+11	1.035E+10	10.44	<.001
Run	2	1.890E+10	9.450E+09	9.54	<.001
Residual	54	5.351E+10	9.910E+08		
Total	83	3.518E+11			

Least significant differences of fungal time to maximum rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	51532.7	16868.0

Table A.17 Analysis of variance for the kinetic parameter maximum value for fungi grown PDB

Variate: Maximum value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	1.4719612	0.0545171	73.64	<.001
Run	2	0.0044636	0.0022318	3.01	0.057
Residual	54	0.0399787	0.0007403		
Total	83	1.5164035			

Least significant differences of fungal maximum value means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	0.05	0.02

Table A.18 Analysis of variance for the kinetic parameter average rate for fungi grown in PDB

Variate: Average rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	2.340E-11	8.667E-13	89.46	<.001
Run	2	1.466E-13	7.330E-14	7.57	0.001
Residual	54	5.232E-13	9.689E-15		
Total	83	2.407E-11			

Least significant differences of fungal average rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	1.61E-07	5.27E-08

Table A.19 Analysis of variance for the kinetic parameter average value for fungi grown in PDB

Variate: Average value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	0.3517797	0.0130289	115.57	<.001
Run	2	0.0020467	0.0010234	9.08	<.001
Residual	54	0.0060876	0.0001127		
Total	83	0.3599141			

Least significant differences of fungal average rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	0.02	0.01

Table A.20 Analysis of variance for the kinetic parameter specific growth rate for fungi grown in PDB

Variate: Growth rate (μ)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	1.308E-02	4.843E-04	4682.14	<.001
Run	2	1.863E-07	9.313E-08	0.90	<.412
Residual	54	5.585E-06	1.034E-07		
Total	83	1.30E-02			

Least significant differences of fungal growth rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	5.3E-04	1.7E-04

Table A.21 Analysis of variance for the kinetic parameter lag phase ends for fungi grown in PDB

Variate: Lag phase ends ($Tlag_f$)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	3889.997	144.074	37.22	<.001
Run	2	66.001	33.001	8.53	<.001
Residual	54	209.034	3.871		
Total	83	4165.032			

Least significant differences of fungal lag phase ends means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	3.221	1.054

Table A.22 Analysis of variance for the kinetic parameter generation rate for fungi grown in PDB

Variate: Generation rate (v)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	2.722E-02	1.008E-03	4682.14	<.001
Run	2	3.877E-07	1.939E-07	0.90	0.412
Residual	54	1.163E-05	2.153E-07		

Least significant differences of fungal generation rate means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	7.6E-04	2.5E-4

Table A 23 Analysis of variance for the kinetic parameter duplication time for fungi grown in PDB

Variate: Duplication time (Td)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	27	2350.0547	87.0391	89.06	<.001
Run	2	2.1704	1.0852	1.11	0.337
Residual	54	52.7723	0.9773		
Total	83	2404.9974			

Least significant differences of fungal duplication time means (5% level)

Table	Isolate	Run
rep.	3	28
d.f.	54	54
l.s.d.	1.618	0.530

Analysis of variance between fungal growth in roots exudates and in PDB

Table A.24 Analysis of variance for the kinetic parameter change

Variate: Change

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	0.041780	0.020890	13.75	<.001
Medium	1	0.733308	0.733308	482.69	<.001
Isolate	27	1.474470	0.054610	35.95	<.001
Medium.Isolate	27	0.533001	0.019741	12.99	<.001
Residual	110	0.167114	0.001519		
Total	167	2.949674			

Tables of means
Grand mean: 0.2339

Medium	PDB	RE50%
	0.2999	0.1678

Least significant differences of fungal change means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.01460	0.01192	0.04460	0.06307

Table A.25 Analysis of variance for the kinetic parameter maximum rate

Variate: Maximum rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	4.404E-09	2.202E-09	2.75	0.068
Medium	1	4.325E-08	4.325E-08	53.97	<.001
Isolate	27	3.846E-07	1.425E-08	17.78	<.001
Medium.Isolate	27	2.550E-07	9.443E-09	11.78	<.001
Residual	110	8.814E-08	8.013E-10		
Total	167	7.754E-07			

Tables of means
Grand mean: 0.0001152

Medium	PDB	RE50%
	1.312357E-04	9.914664E-05

Least significant differences of fungal maximum rate means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.00001060	0.00000866	0.00003239	0.00004580

Table A.26 Analysis of variance for the kinetic parameter slope

Variate: Slope

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	3.126E+08	1.563E+08	11.29	<.001
Medium	1	1.910E+11	1.910E+11	13797.64	<.001
Isolate	27	3.613E+10	1.338E+09	96.68	<.001
Medium.Isolate	27	6.013E+09	2.227E+08	16.09	<.001
Residual	110	1.523E+09	1.384E+07		
Total	167	2.350E+11			

Tables of means
Grand mean: 51872.

Medium	PDB	RE50%
	85589.	18155.

Least significant differences of fungal slope means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	1393.4	1137.7	4256.9	6020.1

Table A.27 Analysis of variance for the kinetic parameter time to maximum rate

Variate: Time to maximum rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	5.694E+08	2.847E+08	0.21	0.812
Medium	1	4.997E+08	4.997E+08	0.37	0.546
Isolate	27	5.739E+11	2.125E+10	15.59	<.001
Medium.Isolate	27	4.166E+10	1.543E+09	1.13	0.318
Residual	110	1.499E+11	1.363E+09		
Total	167	7.665E+11			

Tables of means
Grand mean: 206572.

Medium	PDB	RE50%
	208296.	204847.

RE50%	105450.	299700.	290850.	210450.

Least significant differences of fungal maximum rate means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	13827.0	11289.7	42242.0	59739.3

Table A.28 Analysis of variance for the kinetic parameter maximum value

Variate: Maximum value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	0.030151	0.015076	13.85	<.001
Medium	1	4.046429	4.046429	3718.11	<.001
Isolate	27	1.521158	0.056339	51.77	<.001
Medium.Isolate	27	0.566536	0.020983	19.28	<.001
Residual	110	0.119713	0.001088		
Total	167	6.283987			

Tables of means
Grand mean: 0.3263

Medium	PDB	RE50%
	0.4815	0.1711

Least significant differences of fungal maximum value means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.01236	0.01009	0.03775	0.05338

Table A.29. Analysis of variance for the kinetic parameter average rate

Variate: Average rate

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	5.728E-13	2.864E-13	13.86	<.001
Medium	1	9.170E-12	9.170E-12	443.78	<.001
Isolate	27	2.765E-11	1.024E-12	49.56	<.001
Medium.Isolate	27	1.004E-11	3.719E-13	18.00	<.001
Residual	110	2.273E-12	2.066E-14		
Total	167	4.971E-11			

Tables of means
Grand mean: 9.76127929E-07

Medium	PDB	RE50%
	1.20976202E-06	7.42493832E-07

Least significant differences of fungal average rate means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	5.4E-08	4.4E-08	1.6E-07	2.3E-07

Table A.30 Analysis of variance for the kinetic parameter average value

Variate: Average value

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	0.0045270	0.0022635	11.24	<.001
Medium	1	2.3299937	2.3299937	11567.29	<.001
Isolate	27	0.5011453	0.0185609	92.15	<.001
Medium.Isolate	27	0.0778695	0.0028841	14.32	<.001
Residual	110	0.0221572	0.0002014		
Total	167	2.9356928			

Tables of means
Grand mean: 0.1891

Medium	PDB	RE50%
	0.3068	0.0713

Least significant differences of fungal average value means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.00532	0.00434	0.01624	0.02297

Table A.31 Analysis of variance for the kinetic parameter average value

Variate: growth rate (μ)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	3.204E-05	1.602E-05	7.69	<.001
Medium	1	4.142E-03	4.142E-03	1988.75	<.001
Isolate	27	6.996E-03	2.591E-04	124.41	<.001
Medium.Isolate	27	6.343E-03	2.349E-04	112.80	<.001
Residual	110	2.291E-04	2.083E-06		
Total	167	1.774E-02			

Tables of means
Grand mean: 0.00873

Medium	PDB	RE50%
	0.01370	0.00377

Least significant differences of fungal growth rate means (5% level)

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.000540	0.000441	0.001651	0.002335

Table A.32 Analysis of variance for the kinetic parameter lag phase ends

Variate: Lag phase ends ($Tlag_f$)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	144.75	72.37	6.05	0.003
Medium	1	144.81	144.81	12.10	<.001
Isolate	27	9388.03	347.70	29.05	<.001
Medium.Isolate	27	3504.85	129.81	10.84	<.001
Residual	110	1316.73	11.97		
Total	167	14499.16			

Tables of means
Grand mean: 27.86

Medium	PDB	RE50%
	26.93	28.79

Least significant differences of fungal lag phase ends

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	1.296	1.058	3.959	5.598

Table A.33 Analysis of variance for the kinetic parameter duplication time

Variate: Duplication time (Td)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	59.447	29.724	4.28	0.016
Medium	1	172.874	172.874	24.90	<.001
Isolate	27	1545.402	57.237	8.24	<.001
Medium.Isolate	27	1427.229	52.860	7.61	<.001
Residual	110	763.798	6.944		
Total	167	3968.750			

Tables of means

Grand mean: 3.86

Medium	PDB	RE50%
	2.85	4.88

Least significant differences of fungal duplication time

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.987	0.806	3.015	4.264

Table A.34 Analysis of variance for the kinetic parameter generation rate

Variate: Generation rate (v)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Run	2	6.669E-05	3.334E-05	7.69	<.001
Medium	1	8.622E-03	8.622E-03	1988.75	<.001
Isolate	27	1.456E-02	5.394E-04	124.41	<.001
Medium.Isolate	27	1.320E-02	4.890E-04	112.80	<.001
Residual	110	4.769E-04	4.336E-06		
Total	167	3.693E-02			

Tables of means

Grand mean: 0.01260

Medium	PDB	RE50%
	0.01976	0.00544

Least significant differences of fungal generation rate

Table	Run	Medium	Isolate	Medium Isolate
rep.	56	84	6	3
d.f.	110	110	110	110
l.s.d.	0.000780	0.000637	0.002382	0.003369

Section 2.3.7 *Costelytra giveni* bioassays

Section 2.3.7.1 Mortality and mycosis caused by *Metarhizium spp*. and *Beauveria bassiana* in 20% (w/v) soil moisture

Table A.35 Analysis of variance for the population of alive larvae 20 days post inoculation

Variate: Alive20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.04320	0.02160	1.67	0.201
Isolates	6	0.55695	0.09282	7.18	<.001
conidia/mL	2	0.03585	0.01792	1.39	0.262
Lin	1	0.03231	0.03231	2.50	0.122
Quad	1	0.00353	0.00353	0.27	0.604
Isolates.conidia/mL	12	0.82235	0.06853	5.30	<.001
Isolates.Lin	6	0.48639	0.08106	6.27	<.001
Isolates.Quad	6	0.33596	0.05599	4.33	0.002
Residual	40	0.51701	0.01293		
Total	62	1.97535			

Tables of means
Grand mean: 0.772

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.805	0.917	0.917
Bb21		0.750	0.917	0.917
Bk41		0.583	0.833	0.833
F16		0.750	0.583	0.500
F447		0.917	0.833	0.667
F672		1.000	0.833	0.667
F99		0.833	0.417	0.750

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0709	0.1083	0.0709	0.1876

Table A.36 Analysis of variance for the population of dead larvae 20 days post inoculation

Variate: Dead20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.05045	0.02522	1.98	0.151
Isolates	6	0.24201	0.04033	3.16	0.012
conidia/mL	2	0.08533	0.04266	3.35	0.045
Lin	1	0.07982	0.07982	6.26	0.017
Quad	1	0.00551	0.00551	0.43	0.515
Isolates.conidia/mL	12	0.72654	0.06054	4.75	<.001
Isolates.Lin	6	0.44577	0.07430	5.83	<.001
Isolates.Quad	6	0.28076	0.04679	3.67	0.005
Residual	40	0.50976	0.01274		
Total	62	1.61407			

Tables of means
Grand mean: 0.189

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.139	0.083	0.083
Bb21		0.250	0.083	0.083
Bk41		0.333	0.167	0.167
F16		0.000	0.333	0.333
F447		0.083	0.083	0.333
F672		0.000	0.167	0.333
F99		0.167	0.500	0.250

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0704	0.1076	0.0704	0.1863

Table A.37 Analysis of variance for the population of larvae with mycosis 20 days post inoculation

Variate: Mycosis20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.004195	0.002097	0.40	0.672
Isolates	6	0.181243	0.030207	5.79	<.001
conidia/mL	2	0.010780	0.005390	1.03	0.365
Lin	1	0.010561	0.010561	2.02	0.163
Quad	1	0.000219	0.000219	0.04	0.839
Isolates.conidia/mL	12	0.078677	0.006556	1.26	0.281
Isolates.Lin	6	0.014865	0.002478	0.47	0.823
Isolates.Quad	6	0.063812	0.010635	2.04	0.083
Residual	40	0.208731	0.005218		
Total	62	0.483626			

Tables of means
Grand mean: 0.0383

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.0553	0.0000	0.0000
Bb21		0.0000	0.0000	0.0000
Bk41		0.0833	0.0000	0.0000
F16		0.2500	0.0833	0.1667
F447		0.0000	0.0833	0.0000
F672		0.0000	0.0000	0.0000
F99		0.0000	0.0833	0.0000

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.04506	0.06882	0.04506	0.11921

Table A.38 Analysis of variance for the population of alive larvae 35 days post inoculation

Variate: Alive35

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.23866	0.11933	7.90	0.001
Isolates	6	0.19055	0.03176	2.10	0.074
conidia/mL	2	0.78285	0.39143	25.93	<.001
Lin	1	0.69892	0.69892	46.30	<.001
Quad	1	0.08393	0.08393	5.56	0.023
Isolates.conidia/mL	12	0.54146	0.04512	2.99	0.005
Isolates.Lin	6	0.22603	0.03767	2.50	0.038
Isolates.Quad	6	0.31543	0.05257	3.48	0.007
Residual	40	0.60386	0.01510		
Total	62	2.35738			

Tables of means
Grand mean: 0.147

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.195	0.083	0.055
Bb21		0.167	0.417	0.167
Bk41		0.500	0.083	0.000
F16		0.250	0.083	0.000
F447		0.250	0.000	0.000
F672		0.333	0.000	0.000
F99		0.417	0.000	0.083

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0766	0.1171	0.0766	0.2028

Table A.39. Analysis of variance for the population of dead larvae 35 days post inoculation

Variate: Dead35

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.28212	0.14106	4.31	0.020
Isolates	6	0.24206	0.04034	1.23	0.311
conidia/mL	2	0.36581	0.18291	5.58	0.007
Lin	1	0.23896	0.23896	7.29	0.010
Quad	1	0.12686	0.12686	3.87	0.056
Isolates.conidia/mL	12	0.72211	0.06018	1.84	0.075
Isolates.Lin	6	0.18241	0.03040	0.93	0.485
Isolates.Quad	6	0.53970	0.08995	2.75	0.025
Residual	40	1.31028	0.03276		
Total	62	2.92240			

Tables of means
Grand mean: 0.615

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.666	0.611	0.722
Bb21		0.750	0.583	0.833
Bk41		0.417	0.667	0.833
F16		0.500	0.583	0.583
F447		0.667	0.250	0.667

F672	0.583	0.417	0.750
F99	0.417	0.750	0.667

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.1129	0.1724	0.1129	0.2987

Table A.40 Analysis of variance for the population of larvae with mycosis 35 days post inoculation

Variate: Mycosis35

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.00593	0.00296	0.21	0.815
Isolates	6	0.77576	0.12929	8.96	<.001
conidia/mL	2	0.53770	0.26885	18.62	<.001
Lin	1	0.12054	0.12054	8.35	0.006
Quad	1	0.41716	0.41716	28.90	<.001
Isolates.conidia/mL	12	0.68452	0.05704	3.95	<.001
Isolates.Lin	6	0.09821	0.01637	1.13	0.361
Isolates.Quad	6	0.58631	0.09772	6.77	<.001
Residual	40	0.57741	0.01444		
Total	62	2.58132			

Tables of means

Grand mean: 0.238

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.139	0.306	0.222
Bb21		0.083	0.000	0.000
Bk41		0.083	0.250	0.167
F16		0.250	0.333	0.417
F447		0.083	0.750	0.333
F672		0.083	0.583	0.250
F99		0.167	0.250	0.250

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0749	0.1145	0.0749	0.1983

Section 2.3.7.2 Mortality and mycosis due to *Metarhizium* spp. and *Beauveria bassiana* in 30% (w/v) soil moisture

Table A.41 Analysis of variance for the population of alive larvae 20 days post inoculation

Variate: Alive20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.0019841	0.0009921	1.00	0.377
Isolates	6	1.3018810	0.2169802	218.72	<.001
conidia/mL	2	2.0379286	1.0189643	1027.12	<.001
Lin	1	1.4304286	1.4304286	1441.87	<.001
Quad	1	0.6075000	0.6075000	612.36	<.001

Isolates.conidia/mL	12	1.2814050	0.1067837	107.64	<.001
Isolates.Lin	6	0.7465716	0.1244286	125.42	<.001
Isolates.Quad	6	0.5348334	0.0891389	89.85	<.001
Residual	40	0.0396825	0.0009921		
Total	62	4.6628813			

Tables of means
Grand mean: 0.1746

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.0000	0.0000	0.0830
Bb21		1.0000	0.0000	0.3333
Bk41		0.2500	0.0000	0.0000
F16		0.2500	0.0000	0.0000
F447		0.2500	0.0000	0.0000
F672		0.5000	0.0000	0.0000
F99		0.7500	0.2500	0.0000

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.01965	0.03001	0.01965	0.05198

Table A.42 Analysis of variance for the population of dead larvae 20 days post inoculation

Variate: Dead20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.02929	0.01465	0.88	0.422
Isolates	6	0.43120	0.07187	4.32	0.002
conidia/mL	2	0.21202	0.10601	6.37	0.004
Lin	1	0.20273	0.20273	12.19	0.001
Quad	1	0.00929	0.00929	0.56	0.459
Isolates.conidia/mL	12	1.97025	0.16419	9.87	<.001
Isolates.Lin	6	1.41656	0.23609	14.20	<.001
Isolates.Quad	6	0.55369	0.09228	5.55	<.001
Residual	40	0.66526	0.01663		
Total	62	3.30803			

Tables of means
Grand mean: 0.574

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.722	0.639	0.778
Bb21		0.000	0.750	0.667
Bk41		0.750	0.750	0.417
F16		0.500	0.333	0.583
F447		0.750	0.667	0.417
F672		0.500	0.500	0.750
F99		0.250	0.500	0.833

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0804	0.1229	0.0804	0.2128

Table A.43 Analysis of variance for the population of larvae with mycosis 20 days post inoculation

Variate: Mycosis20

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.04582	0.02291	1.51	0.233
Isolates	6	0.73675	0.12279	8.09	<.001
conidia/mL	2	1.02267	0.51134	33.69	<.001
Lin	1	0.55614	0.55614	36.64	<.001
Quad	1	0.46653	0.46653	30.74	<.001
Isolates.conidia/mL	12	0.94192	0.07849	5.17	<.001
Isolates.Lin	6	0.67076	0.11179	7.37	<.001
Isolates.Quad	6	0.27116	0.04519	2.98	0.017
Residual	40	0.60707	0.01518		
Total	62	3.35423			

Tables of means

Grand mean: 0.251

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.278	0.361	0.139
Bb21		0.000	0.250	0.000
Bk41		0.000	0.250	0.583
F16		0.250	0.667	0.417
F447		0.000	0.333	0.583
F672		0.000	0.500	0.250
F99		0.000	0.250	0.167

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0768	0.1174	0.0768	0.2033

Table A.44 Analysis of variance for the population of alive larvae 35 days post inoculation

Variate: Alive35

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.001984	0.000992	0.32	0.726
Isolates	6	0.388889	0.064815	21.08	<.001
conidia/mL	2	0.126984	0.063492	20.65	<.001
Lin	1	0.095238	0.095238	30.97	<.001
Quad	1	0.031746	0.031746	10.32	0.003
Isolates.conidia/mL	12	0.539683	0.044974	14.62	<.001
Isolates.Lin	6	0.238095	0.039683	12.90	<.001
Isolates.Quad	6	0.301587	0.050265	16.34	<.001
Residual	40	0.123016	0.003075		
Total	62	1.180556			

Tables of means

Grand mean: 0.0556

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.0000	0.0000	0.0000

Bb21	0.5000	0.0000	0.1667
Bk41	0.0000	0.0000	0.0000
F16	0.0000	0.0000	0.0000
F447	0.0000	0.0000	0.0000
F672	0.3333	0.0000	0.0000
F99	0.0000	0.1667	0.0000

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.03459	0.05284	0.03459	0.09151

Table A.45 Analysis of variance for the population of dead larvae 35 days post inoculation

Variate: Dead35

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.06608	0.03304	1.54	0.227
Isolates	6	0.44444	0.07407	3.45	0.008
conidia/mL	2	0.09793	0.04896	2.28	0.116
Lin	1	0.08000	0.08000	3.72	0.061
Quad	1	0.01793	0.01793	0.83	0.367
Isolates.conidia/mL	12	1.19861	0.09988	4.65	<.001
Isolates.Lin	6	0.45240	0.07540	3.51	0.007
Isolates.Quad	6	0.74621	0.12437	5.79	<.001
Residual	40	0.85977	0.02149		
Total	62	2.66683			

Tables of means

Grand mean: 0.583

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.722	0.583	0.695
Bb21		0.500	0.667	0.833
Bk41		0.500	0.750	0.417
F16		0.750	0.167	0.500
F447		0.583	0.667	0.333
F672		0.667	0.500	0.333
F99		0.750	0.583	0.750

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0914	0.1397	0.0914	0.2419

Table A.46 Analysis of variance for the population of larvae with mycosis 35 days post inoculation

Variate: Mycosis35

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Time_Rep	2	0.08790	0.04395	2.47	0.098
Isolates	6	1.11111	0.18519	10.39	<.001
conidia/mL	2	0.44720	0.22360	12.54	<.001
Lin	1	0.34981	0.34981	19.63	<.001
Quad	1	0.09739	0.09739	5.46	0.025

Isolates.conidia/mL	12	1.40490	0.11707	6.57	<.001
Isolates.Lin	6	0.51592	0.08599	4.82	<.001
Isolates.Quad	6	0.88897	0.14816	8.31	<.001
Residual	40	0.71295	0.01782		
Total	62	3.76406			

Tables of means
Grand mean: 0.361

Isolates	conidia/mL	$1x10^5$	$1x10^7$	$1x10^9$
A1080		0.278	0.417	0.305
Bb21		0.000	0.333	0.000
Bk41		0.500	0.250	0.583
F16		0.250	0.833	0.500
F447		0.417	0.333	0.667
F672		0.000	0.500	0.667
F99		0.250	0.250	0.250

Least significant differences of means (5% level)

Table	Time_Rep	Isolates	conidia/mL	Isolates conidia/mL
rep.	21	9	21	3
d.f.	40	40	40	40
l.s.d.	0.0833	0.1272	0.0833	0.2203

B.2 Chapter 3 – Fungal interactions with maize

Section 3.5.1 Effect of conidia coated onto seeds on maize germination

Germination test between paper method

Table A. 47 Analysis of variance of normal seedlings

Variate: Normal seedlings

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	32.333	16.167	4.62	0.178
Trial	1	13.500	13.500	3.86	0.188
Residual	2	7.000	3.500		
Total	5	52.833			

Tables of means
Grand mean: 86.2

Treatment	CC	PC	PS
	87.00	83.00	88.50

Trial	1	2
	87.67	84.67

Least significant differences of means (5% level)

Table	Treatment	Trial
rep.	2	3
d.f.	2	2
l.s.d.	8.050	6.572

Table A.48 Analysis of variance of abnormal seedlings

Variate: Abnormal seedlings

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	7.000	3.500	3.00	0.250
Trial	1	10.667	10.667	9.14	0.094
Residual	2	2.333	1.167		
Total	5	20.000			

Tables of means
Grand mean: 12.00

Treatment	CC	PC	PS
	11.00	13.50	11.50

Trial	1	2
	10.67	13.33

Least significant differences of means (5% level)

Table	Treatment	Trial
rep.	2	3
d.f.	2	2
l.s.d.	4.647	3.795

Table A.49 Analysis of variance of ungerminated seeds

Variate: Ungerminated seeds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Treatment	2	12.333	6.167	5.29	0.159
Trial	1	0.167	0.167	0.14	0.742
Residual	2	2.333	1.167		
Total	5	14.833			

Tables of means
Grand mean: 1.83

Treatment	CC	PC	PS
	2.00	3.50	0.00

Trial	1	2
	1.67	2.00

Least significant differences of means (5% level)

Table	Treatment	Trial
rep.	2	3
d.f.	2	2
l.s.d.	4.647	3.795

Germination test in sand

Table A.50 Analysis of variance of normal seedlings

Variate: Normal seedlings

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	68.679	9.811	1.10	0.417
Cond/mL	3	1.500	0.500	0.06	0.982
Isolate.Cond/mL	3	83.000	27.667	3.11	0.064
Trial	1	15.750	15.750	1.77	0.206

Residual		13	115.750	8.904			
Total		27	284.679				

Tables of means
Grand mean: 93.1

Isolate	CS	F11	F133	F138	F16	F672	F99
	89.50	94.00	92.50	93.50	92.75	93.75	92.00
rep.	2	4	4	4	4	4	4

Isolate	PC
	97.00
rep.	2

Cond/mL	100000	100000000	CS	PC
	93.36	92.86	93.11	93.11
rep.	12	12	2	2

Isolate	Cond/mL	100000	100000000	CS	PC
CS				89.50	
F11		93.50	94.50		
F133		91.00	94.00		
F138		95.50	91.50		
F16		90.50	95.00		
F672		94.50	93.00		
F99		95.00	89.00		
PC					97.00

Trial	1	2
	93.86	92.36

Least significant differences of means (5% level)

Table	Isolate	Cond/mL	Isolate Cond/mL	Trial	
rep.	unequal	unequal	2	14	
d.f.	13	13	13	13	
l.s.d.	6.446	6.446			min.rep
	5.583	4.924	6.446	2.437	max-min
	4.558	2.632			max.rep

Table A.51 Analysis of variance abnormal seedlings

Variate: Abnormal seedlings

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	7.929	1.133	0.42	0.876
Cond/mL	3	0.667	0.222	0.08	0.969
Isolate.Cond/mL	3	17.833	5.944	2.18	0.139
Trial	1	3.571	3.571	1.31	0.273
Residual	13	35.429	2.725		
Total	27	65.429			

Tables of means
Grand mean: 2.96

Isolate	CS	F11	F133	F138	F16	F672	F99
	1.50	2.50	3.00	2.75	2.75	3.50	3.50
rep.	2	4	4	4	4	4	4

Isolate	PC
	2.50
rep.	2

Cond/mL	100000	100000000	CS	PC
	2.69	3.02	2.86	2.86
rep.	12	12	2	2

Isolate	Cond/mL	100000	100000000	CS	PC
CS				1.50	
F11		2.00	3.00		
F133		2.50	3.50		
F138		2.00	3.50		
F16		4.50	1.00		
F672		3.00	4.00		
F99		3.00	4.00		
PC					2.50

Trial	1	2
	3.21	2.50

Least significant differences of means (5% level)

Table	Isolate	Cond/mL	Isolate Cond/mL	Trial	
rep.	unequal	unequal	2	14	
d.f.	13	13	13	13	
l.s.d.	3.566	3.566			min.rep
	3.089	2.724	3.566	1.348	max-min
	2.522	1.456			max.rep

Table A 52. Analysis of variance ungerminated seeds

Variate: Ungerminated seedlings

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	84.964	12.138	1.81	0.169
Cond/mL	3	0.167	0.056	0.01	0.999
Isolate.Cond/mL	3	52.333	17.444	2.60	0.097
Trial	1	34.321	34.321	5.12	0.041
Residual	13	87.179	6.706		
Total	27	258.964			

Tables of means

Grand mean: 4.04

Isolate	CS	F11	F133	F138	F16	F672	F99
	9.00	3.50	4.50	3.75	4.50	2.75	4.50
rep.	2	4	4	4	4	4	4

Isolate	PC
	0.50
rep.	2

Cond/mL	100000	100000000	CS	PC

	3.95	4.12	4.04	4.04	
rep.	12	12	2	2	

Isolate	Cond/mL	100000	100000000	CS	PC
CS				9.00	
F11		4.50	2.50		
F133		6.50	2.50		
F138		2.50	5.00		
F16		5.00	4.00		
F672		2.50	3.00		
F99		2.00	7.00		
PC					0.50

Trial	1	2
	2.93	5.14

Least significant differences of means (5% level)

Table	Isolate	Cond/mL	Isolate Cond/mL	Trial	
rep.	unequal	unequal	2	14	
d.f.	13	13	13	13	
l.s.d.	5.594	5.594			min.rep
	4.845	4.273	5.594	2.115	max-min
	3.956	2.284			max.rep

Table A.53 Analysis of variance of maize seedlings

Variate: Emergence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	1	10.32	10.32	0.49	0.495
Isolate	7	244.00	34.86	1.66	0.203
Cond	3	9.38	3.12	0.15	0.928
Isolate.Cond	3	173.38	57.79	2.76	0.084
Residual	13	272.18	20.94		
Total	27	709.25			

Tables of means

Grand mean: 88.75

Trial	1	2
	89.36	88.14

Isolate	CS	F11	F133	F138	F16	F672	F99
	82.00	92.25	85.75	89.00	86.75	91.50	89.00
rep.	2	4	4	4	4	4	4

Isolate	PC
	92.00
rep.	2

Cond	100000	100000000	CS	PC
	88.12	89.38	88.75	88.75
rep.	12	12	2	2

Isolate	Cond	100000	100000000	CS	PC
CS				82.00	

F11	91.50	93.00		
F133	82.00	89.50		
F138	88.50	89.50		
F16	83.50	90.00		
F672	91.50	91.50		
F99	93.50	84.50		
PC			92.00	

Least significant differences of means (5% level)

Table	Trial	Isolate	Cond	Isolate Cond	
rep.	14	unequal	unequal	2	
d.f.	13	13	13	13	
l.s.d.		9.885	9.885		min.rep
	3.736	8.561	7.550	9.885	max-min
		6.990	4.036		max.rep

Variate: Maize Shoot

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	1	0.0175	0.0175	0.11	0.745
Isolate	7	9.5818	1.3688	8.61	<.001
Cond	3	0.0600	0.0200	0.13	0.943
Isolate.Cond	3	0.5400	0.1800	1.13	0.372
Residual	13	2.0675	0.1590		
Total	27	12.2668			

Tables of means

Grand mean: 3.661

Trial	1	2					
	3.686	3.636					
Isolate	CS	F11	F133	F138	F16	F672	F99
	2.500	4.225	3.250	3.325	3.725	4.375	4.100
rep.	2	4	4	4	4	4	4
Isolate	PC						
	2.750						
rep.	2						
Cond	100000	100000000	CS	PC			
	3.611	3.711	3.661	3.661			
rep.	12	12	2	2			

Isolate	Cond	100000	100000000	CS	PC
CS				2.500	
F11		4.350	4.100		
F133		3.000	3.500		
F138		3.250	3.400		
F16		3.500	3.950		
F672		4.500	4.250		
F99		4.100	4.100		
PC					2.750

Least significant differences of means (5% level)

Table	Trial	Isolate	Cond	Isolate

				Cond	
rep.	14	unequal	unequal	2	
d.f.	13	13	13	13	
l.s.d.		0.8615	0.8615		min.rep
	0.3256	0.7461	0.6580	0.8615	max-min
		0.6092	0.3517		max.rep

Section 3.5.2 Evaluation of maize plant performance after coating with conidia from entomopathogenic fungi

Table A.54 Analysis of variance effect of conidia concentration on maize plant performance

Variate: Height (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum	2	226.11	113.05	10.29	
Block.*Units* stratum					
Treatment	7	348.10	49.73	4.53	<.001
Conidia/mL	2	23.38	11.69	1.06	0.346
Treatment.Conidia/mL	4	157.08	39.27	3.57	0.007
Trial	1	9314.80	9314.80	847.90	<.001
Residual	319	3504.43	10.99		
Total	335	13573.89			

Tables of means
Variate: Height

Grand mean: 24.17

Treatment	CS	F11	F133	F138	F16	F672	F99
	24.15	23.21	22.93	23.61	24.48	24.35	25.13
rep.	24	48	48	48	48	48	48

Treatment	PC
	26.79
rep.	24

Conidia/mL	0	10 5	10 8
	24.17	24.45	23.88
rep.	48	144	144

Treatment	Conidia/mL	0	10 5	10 8
CS		24.15		
F11			22.74	23.68
F133			22.88	22.98
F138			23.23	24.00
F16			24.52	24.44
F672			25.85	22.85
F99			26.20	24.07
PC		26.79		

Trial	1	2
	18.90	29.43

Least significant differences of means (5% level)

Table	Treatment	Conidia/mL	Treatment Conidia/mL	Trial
rep.	unequal	unequal	24	168
d.f.	319	319	319	319

l.s.d.	1.882	1.331X			min.rep
	1.630	1.087	1.882	0.711	max-min
	1.331	0.769			max.rep

Section 3.5.4 Effect of the entomopathogenic fungal isolates in maize plant performance in the presence of *C. giveni* and *F. graminearum*

Table A.55 Analysis of variance emergence of maize treated plants

Variate: Emergence

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial stratum	2	22.7111	11.3556	46.45	
Trial.MainPlot stratum					
Insect	1	1.6056	1.6056	6.57	0.043
Fusarium	1	0.1389	0.1389	0.57	0.480
Insect.*Fusarium*	1	0.0056	0.0056	0.02	0.885
Residual	6	1.4667	0.2444	0.37	
Trial.MainPlot.*Units* stratum					
Isolate	8	1.5333	0.1917	0.29	0.967
Insect.Isolate	8	2.2000	0.2750	0.42	0.907
Fusarium.Isolate	8	2.7778	0.3472	0.53	0.832
Insect.*Fusarium*.Isolate	8	6.6889	0.8361	1.28	0.260
Residual	136	88.9333	0.6539		
Total	179	128.0611			

Tables of means
Grand mean: 2.628

Insect	+	-
	2.533	2.722

Fusarium	+	-
	2.600	2.656

Isolate	A1080	Bb21	BK41	Coated Seeds	F16
	2.556	2.917	2.583	2.583	2.583
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	2.583	2.750	2.639	2.667
rep.	12	12	36	12

Least significant differences of means (5% level)

Table	Insect	*Fusarium*	Isolate	Insect *Fusarium*	
rep.	90	90	unequal	45	
d.f.	6	6	136	6	
l.s.d.			0.6529		min.rep
	0.1803	0.1803	0.5331	0.2550	max-min
			0.3769		max.rep

Table A.56 Analysis of variance total length maize plants

Variate: log Length (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.MainPlot stratum					
Trial	2	1.289004	0.644502	65.88	<.001
Fusarium	1	0.034776	0.034776	3.55	0.108
C. giveni	1	0.188863	0.188863	19.31	0.005
Fusarium.*C. giveni*	1	0.006314	0.006314	0.65	0.452
Residual	6	0.058695	0.009782	2.15	
Trial.MainPlot.*Units* stratum					
Isolate	8	0.043698	0.005462	1.20	0.304
Isolate.*Fusarium*	8	0.071653	0.008957	1.97	0.055
Isolate.*C. giveni*	8	0.021974	0.002747	0.60	0.774
Isolate.*Fusarium*.*C. giveni*	8	0.024686	0.003086	0.68	0.710
Residual	136	0.619165	0.004553		
Total	179	2.358828			

Tables of means

Grand mean: 1.8053

Trial	1	2	4
	1.8724	1.8574	1.6859

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	1.8022	1.7763	1.7792	1.8176	1.8267
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	1.7905	1.8006	1.8196	1.7873
rep.	12	12	36	12

Fusarium	+	-
	1.7914	1.8192

C. giveni	+	-
	1.7729	1.8376

Isolate	*Fusarium*	+	-
A1080		1.8038	1.8006
	rep.	18	18
Bb21		1.7064	1.8461
	rep.	6	6
BK41		1.7827	1.7757
	rep.	6	6
Control Seeds		1.8128	1.8224
	rep.	18	18
F16		1.8350	1.8184
	rep.	6	6
F672		1.7633	1.8178
	rep.	6	6
F99		1.7887	1.8126
	rep.	6	6
FCC327		1.7996	1.8396
	rep.	18	18
FCC447		1.7456	1.8291
	rep.	6	6

Isolate	*C. giveni*	+	-
A1080		1.7862	1.8182
	rep.	18	18
Bb21		1.7303	1.8222
	rep.	6	6
BK41		1.7544	1.8040
	rep.	6	6
Control Seeds		1.7810	1.8542
	rep.	18	18
F16		1.8100	1.8434
	rep.	6	6
F672		1.7507	1.8303
	rep.	6	6
F99		1.7660	1.8353
	rep.	6	6
FCC327		1.7754	1.8639
	rep.	18	18
FCC447		1.7538	1.8209
	rep.	6	6

Fusarium	*C. giveni*	+	-
+		1.7530	1.8297
-		1.7927	1.8456

	Fusarium	+		-	
Isolate	*C. giveni*	+	-	+	-
A1080		1.7897	1.8180	1.7828	1.8184
	rep.	9	9	9	9
Bb21		1.6314	1.7814	1.8292	1.8630
	rep.	3	3	3	3
BK41		1.7362	1.8292	1.7726	1.7788
	rep.	3	3	3	3
Control Seeds		1.7785	1.8470	1.7834	1.8613
	rep.	9	9	9	9
F16		1.8167	1.8533	1.8033	1.8335
	rep.	3	3	3	3
F672		1.6939	1.8326	1.8076	1.8279
	rep.	3	3	3	3
F99		1.7654	1.8121	1.7665	1.8586
	rep.	3	3	3	3
FCC327		1.7474	1.8518	1.8033	1.8759
	rep.	9	9	9	9
FCC447		1.7053	1.7859	1.8022	1.8559
	rep.	3	3	3	3

Least significant differences of means (5% level)

Table	Trial	Isolate	*Fusarium*	*C. giveni*	
rep.	60	unequal	90	90	
d.f.	6	136	6	6	
l.s.d.		0.05447			min.rep
	0.04419	0.04448	0.03608	0.03608	max-min
		0.03145			max.rep

Table	Isolate *Fusarium*	Isolate *C. giveni*	*Fusarium* *C. giveni*	Isolate *Fusarium* *C. giveni*
rep.	unequal	unequal	45	unequal

l.s.d.	0.08004	0.08004		0.11320	min.rep
d.f.	117.96	117.96		117.96	
l.s.d.	0.06671	0.06671	0.05102	0.09434	max-min
d.f.	91.05	91.05	6	91.05	
l.s.d.	0.05031	0.05031		0.07115	max.rep
d.f.	42.62	42.62		42.62	
Except when comparing means with the same level(s) of					
Fusarium	0.07704				min.rep
d.f.	136				
	0.06290				max-min
d.f.	136				
	0.04448				max.rep
d.f.	136				
C. giveni		0.07704			min.rep
d.f.		136			
		0.06290			max-min
d.f.		136			
		0.04448			max.rep
d.f.		136			
Fusarium.*C. giveni*				0.10895	min.rep
d.f.				136	
				0.08896	max-min
d.f.				136	
				0.06290	max.rep
d.f.				136	

Table A.57. Analysis of variance shoot length treated maize plants

Variate: log Shoot (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.MainPlot stratum					
Trial	2	6.470734	3.235367	234.06	<.001
Fusarium	1	0.019099	0.019099	1.38	0.284
C. giveni	1	0.070635	0.070635	5.11	0.064
Fusarium.*C. giveni*	1	0.006230	0.006230	0.45	0.527
Residual	6	0.082935	0.013823	4.86	
Trial.MainPlot.*Units* stratum					
Isolate	8	0.068061	0.008508	2.99	0.004
Isolate.*Fusarium*	8	0.071334	0.008917	3.13	0.003
Isolate.*C. giveni*	8	0.010368	0.001296	0.46	0.885
Isolate.*Fusarium*.*C. giveni*	8	0.027473	0.003434	1.21	0.300
Residual	136	0.387069	0.002846		
Total	179	7.213938			

Tables of means

Grand mean: 1.4533

Trial	1	2	4
	1.5924	1.5823	1.1852

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	1.4492	1.3894	1.4375	1.4638	1.4703
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	1.4668	1.4524	1.4656	1.4473
rep.	12	12	36	12

Fusarium	+	-
	1.4430	1.4636

C. giveni	+	-
	1.4335	1.4731

Isolate	*Fusarium*	+	-
A1080		1.4534	1.4450
	rep.	18	18
Bb21		1.3195	1.4593
	rep.	6	6
BK41		1.4550	1.4201
	rep.	6	6
Control Seeds		1.4547	1.4729
	rep.	18	18
F16		1.4761	1.4646
	rep.	6	6
F672		1.4580	1.4756
	rep.	6	6
F99		1.4422	1.4626
	rep.	6	6
FCC327		1.4532	1.4780
	rep.	18	18
FCC447		1.4104	1.4843
	rep.	6	6

Isolate	*C. giveni*	+	-
A1080		1.4371	1.4613
	rep.	18	18
Bb21		1.3648	1.4140
	rep.	6	6
BK41		1.4246	1.4504
	rep.	6	6
Control Seeds		1.4432	1.4845
	rep.	18	18
F16		1.4418	1.4988
	rep.	6	6
F672		1.4407	1.4929
	rep.	6	6
F99		1.4381	1.4667
	rep.	6	6
FCC327		1.4365	1.4946
	rep.	18	18
FCC447		1.4420	1.4526
	rep.	6	6

Fusarium	*C. giveni*	+	-
+		1.4173	1.4687
-		1.4497	1.4775

	Fusarium	+		-	
Isolate	*C. giveni*	+	-	+	-
A1080		1.4376	1.4692	1.4366	1.4534
	rep.	9	9	9	9
Bb21		1.2690	1.3700	1.4607	1.4580
	rep.	3	3	3	3
BK41		1.4103	1.4998	1.4390	1.4011
	rep.	3	3	3	3

Control Seeds		1.4398	1.4696	1.4466	1.4993
	rep.	9	9	9	9
F16		1.4438	1.5084	1.4399	1.4892
	rep.	3	3	3	3
F672		1.4019	1.5142	1.4796	1.4716
	rep.	3	3	3	3
F99		1.4339	1.4504	1.4422	1.4830
	rep.	3	3	3	3
FCC327		1.4212	1.4852	1.4518	1.5041
	rep.	9	9	9	9
FCC447		1.4052	1.4156	1.4789	1.4896
	rep.	3	3	3	3

Least significant differences of means (5% level)

Table	Trial	Isolate	*Fusarium*	*C. giveni*	
rep.	60	unequal	90	90	
d.f.	6	136	6	6	
l.s.d.		0.04307			min.rep
	0.05252	0.03517	0.04288	0.04288	max-min
		0.02487			max.rep

Table	Isolate *Fusarium*	Isolate *C. giveni*	*Fusarium* *C. giveni*	Isolate *Fusarium* *C. giveni*	
rep.	unequal	unequal	45	unequal	
l.s.d.	0.06895	0.06895		0.09750	min.rep
d.f.	66.19	66.19		66.19	
l.s.d.	0.05973	0.05973	0.06065	0.08446	max-min
d.f.	42.42	42.42	6	42.42	
l.s.d.	0.04947	0.04947		0.06996	max.rep
d.f.	19.37	19.37		19.37	
Except when comparing means with the same level(s) of					
Fusarium	0.06091				min.rep
d.f.	136				
	0.04973				max-min
d.f.	136				
	0.03517				max.rep
d.f.	136				
C. giveni		0.06091			min.rep
d.f.		136			
		0.04973			max-min
d.f.		136			
		0.03517			max.rep
d.f.		136			
Fusarium.*C. giveni*				0.08614	min.rep
d.f.				136	
				0.07033	max-min
d.f.				136	
				0.04973	max.rep
d.f.				136	

Table A.58 Analysis of variance root length treated maize plants

Variate: log Root (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.MainPlot stratum					
Trial	2	0.01925	0.00963	0.75	0.510
Fusarium	1	0.05446	0.05446	4.27	0.084
C. giveni	1	0.42929	0.42929	33.62	0.001
Fusarium.*C. giveni*	1	0.01194	0.01194	0.94	0.371
Residual	6	0.07660	0.01277	0.92	
Trial.MainPlot.*Units* stratum					
Isolate	8	0.08843	0.01105	0.80	0.605
Isolate.*Fusarium*	8	0.12536	0.01567	1.13	0.346
Isolate.*C. giveni*	8	0.08090	0.01011	0.73	0.664
Isolate.*Fusarium*.*C. giveni*	8	0.05347	0.00668	0.48	0.867
Residual	136	1.88219	0.01384		
Total	179	2.82190			

Tables of means

Grand mean: 1.5259

Trial	1	2	4
	1.5405	1.5192	1.5179

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	1.5270	1.5103	1.4994	1.5376	1.5539
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	1.4769	1.5217	1.5478	1.4889
rep.	12	12	36	12

Fusarium	+	-
	1.5085	1.5433

C. giveni	+	-
	1.4770	1.5747

Isolate	*Fusarium*	+	-
A1080		1.5298	1.5241
	rep.	18	18
Bb21		1.4241	1.5965
	rep.	6	6
BK41		1.4887	1.5102
	rep.	6	6
Control Seeds		1.5399	1.5353
	rep.	18	18
F16		1.5688	1.5389
	rep.	6	6
F672		1.4301	1.5236
	rep.	6	6
F99		1.5020	1.5413
	rep.	6	6
FCC327		1.5223	1.5733
	rep.	18	18
FCC447		1.4374	1.5404
	rep.	6	6

Isolate	*C. giveni*	+	-
A1080		1.5045	1.5495
	rep.	18	18
Bb21		1.4270	1.5935
	rep.	6	6
BK41		1.4635	1.5354
	rep.	6	6
Control Seeds		1.4815	1.5937
	rep.	18	18
F16		1.5468	1.5610
	rep.	6	6
F672		1.4014	1.5523
	rep.	6	6
F99		1.4723	1.5710
	rep.	6	6
FCC327		1.4889	1.6066
	rep.	18	18
FCC447		1.4199	1.5578
	rep.	6	6

Fusarium	*C. giveni*	+	-
+		1.4515	1.5654
-		1.5026	1.5839

	Fusarium	+		-	
Isolate	*C. giveni*	+	-	+	-
A1080		1.5155	1.5442	1.4934	1.5548
	rep.	9	9	9	9
Bb21		1.2854	1.5628	1.5687	1.6242
	rep.	3	3	3	3
BK41		1.4323	1.5450	1.4946	1.5257
	rep.	3	3	3	3
Control Seeds		1.4851	1.5947	1.4779	1.5926
	rep.	9	9	9	9
F16		1.5624	1.5751	1.5311	1.5468
	rep.	3	3	3	3
F672		1.3210	1.5392	1.4818	1.5655
	rep.	3	3	3	3
F99		1.4587	1.5452	1.4858	1.5968
	rep.	3	3	3	3
FCC327		1.4524	1.5922	1.5254	1.6211
	rep.	9	9	9	9
FCC447		1.3536	1.5212	1.4863	1.5945
	rep.	3	3	3	3

Least significant differences of means (5% level)

Table	Trial	Isolate	*Fusarium*	*C. giveni*	
rep.	60	unequal	90	90	
d.f.	6	136	6	6	
l.s.d.		0.09498			min.rep
	0.05048	0.07755	0.04122	0.04122	max-min
		0.05483			max.rep

Table	Isolate *Fusarium*	Isolate *C. giveni*	*Fusarium* *C. giveni*	Isolate *Fusarium* *C. giveni*	
rep.	unequal	unequal	45	unequal	
l.s.d.	0.13393	0.13393		0.18941	min.rep

d.f.	140.67	140.67		140.67	
l.s.d.	0.10926	0.10926	0.05829	0.15452	max-min
d.f.	133.51	133.51	6	133.51	
l.s.d.	0.07726	0.07726		0.10926	max.rep
d.f.	93.38	93.38		93.38	
Except when comparing means with the same level(s) of					
Fusarium	0.13432				min.rep
d.f.	136				
	0.10967				max-min
d.f.	136				
	0.07755				max.rep
d.f.	136				
C. giveni		0.13432			min.rep
d.f.		136			
		0.10967			max-min
d.f.		136			
		0.07755			max.rep
d.f.		136			
Fusarium.*C. giveni*				0.18995	min.rep
d.f.				136	
				0.15510	max-min
d.f.				136	
				0.10967	max.rep
d.f.				136	

Table A 59. Analysis of variance total weight treated maize plants

Variate: log Total Weight (mg)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.MainPlot stratum					
Trial	2	0.029766	0.014883	0.37	0.708
Fusarium	1	0.013651	0.013651	0.34	0.583
C. giveni	1	0.447518	0.447518	11.02	0.016
Fusarium.*C. giveni*	1	0.005677	0.005677	0.14	0.721
Residual	6	0.243552	0.040592	5.25	
Trial.MainPlot.*Units* stratum					
Isolate	8	0.060308	0.007539	0.98	0.458
Isolate.*Fusarium*	8	0.093963	0.011745	1.52	0.156
Isolate.*C. giveni*	8	0.039342	0.004918	0.64	0.746
Isolate.*Fusarium*.*C. giveni*	8	0.070294	0.008787	1.14	0.343
Residual	136	1.051173	0.007729		
Total	179	2.055246			

Tables of means

Variate: log Weight (mg)

Grand mean: 2.3522

Trial	1	2	4
	2.3346	2.3651	2.3568

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	2.3546	2.3506	2.3334	2.3542	2.3906
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	2.3716	2.3028	2.3572	2.3357
rep.	12	12	36	12

Fusarium	+	-
	2.3435	2.3609

C. giveni	+	-
	2.3023	2.4020

Isolate	*Fusarium*	+	-
A1080		2.3620	2.3472
	rep.	18	18
Bb21		2.3341	2.3672
	rep.	6	6
BK41		2.3702	2.2967
	rep.	6	6
Control Seeds		2.3386	2.3698
	rep.	18	18
F16		2.4281	2.3531
	rep.	6	6
F672		2.3419	2.4013
	rep.	6	6
F99		2.2970	2.3086
	rep.	6	6
FCC327		2.3258	2.3885
	rep.	18	18
FCC447		2.3013	2.3700
	rep.	6	6

Isolate	*C. giveni*	+	-
A1080		2.3143	2.3949
	rep.	18	18
Bb21		2.3216	2.3797
	rep.	6	6
BK41		2.2973	2.3696
	rep.	6	6
Control Seeds		2.2945	2.4140
	rep.	18	18
F16		2.3514	2.4298
	rep.	6	6
F672		2.3290	2.4143
	rep.	6	6
F99		2.2422	2.3633
	rep.	6	6
FCC327		2.2864	2.4280
	rep.	18	18
FCC447		2.3079	2.3635
	rep.	6	6

Fusarium	*C. giveni*	+	-
+		2.2992	2.3877
-		2.3054	2.4164

	Fusarium	+		-	
Isolate	*C. giveni*	+	-	+	-
A1080		2.3220	2.4021	2.3066	2.3877
	rep.	9	9	9	9

Bb21		2.2976	2.3706	2.3456	2.3887
	rep.	3	3	3	3
BK41		2.3363	2.4041	2.2583	2.3351
	rep.	3	3	3	3
Control Seeds		2.2928	2.3844	2.2961	2.4435
	rep.	9	9	9	9
F16		2.4096	2.4466	2.2931	2.4131
	rep.	3	3	3	3
F672		2.2640	2.4199	2.3940	2.4087
	rep.	3	3	3	3
F99		2.2861	2.3079	2.1984	2.4188
	rep.	3	3	3	3
FCC327		2.2467	2.4049	2.3260	2.4511
	rep.	9	9	9	9
FCC447		2.3101	2.2925	2.3057	2.4344
	rep.	3	3	3	3

Least significant differences of means (5% level)

Table	Trial	Isolate	*Fusarium*	*C. giveni*	
rep.	60	unequal	90	90	
d.f.	6	136	6	6	
l.s.d.		0.07098			min.rep
	0.09001	0.05795	0.07349	0.07349	max-min
		0.04098			max.rep

Table	Isolate *Fusarium*	Isolate *C. giveni*	*Fusarium* *C. giveni*	Isolate *Fusarium* *C. giveni*	
rep.	unequal	unequal	45	unequal	
l.s.d.	0.11497	0.11497		0.16259	min.rep
d.f.	61.38	61.38		61.38	
l.s.d.	0.10007	0.10007	0.10393	0.14151	max-min
d.f.	39.12	39.12	6	39.12	
l.s.d.	0.08370	0.08370		0.11837	max.rep
d.f.	18.16	18.16		18.16	
Except when comparing means with the same level(s) of					
Fusarium	0.10038				min.rep
d.f.	136				
	0.08196				max-min
d.f.	136				
	0.05795				max.rep
d.f.	136				
C. giveni		0.10038			min.rep
d.f.		136			
		0.08196			max-min
d.f.		136			
		0.05795			max.rep
d.f.		136			
Fusarium.*C. giveni*				0.14196	min.rep
d.f.				136	
				0.11591	max-min
d.f.				136	
				0.08196	max.rep
d.f.				136	

Table A.60 Analysis of variance shoot weight treated maize plants

Variate: log Shoot (mg)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.MainPlot stratum					
Trial	2	1.232401	0.616201	18.93	0.003
Fusarium	1	0.044792	0.044792	1.38	0.285
C. giveni	1	0.052398	0.052398	1.61	0.251
Fusarium.*C. giveni*	1	0.005855	0.005855	0.18	0.686
Residual	6	0.195259	0.032543	3.37	
Trial.MainPlot.*Units* stratum					
Isolate	8	0.112360	0.014045	1.45	0.180
Isolate.*Fusarium*	8	0.132817	0.016602	1.72	0.100
Isolate.*C. giveni*	8	0.076499	0.009562	0.99	0.447
Isolate.*Fusarium*.*C. giveni*	8	0.092488	0.011561	1.20	0.306
Residual	136	1.314513	0.009666		
Total	179	3.259383			

Tables of means

Grand mean: 2.0533

Trial	1	2	4
	2.0963	2.1261	1.9376

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	2.0461	1.9913	2.0316	2.0670	2.0735
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	2.0906	2.0309	2.0730	2.0238
rep.	12	12	36	12

Fusarium	+	-
	2.0376	2.0691

C. giveni	+	-
	2.0363	2.0704

Isolate	*Fusarium*	+	-
A1080		2.0560	2.0362
	rep.	18	18
Bb21		1.9356	2.0471
	rep.	6	6
BK41		2.0592	2.0040
	rep.	6	6
Control Seeds		2.0471	2.0869
	rep.	18	18
F16		2.0897	2.0573
	rep.	6	6
F672		2.0512	2.1301
	rep.	6	6
F99		2.0063	2.0556
	rep.	6	6
FCC327		2.0556	2.0904
	rep.	18	18
FCC447		1.9456	2.1020
	rep.	6	6

Isolate	*C. giveni*	+	-
A1080		2.0333	2.0589
	rep.	18	18
Bb21		1.9787	2.0040
	rep.	6	6
BK41		2.0409	2.0224
	rep.	6	6
Control Seeds		2.0293	2.1047
	rep.	18	18
F16		2.0794	2.0676
	rep.	6	6
F672		2.0672	2.1140
	rep.	6	6
F99		2.0431	2.0188
	rep.	6	6
FCC327		2.0340	2.1120
	rep.	18	18
FCC447		2.0451	2.0025
	rep.	6	6

Fusarium	*C. giveni*	+	-
+		2.0262	2.0489
-		2.0463	2.0919

	Fusarium	+		-	
Isolate	*C. giveni*	+	-	+	-
A1080		2.0632	2.0487	2.0033	2.0691
	rep.	9	9	9	9
Bb21		1.9012	1.9700	2.0562	2.0379
	rep.	3	3	3	3
BK41		2.0625	2.0560	2.0193	1.9888
	rep.	3	3	3	3
Control Seeds		2.0308	2.0633	2.0278	2.1461
	rep.	9	9	9	9
F16		2.0897	2.0897	2.0692	2.0454
	rep.	3	3	3	3
F672		1.9722	2.1302	2.1623	2.0978
	rep.	3	3	3	3
F99		2.0341	1.9785	2.0521	2.0591
	rep.	3	3	3	3
FCC327		2.0144	2.0967	2.0537	2.1272
	rep.	9	9	9	9
FCC447		2.0082	1.8830	2.0820	2.1220
	rep.	3	3	3	3

Least significant differences of means (5% level)

Table	Trial	Isolate	*Fusarium*	*C. giveni*	
rep.	60	unequal	90	90	
d.f.	6	136	6	6	
l.s.d.		0.07937			min.rep
	0.08059	0.06481	0.06580	0.06580	max-min
		0.04583			max.rep

Table	Isolate *Fusarium*	Isolate *C. giveni*	*Fusarium* *C. giveni*	Isolate *Fusarium* *C. giveni*	
rep.	unequal	unequal	45	unequal	
l.s.d.	0.12133	0.12133		0.17158	min.rep

d.f.	90.56	90.56		90.56	
l.s.d.	0.10304	0.10304	0.09306	0.14572	max-min
d.f.	61.55	61.55	6	61.55	
l.s.d.	0.08161	0.08161		0.11542	max.rep
d.f.	27.04	27.04		27.04	
Except when comparing means with the same level(s) of					
Fusarium	0.11225				min.rep
d.f.	136				
	0.09165				max-min
d.f.	136				
	0.06481				max.rep
d.f.	136				
C. giveni		0.11225			min.rep
d.f.		136			
		0.09165			max-min
d.f.		136			
		0.06481			max.rep
d.f.		136			
Fusarium.*C. giveni*				0.15874	min.rep
d.f.				136	
				0.12961	max-min
d.f.				136	
				0.09165	max.rep
d.f.				136	

Table A.61 Analysis of variance root weight treated maize plants

Variate: log Root (mg)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.MainPlot stratum					
Trial	2	1.28601	0.64300	11.19	0.009
Fusarium	1	0.00003	0.00003	0.00	0.982
C. giveni	1	1.27293	1.27293	22.15	0.003
Fusarium.*C. giveni*	1	0.00729	0.00729	0.13	0.734
Residual	6	0.34482	0.05747	3.74	
Trial.MainPlot.*Units* stratum					
Isolate	8	0.22219	0.02777	1.81	0.081
Isolate.*Fusarium*	8	0.19329	0.02416	1.57	0.139
Isolate.*C. giveni*	8	0.09567	0.01196	0.78	0.623
Isolate.*Fusarium*.*C. giveni*	8	0.24022	0.03003	1.95	0.057
Residual	136	2.09065	0.01537		
Total	179	5.75310			

Tables of means

Grand mean: 2.0188

Trial	1	2	4
	1.9445	1.9749	2.1371

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	2.0293	2.0794	2.0100	2.0127	2.0812
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	2.0286	1.9191	2.0072	2.0168
rep.	12	12	36	12

Fusarium	+	-
	2.0193	2.0184

C. giveni	+	-
	1.9347	2.1029

Isolate	*Fusarium*	+	-
A1080		2.0377	2.0208
	rep.	18	18
Bb21		2.1037	2.0550
	rep.	6	6
BK41		2.0586	1.9613
	rep.	6	6
Control Seeds		2.0042	2.0211
	rep.	18	18
F16		2.1468	2.0156
	rep.	6	6
F672		2.0164	2.0407
	rep.	6	6
F99		1.9403	1.8979
	rep.	6	6
FCC327		1.9565	2.0579
	rep.	18	18
FCC447		2.0275	2.0061
	rep.	6	6

Isolate	*C. giveni*	+	-
A1080		1.9598	2.0987
	rep.	18	18
Bb21		2.0329	2.1259
	rep.	6	6
BK41		1.9284	2.0915
	rep.	6	6
Control Seeds		1.9318	2.0935
	rep.	18	18
F16		1.9919	2.1705
	rep.	6	6
F672		1.9678	2.0893
	rep.	6	6
F99		1.7737	2.0646
	rep.	6	6
FCC327		1.9018	2.1126
	rep.	18	18
FCC447		1.9462	2.0874
	rep.	6	6

Fusarium	*C. giveni*	+	-
+		1.9415	2.0970
-		1.9279	2.1089

	Fusarium	+		-	
Isolate	*C. giveni*	+	-	+	-
A1080		1.9407	2.1348	1.9789	2.0627
	rep.	9	9	9	9
Bb21		2.0654	2.1420	2.0003	2.1098
	rep.	3	3	3	3
BK41		1.9960	2.1213	1.8608	2.0618
	rep.	3	3	3	3
Control Seeds		1.9272	2.0812	1.9364	2.1058

	rep.	9	9	9	9
F16		2.1112	2.1825	1.8726	2.1586
	rep.	3	3	3	3
F672		1.9416	2.0912	1.9940	2.0874
	rep.	3	3	3	3
F99		1.9024	1.9783	1.6450	2.1509
	rep.	3	3	3	3
FCC327		1.8328	2.0803	1.9708	2.1450
	rep.	9	9	9	9
FCC447		2.0042	2.0508	1.8881	2.1240
	rep.	3	3	3	3

Least significant differences of means (5% level)

Table	Trial	Isolate	*Fusarium*	*C. giveni*	
rep.	60	unequal	90	90	
d.f.	6	136	6	6	
l.s.d.		0.10010			min.rep
	0.10710	0.08173	0.08744	0.08744	max-min
		0.05779			max.rep

Table	Isolate *Fusarium*	Isolate *C. giveni*	*Fusarium* *C. giveni*	Isolate *Fusarium* *C. giveni*	
rep.	unequal	unequal	45	unequal	
l.s.d.	0.15482	0.15482		0.21894	min.rep
d.f.	83.45	83.45		83.45	
l.s.d.	0.13217	0.13217	0.12366	0.18692	max-min
d.f.	55.48	55.48	6	55.48	
l.s.d.	0.10601	0.10601		0.14992	max.rep
d.f.	24.47	24.47		24.47	
Except when comparing means with the same level(s) of					
Fusarium	0.14156				min.rep
d.f.	136				
	0.11558				max-min
d.f.	136				
	0.08173				max.rep
d.f.	136				
C. giveni		0.14156			min.rep
d.f.		136			
		0.11558			max-min
d.f.		136			
		0.08173			max.rep
d.f.		136			
Fusarium.*C. giveni*				0.20020	min.rep
d.f.				136	
				0.16346	max-min
d.f.				136	
				0.11558	max.rep
d.f.				136	

Section 3.5.5 Effect of the entomopathogenic fungal seed coating on incidence of *Fusarium* root rot in maize plants

Table A. 62 Analysis of variance of maize root *Fusarium graminearum* symptom

Variate: Symptom

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial stratum	2	0.03457	0.01728	0.38	
Trial.MainPlot stratum					
C. giveni	1	1.39275	1.39275	30.49	0.001
Fusarium	1	8.81497	8.81497	192.98	<.001
C. giveni.*Fusarium*	1	1.39275	1.39275	30.49	0.001
Residual	6	0.27407	0.04568	1.05	
Trial.MainPlot.*Units* stratum					
Isolate	8	1.01836	0.12730	2.91	0.005
C. giveni.Isolate	8	0.41975	0.05247	1.20	0.303
Fusarium.Isolate	8	1.01836	0.12730	2.91	0.005
C. giveni.*Fusarium*.Isolate	8	0.41975	0.05247	1.20	0.303
Residual	136	5.94136	0.04369		
Total	179	20.72670			

Table A.63 Analysis of variance of effect of entomopathogenic fungal isolate on *Fusarium graminearum* symptom on maize root

Variate: Sympton

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial stratum	2	0.06914	0.03457	0.29	
Trial.MainPlot stratum					
C. giveni	1	2.78549	2.78549	23.26	0.040
Residual	2	0.23951	0.11975	1.37	
Trial.MainPlot.*Units* stratum					
Isolate	8	2.03673	0.25459	2.91	0.007
Isolate.*C. giveni*	8	0.83951	0.10494	1.20	0.312
Residual	68	5.94136	0.08737		
Total	89	11.91173			

Tables of means

Grand mean: 0.443

Isolate	A1080	Bb21	BK41	CS	F16
	0.356	0.264	0.333	0.662	0.222
rep.	18	6	6	18	6

Isolate	F672	F99	FCC327	FCC447
	0.597	0.278	0.426	0.611
rep.	6	6	18	6

C. giveni	0	1
	0.619	0.267

Isolate	*C. giveni*	0	1
A1080		0.611	0.102
	rep.	9	9
Bb21		0.278	0.250
	rep.	3	3
BK41		0.667	0.000
	rep.	3	3
CS		0.750	0.574
	rep.	9	9
F16		0.444	0.000
	rep.	3	3
F672		0.611	0.583
	rep.	3	3
F99		0.389	0.167
	rep.	3	3
FCC327		0.639	0.213
	rep.	9	9
FCC447		0.889	0.333
	rep.	3	3

Least significant differences of means (5% level)

Table	Isolate	*C. giveni*	Isolate *C. giveni*	
rep.	unequal	45	unequal	
l.s.d.	0.3405		0.4884	min.rep
d.f.	68		61.82	
l.s.d.	0.2781	0.3139	0.4030	max-min
d.f.	68	2	50.48	
l.s.d.	0.1966		0.2977	max.rep
d.f.	68		24.56	
Except when comparing means with the same level(s) of *C. giveni*			0.4816	min.rep
d.f.			68	
			0.3932	max-min
d.f.			68	
			0.2781	max.rep
d.f.			68	

Section 3.5.6 Costelytra giveni mortality in presence of maize plants treated with entomopathogenic fungal isolates

Table A 64. Analysis of variance of *C. giveni* mortality

Variate: Mortality

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial stratum	2	0.10556	0.05278	19.00	
Trial.MainPlot stratum					
Fusarium	1	0.04444	0.04444	16.00	0.057
Residual	2	0.00556	0.00278	0.07	
Trial.MainPlot.*Units* stratum					
Isolate	8	5.17778	0.64722	16.86	<.001
Isolate.*Fusarium*	8	1.17778	0.14722	3.83	<.001
Residual	68	2.61111	0.03840		
Total	89	9.12222			

Tables of means
Grand mean: 0.244

Isolate	A1080	Bb21	BK41	Control Seeds	F16
	0.444	0.000	0.333	0.000	0.500
rep.	18	6	6	18	6

Isolate	F672	F99	FCC327	FCC447
	0.667	0.500	0.000	0.333
rep.	6	6	18	6

Fusarium	+	-
	0.222	0.267

Isolate	*Fusarium*	+	-
A1080		0.333	0.556
	rep.	9	9
Bb21		0.000	0.000
	rep.	3	3
BK41		0.333	0.333
	rep.	3	3
Control Seeds		0.000	0.000
	rep.	9	9
F16		0.333	0.667
	rep.	3	3
F672		0.667	0.667
	rep.	3	3
F99		0.333	0.667
	rep.	3	3
FCC327		0.000	0.000
	rep.	9	9
FCC447		0.667	0.000
	rep.	3	3

Least significant differences of means (5% level)

Table	Isolate	*Fusarium*	Isolate *Fusarium*	
rep.	unequal	45	unequal	
l.s.d.	0.2258		0.3092	min.rep
d.f.	68		68.64	
l.s.d.	0.1843	0.0478	0.2482	max-min
d.f.	68	2	68.95	
l.s.d.	0.1303		0.1663	max.rep
d.f.	68		69.71	
Except when comparing means with the same level(s) of *Fusarium*			0.3193	min.rep
d.f.			68	
			0.2607	max-min
d.f.			68	
			0.1843	max.rep
d.f.			68	

Section 3.5.7 Rhizosphere and endophytic colonization of maize plants by entomopathogenic fungi at 25 and 28°C

Table A.65 Analysis of variance rhizosphere colonization per gram of rhizosphere at 25°C

Variate: AvRhiz/g

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial stratum	2	1332187.	666093.	1.79	
Trial.Mainplot stratum					
C. giveni	1	98927034.	98927034.	266.30	<.001
Fusarium	1	802710.	802710.	2.16	0.192
C. giveni.*Fusarium*	1	7289218.	7289218.	19.62	0.004
Residual	6	2228942.	371490.	0.95	
Trial.Mainplot.*Units* stratum					
Isolate	8	340254079.	42531760.	109.12	<.001
C. giveni.Isolate	8	256306907.	32038363.	82.20	<.001
Fusarium.Isolate	8	70325935.	8790742.	22.55	<.001
C. giveni.*Fusarium*.Isolate	8	60348241.	7543530.	19.35	<.001
Residual	136	53008763.	389770.		
Total	179	890824017.			

Tables of means
Grand mean: 1410.

C. giveni	+	-
	668.	2151.

Fusarium	+	-
	1343.	1476.

Isolate	A1080	Bb21	BK41	Coated Seeds	F16
	3138.	162.	268.	0.	780.
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	2728.	637.	1010.	4124.
rep.	12	12	36	12

C. giveni	*Fusarium*	+	-
+		803.	534.
-		1883.	2419.

C. giveni	Isolate	A1080	Bb21	BK41	Coated Seeds
+		787.	223.	250.	0.
	rep.	18	6	6	18
-		5489.	102.	286.	0.
	rep.	18	6	6	18

C. giveni	Isolate	F16	F672	F99	FCC327
+		252.	2676.	0.	1211.
	rep.	6	6	6	18
-		1308.	2779.	1274.	810.
	rep.	6	6	6	18

C. giveni	Isolate	FCC447
+		630.
	rep.	6

-		7618.
	rep.	6

Fusarium	Isolate	A1080	Bb21	BK41	Coated Seeds
+		2711.	198.	202.	0.
	rep.	18	6	6	18
-		3564.	127.	335.	0.
	rep.	18	6	6	18

Fusarium	Isolate	F16	F672	F99	FCC327
+		861.	1412.	1213.	1758.
	rep.	6	6	6	18
-		700.	4044.	61.	262.
	rep.	6	6	6	18

Fusarium	Isolate	FCC447
+		2849.
	rep.	6
-		5399.
	rep.	6

C. giveni	*Fusarium*	Isolate	A1080	Bb21	BK41	Coated Seeds
+	+		1284.	267.	0.	0.
		rep.	9	3	3	9
	-		289.	178.	501.	0.
		rep.	9	3	3	9
-	+		4137.	128.	404.	0.
		rep.	9	3	3	9
	-		6840.	75.	168.	0.
		rep.	9	3	3	9

C. giveni	*Fusarium*	Isolate	F16	F672	F99	FCC327
+	+		105.	511.	0.	2209.
		rep.	3	3	3	9
	-		399.	4842.	0.	212.
		rep.	3	3	3	9
-	+		1616.	2312.	2426.	1308.
		rep.	3	3	3	9
	-		1000.	3247.	122.	312.
		rep.	3	3	3	9

C. giveni	*Fusarium*	Isolate	FCC447
+	+		676.
		rep.	3
	-		584.
		rep.	3
-	+		5021.
		rep.	3
	-		10215.
		rep.	3

Least significant differences of means (5% level)

Table	*C. giveni*	*Fusarium*	Isolate	*C. giveni* *Fusarium*	
rep.	90	90	unequal	45	
d.f.	6	6	136	6	
l.s.d.			504.0		min.rep
	222.3	222.3	411.5	314.4	max-min
			291.0		max.rep

Table	*C. giveni* Isolate	*Fusarium* Isolate	*C. giveni* *Fusarium* Isolate	
rep.	unequal	unequal	unequal	
l.s.d.	711.5	711.5	1006.2	min.rep
d.f.	140.40	140.40	140.40	
l.s.d.	580.8	580.8	821.3	max-min
d.f.	132.62	132.62	132.62	
l.s.d.	411.4	411.4	581.8	max.rep
d.f.	91.19	91.19	91.19	
Except when comparing means with the same level(s) of				
C. giveni	712.8			min.rep
d.f.	136			
	582.0			max-min
d.f.	136			
	411.5			max.rep
d.f.	136			
Fusarium		712.8		min.rep
d.f.		136		
		582.0		max-min
d.f.		136		
		411.5		max.rep
d.f.		136		
C. giveni.*Fusarium*			1008.1	min.rep
d.f.			136	
			823.1	max-min
d.f.			136	
			582.0	max.rep
d.f.			136	

Table A.66 Analysis of variance rhizosphere colonization per gram of rhizosphere at 28°C

Variate: AvRhiz_g

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.Mainplot stratum					
Fusarium	1	9208.	9208.	0.00	0.985
Residual	2	41409522.	20704761.	8.20	
Trial.Mainplot.*Units* stratum					
Isolate	8	108303930.	13537991.	5.36	<.001
Fusarium.Isolate	8	21355255.	2669407.	1.06	0.411
Residual	40	100986010.	2524650.		
Total	59	272063925.			

Tables of means
Grand mean: 1363.

Fusarium	+	-
	1350.	1375.

Isolate	A1080	Bb21	BK41	Coated Seeds	F16
	3116.	62.	279.	0.	697.
rep.	12	4	4	12	4

Isolate	F672	F99	FCC327	FCC447
	2090.	585.	1086.	4121.

rep.		4	4	12	4
Fusarium	Isolate	A1080	Bb21	BK41	Coated Seeds
+		2662.	124.	331.	0.
	rep.	6	2	2	6
-		3571.	0.	227.	0.
	rep.	6	2	2	6
Fusarium	Isolate	F16	F672	F99	FCC327
+		776.	1439.	1170.	1901.
	rep.	2	2	2	6
-		617.	2741.	0.	271.
	rep.	2	2	2	6
Fusarium	Isolate	FCC447			
+		2724.			
	rep.	2			
-		5517.			
	rep.	2			

Least significant differences of means (5% level)

Table	*Fusarium*	Isolate	*Fusarium* Isolate	
rep.	30	unequal	unequal	
d.f.	2	40	8.30	
l.s.d.		2270.7	4183.0	min.rep
	5055.1	1854.1	3899.2	max-min
		1311.0	3847.8	max.rep
Except when comparing means with the same level(s) of *Fusarium*			3211.3	min.rep
			2622.0	max-min
			1854.1	max.rep

Section 3.5.9 Ability of the entomopathogenic fungal isolates to endophytically colonize maize plants at 25 and 28°C

Table A.67 Analysis of variance endophytic colonization of maize plants by entomopathogenic fungi at 25°C

Variate: AvEndo

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial stratum	2	0.1583	0.0792	0.56	
Trial.Mainplot stratum					
Insect	1	692.2722	692.2722	4935.01	<.001
Fusarium	1	228.9389	228.9389	1632.04	<.001
Insect.*Fusarium*	1	1.0889	1.0889	7.76	0.032
Residual	6	0.8417	0.1403	0.34	
Trial.Mainplot.*Units* stratum					
Isolate	8	1081.0222	135.1278	331.12	<.001
Insect.Isolate	8	363.9222	45.4903	111.47	<.001
Fusarium.Isolate	8	113.2000	14.1500	34.67	<.001
Insect.*Fusarium*.Isolate	8	312.8556	39.1069	95.83	<.001
Residual	136	55.5000	0.4081		
Total	179	2849.8000			

Tables of means
Grand mean: 3.133

Insect	+	-
	1.172	5.094

Fusarium	+	-
	4.261	2.006

Isolate	A1080	Bb21	BK41	Coated Seeds	F16
	5.681	0.000	1.792	0.000	1.417
rep.	36	12	12	36	12

Isolate	F672	F99	FCC327	FCC447
	2.750	1.833	5.903	4.458
rep.	12	12	36	12

Insect	*Fusarium*	+	-
+		2.222	0.122
-		6.300	3.889

Insect	Isolate	A1080	Bb21	BK41	Coated Seeds
+		2.611	0.000	0.000	0.000
	rep.	18	6	6	18
-		8.750	0.000	3.583	0.000
	rep.	18	6	6	18

Insect	Isolate	F16	F672	F99	FCC327
+		0.000	0.000	0.917	2.944
	rep.	6	6	6	18
-		2.833	5.500	2.750	8.861
	rep.	6	6	6	18

Insect	Isolate	FCC447
+		0.000
	rep.	6
-		8.917
	rep.	6

Fusarium	Isolate	A1080	Bb21	BK41	Coated Seeds
+		7.250	0.000	3.583	0.000
	rep.	18	6	6	18
-		4.111	0.000	0.000	0.000
	rep.	18	6	6	18

Fusarium	Isolate	F16	F672	F99	FCC327
+		2.833	5.500	2.750	7.417
	rep.	6	6	6	18
-		0.000	0.000	0.917	4.389
	rep.	6	6	6	18

Fusarium	Isolate	FCC447
+		5.250
	rep.	6
-		3.667
	rep.	6

Insect	*Fusarium*	Isolate	A1080	Bb21	BK41	Coated Seeds
+	+		5.222	0.000	0.000	0.000
		rep.	9	3	3	9
	-		0.000	0.000	0.000	0.000
		rep.	9	3	3	9
-	+		9.278	0.000	7.167	0.000
		rep.	9	3	3	9
	-		8.222	0.000	0.000	0.000
		rep.	9	3	3	9

Insect	*Fusarium*	Isolate	F16	F672	F99	FCC327
+	+		0.000	0.000	0.000	5.889
		rep.	3	3	3	9
	-		0.000	0.000	1.833	0.000
		rep.	3	3	3	9
-	+		5.667	11.000	5.500	8.944
		rep.	3	3	3	9
	-		0.000	0.000	0.000	8.778
		rep.	3	3	3	9

Insect	*Fusarium*	Isolate	FCC447
+	+		0.000
		rep.	3
	-		0.000
		rep.	3
-	+		10.500
		rep.	3
	-		7.333
		rep.	3

Least significant differences of means (5% level)

Table	Insect	*Fusarium*	Isolate	Insect *Fusarium*	
rep.	90	90	unequal	45	
d.f.	6	6	136	6	
l.s.d.			0.5157		min.rep
	0.1366	0.1366	0.4211	0.1932	max-min
			0.2978		max.rep

Table	Insect Isolate	*Fusarium* Isolate	Insect *Fusarium* Isolate		
rep.	unequal	unequal	unequal		
l.s.d.	0.7130	0.7130	1.0084		min.rep
d.f.	140.84	140.84	140.84		
l.s.d.	0.5754	0.5754	0.8138		max-min
d.f.	141.90	141.90	141.90		
l.s.d.	0.3925	0.3925	0.5550		max.rep
d.f.	137.38	137.38	137.38		
Except when comparing means with the same level(s) of					
Insect	0.7294				min.rep
d.f.	136				
	0.5955				max-min
d.f.	136				
	0.4211				max.rep
d.f.	136				
Fusarium		0.7294			min.rep
d.f.		136			
		0.5955			max-min

d.f.	136		
	0.4211		max.rep
d.f.	136		
Insect.*Fusarium*		1.0315	min.rep
d.f.		136	
		0.8422	max-min
d.f.		136	
		0.5955	max.rep
d.f.		136	

Table A.68 Analysis of variance endophytic colonization of maize plants by entomopathogenic fungi at 28°C

Variate: AvEndo

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial.Mainplot stratum					
Fusarium	1	80.504	80.504	0.69	0.493
Residual	2	232.308	116.154	20.51	
Trial.Mainplot.*Units* stratum					
Isolate	8	333.942	41.743	7.37	<.001
Fusarium.Isolate	8	40.225	5.028	0.89	0.535
Residual	40	226.567	5.664		
Total	59	913.546			

Tables of means

Grand mean: 3.06

Fusarium	+	-
	4.22	1.90

Isolate	A1080	Bb21	BK41	Coated Seeds	F16
	5.38	0.00	1.88	0.00	1.38
rep.	12	4	4	12	4

Isolate	F672	F99	FCC327	FCC447
	2.75	2.00	5.83	4.25
rep.	4	4	12	4

Fusarium	Isolate	A1080	Bb21	BK41	Coated Seeds
+		7.17	0.00	3.75	0.00
	rep.	6	2	2	6
-		3.58	0.00	0.00	0.00
	rep.	6	2	2	6

Fusarium	Isolate	F16	F672	F99	FCC327
+		2.75	5.50	3.00	7.25
	rep.	2	2	2	6
-		0.00	0.00	1.00	4.42
	rep.	2	2	2	6

Fusarium	Isolate	FCC447
+		5.00
	rep.	2
-		3.50
	rep.	2

Least significant differences of means (5% level)

Table	*Fusarium*	Isolate	*Fusarium* Isolate	
rep.	30	unequal	unequal	
l.s.d.		3.401	9.016	min.rep
d.f.		40	5.53	
l.s.d.	11.973	2.777	9.178	max-min
d.f.	2	40	4.10	
l.s.d.		1.964	9.974	max.rep
d.f.		40	2.85	
Except when comparing means with the same level(s) of *Fusarium*			4.810	min.rep
d.f.			40	
			3.927	max-min
d.f.			40	
			2.777	max.rep
d.f.			40	

Section 3.5.10 Effect of Metarhizium anisopliae seed-coating on jasmonic acid (JA) and salicylic acid (SA) content in maize in the presence of *Costelytra giveni*

Table A.69 Analysis of variance salicylic acid in maize shoots and roots after coating with entomopathogenic fungi

Variate: Log SA (ng/g)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
C. giveni	1	0.006791	0.006791	1.25	0.345
Trial	1	0.003204	0.003204	0.59	0.498
Residual	3	0.016302	0.005434	1.90	
Block.*Units* stratum					
Sample	1	0.215047	0.215047	75.08	<.001
Treatment	2	0.003841	0.001920	0.67	0.523
C. giveni.Sample	1	0.004227	0.004227	1.48	0.239
C. giveni.Treatment	2	0.042387	0.021193	7.40	0.004
Sample.Treatment	2	0.001909	0.000955	0.33	0.720
C. giveni.Sample.Treatment	2	0.133647	0.066823	23.33	<.001
Residual	20	0.057288	0.002864		
Total	35	0.484642			

Tables of means

Grand mean: 2.1586

C. giveni	0	1
	2.1780	2.1489
rep.	12	24

Sample	Root	Shoot
	2.2359	2.0813

Treatment	A1080	CS	F672
	2.1703	2.1452	2.1603

C. giveni	Sample	Root	Shoot
0		2.2400	2.1161
	rep.	6	6
1		2.2338	2.0639
	rep.	12	12

C. giveni	Treatment	A1080	CS	F672
0		2.2336	2.1883	2.1121
	rep.	4	4	4
1		2.1386	2.1236	2.1844
	rep.	8	8	8

Sample	Treatment	A1080	CS	F672
Root		2.2522	2.2122	2.2433
Shoot		2.0884	2.0782	2.0774

	Sample	Root			Shoot		
C. giveni	Treatment	A1080	CS	F672	A1080	CS	F672
0		2.4190	2.2042	2.0968	2.0483	2.1725	2.1274
	rep.	2	2	2	2	2	2
1		2.1688	2.2162	2.3165	2.1085	2.0310	2.0524
	rep.	4	4	4	4	4	4

Trial	1	2
	2.1492	2.1680

Least significant differences of means (5% level)

Table	*C. giveni*	Sample	Treatment	*C. giveni* Sample	
rep.	unequal	18	12	unequal	
l.s.d.				0.08870	min.rep
d.f.				6.72	
l.s.d.	0.08294	0.03721	0.04558	0.07681	max-min
d.f.	3	20	20	6.72	
l.s.d.				0.06272	max.rep
d.f.				6.72	
Except when comparing means with the same level(s) of					
C. giveni				0.06446	min.rep
d.f.				20	
				0.05582	max-min
d.f.				20	
				0.04558	max.rep
d.f.				20	

Table	*C. giveni* Treatment	Sample Treatment	*C. giveni* Sample Treatment	Trial	
rep.	unequal	6	unequal	18	
l.s.d.	0.09509		0.11993		min.rep
d.f.	10.85		19.42		
l.s.d.	0.08235	0.06446	0.10386	0.07820	max-min
d.f.	10.85	20	19.42	3	
l.s.d.	0.06724		0.08480		max.rep
d.f.	10.85		19.42		
Except when comparing means with the same level(s) of					
C. giveni	0.07894		0.11164		min.rep
d.f.	20		20		
	0.06837		0.09668		max-min
d.f.	20		20		

	0.05582	0.07894	max.rep
d.f.	20	20	
C. giveni.Sample			
		0.11164	min.rep
d.f.		20	
		0.09668	max-min
d.f.		20	
		0.07894	max.rep
d.f.		20	
C. giveni.Treatment			
		0.11164	min.rep
d.f.		20	
		0.09668	max-min
d.f.		20	
		0.07894	max.rep
d.f.		20	

Table A.70 Analysis of variance jasmonic acid in maize shoots and roots after coating with entomopathogenic fungi

Variate: Log JA (ng/g)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Block stratum					
C. giveni	1	0.01811	0.01811	1.83	0.269
Trial	1	0.00598	0.00598	0.60	0.494
Residual	3	0.02975	0.00992	0.51	
Block.*Units* stratum					
Sample	1	1.99296	1.99296	103.47	<.001
Treatment	2	0.14045	0.07023	3.65	0.045
C. giveni.Sample	1	0.04520	0.04520	2.35	0.141
C. giveni.Treatment	2	0.16941	0.08470	4.40	0.026
Sample.Treatment	2	0.06507	0.03253	1.69	0.210
C. giveni.Sample.Treatment					
	2	0.02284	0.01142	0.59	0.562
Residual	20	0.38523	0.01926		
Total	35	2.87499			

Tables of means

Grand mean: 2.301

C. giveni	0	1
	2.270	2.317
rep.	12	24

Sample	Root	Shoot
	2.537	2.066

Treatment	A1080	CS	F672
	2.356	2.214	2.335

C. giveni	Sample	Root	Shoot
0		2.555	1.984
	rep.	6	6
1		2.527	2.107
	rep.	12	12

C. giveni	Treatment	A1080	CS	F672
0		2.456	2.085	2.267
	rep.	4	4	4
1		2.305	2.278	2.368
	rep.	8	8	8

Sample	Treatment	A1080	CS	F672
Root		2.568	2.413	2.629
Shoot		2.144	2.015	2.040

	Sample	Root			Shoot		
C. giveni	Treatment	A1080	CS	F672	A1080	CS	F672
0		2.681	2.382	2.601	2.232	1.788	1.933
	rep.	2	2	2	2	2	2
1		2.511	2.428	2.643	2.100	2.128	2.093
	rep.	4	4	4	4	4	4

Trial	1	2
	2.288	2.314

Least significant differences of means (5% level)

Table	*C. giveni*	Sample	Treatment	*C. giveni* Sample	
rep.	unequal	18	12	unequal	
l.s.d.				0.1474	min.rep
d.f.				16.59	
l.s.d.	0.1120	0.0965	0.1182	0.1277	max-min
d.f.	3	20	20	16.59	
l.s.d.				0.1042	max.rep
d.f.				16.59	
Except when comparing means with the same level(s) of					
C. giveni				0.1671	min.rep
d.f.				20	
				0.1448	max-min
d.f.				20	
				0.1182	max.rep
d.f.				20	

Table	*C. giveni* Treatment	Sample Treatment	*C. giveni* Sample Treatment	Trial	
rep.	unequal	6	unequal	18	
l.s.d.	0.1864		0.2754		min.rep
d.f.	21.93		22.72		
l.s.d.	0.1614	0.1671	0.2385	0.1056	max-min
d.f.	21.93	20	22.72	3	
l.s.d.	0.1318		0.1948		max.rep
d.f.	21.93		22.72		
Except when comparing means with the same level(s) of					
C. giveni	0.2047		0.2895		min.rep
d.f.	20		20		
	0.1773		0.2507		max-min
d.f.	20		20		
	0.1448		0.2047		max.rep
d.f.	20		20		
C. giveni.Sample					
			0.2895		min.rep

d.f.	20	
	0.2507	max-min
d.f.	20	
	0.2047	max.rep
d.f.	20	
C. giveni.Treatment		
	0.2895	min.rep
d.f.	20	
	0.2507	max-min
d.f.	20	
	0.2047	max.rep
d.f.	20	

B.3 Chapter 4 Production of fungal microsclerotia and use in delivery of fungal biocontrol

Section 4.3.1 Microsclerotia production from entomopathogenic fungal isolates in liquid substrate fermentation

Section 4.3.1..1 Primary inoculum evaluation

Table A.71 Analysis of variance biomass production during inoculum production

Variate: Log Biomass/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	0.0975567	0.0487784	144.60	<.001
Isolate	7	0.0958628	0.0136947	40.60	<.001
Day	1	0.0269840	0.0269840	79.99	<.001
Isolate.Day	7	0.0034031	0.0004862	1.44	0.226
Residual	30	0.0101199	0.0003373		
Total	47	0.2339265			

Tables of means
Grand mean: 1.9042

Trial	2015	2016	2017
	1.8439	1.9523	1.9164

Isolate	A1080	Bb21	Bk41	F16	F327	F447	F672
	1.8945	1.9005	1.9094	1.9292	1.7971	1.9318	1.9135

Isolate	F99
	1.9580

Day	3	4
	1.9279	1.8805

Isolate	Day	3	4
A1080		1.8983	1.8906
Bb21		1.9244	1.8767
Bk41		1.9316	1.8872
F16		1.9562	1.9022
F327		1.8198	1.7743
F447		1.9584	1.9051
F672		1.9419	1.8852
F99		1.9930	1.9229

Least significant differences of means (5% level)

Table	Trial	Isolate	Day	Isolate Day
rep.	16	6	24	3
d.f.	30	30	30	30
l.s.d.	0.01326	0.02166	0.01083	0.03063

Table A.72 Analysis of variance blastospore production during inoculum production

Variate: Log Blastospore/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	0.4201	0.2101	1.70	0.200
Isolate	7	261.1776	37.3111	301.73	<.001
Day	1	0.1699	0.1699	1.37	0.250
Isolate.Day	7	2.2775	0.3254	2.63	0.030
Residual	30	3.7098	0.1237		
Total	47	267.7549			

Tables of means

Grand mean: 5.900

Trial	2015	2016	2017
	5.769	5.980	5.952

Isolate	A1080	Bb21	Bk41	F16	F327	F447	F672
	6.292	8.168	6.874	6.948	0.000	5.715	7.024

Isolate	F99
	6.182

Day	3	4
	5.841	5.960

Isolate	Day	3	4
A1080		6.459	6.126
Bb21		7.867	8.468
Bk41		6.856	6.892
F16		7.260	6.636
F327		0.000	0.000
F447		5.311	6.119
F672		6.942	7.106
F99		6.033	6.332

Least significant differences of means (5% level)

Table	Trial	Isolate	Day	Isolate Day
rep.	16	6	24	3
d.f.	30	30	30	30
l.s.d.	0.2539	0.4146	0.2073	0.5864

Table A.73 Analysis of variance microsclerotia production during inoculum production

Variate: Log MS/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	5.1210	2.5605	3.20	0.055
Isolate	7	23.7529	3.3933	4.24	0.002
Day	1	5.4070	5.4070	6.75	0.014
Isolate.Day	7	4.2289	0.6041	0.75	0.629
Residual	30	24.0368	0.8012		
Total	47	62.5466			

Tables of means
Grand mean: 0.91

Trial	2015	2016	2017
	0.75	0.60	1.36

Isolate	A1080	Bb21	Bk41	F16	F327	F447	F672
	0.69	0.00	1.80	1.52	1.90	0.67	0.67

Isolate	F99
	0.00

Day	3	4
	0.57	1.24

Isolate	Day	3	4
A1080		0.00	1.38
Bb21		0.00	0.00
Bk41		1.34	2.26
F16		1.44	1.60
F327		1.79	2.01
F447		0.00	1.34
F672		0.00	1.34
F99		0.00	0.00

Least significant differences of means (5% level)

Table				Trial	Isolate	Day	Isolate Day
rep.				16	6	24	3
d.f.				30	30	30	30
l.s.d.	0.646	1.055	0.528	1.493			

Section 4.3.1..2 Fungal growth evaluation during microsclerotia production in liquid fermentation

Table A.74 Analysis of variance biomass production during liquid fermentation of entomopathogenic fungi

Variate: Log Biomass/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	0.463973	0.066282	31.11	<.001
Day	4	1.359120	0.339780	159.46	<.001
Isolate.Day	28	0.304356	0.010870	5.10	<.001
Year	2	0.004529	0.002264	1.06	0.350
Residual	78	0.166208	0.002131		
Total	119	2.298185			

Tables of means
Grand mean: 1.8234

Isolate	A1080	Bb21	Bk41	F16	F327	F447	F672
	1.8366	1.8319	1.8132	1.8059	1.6801	1.8413	1.8700

Isolate	F99
	1.9086

Day	3	4	6	7	8
	1.9446	1.9315	1.8366	1.7214	1.6831

Isolate	Day	3	4	6	7	8
A1080		1.9515	1.9503	1.8425	1.7638	1.6751
Bb21		1.9677	1.9459	1.8305	1.6100	1.8052
Bk41		1.8948	1.8893	1.8092	1.7341	1.7387
F16		1.9211	1.9272	1.8506	1.6756	1.6550
F327		1.7670	1.7490	1.6336	1.5808	1.6700
F447		1.9872	1.9731	1.8757	1.7212	1.6489
F672		2.0129	1.9849	1.8972	1.8351	1.6197
F99		2.0543	2.0325	1.9532	1.8504	1.6524

Year	2015	2016	2017
	1.8160	1.8232	1.8311

Least significant differences of means (5% level)

Table	Isolate	Day	Isolate Day	Year
rep.	15	24	3	40
d.f.	78	78	78	78
l.s.d.	0.03356	0.02653	0.07504	0.02055

Table A.75 Analysis of variance blastospore production during liquid fermentation of entomopathogenic fungi

Variate: Log Blst/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	72.3104	10.3301	88.04	<.001
Day	4	9.1480	2.2870	19.49	<.001
Isolate.Day	28	3.8479	0.1374	1.17	0.288
Year	2	4.9743	2.4872	21.20	<.001
Residual	78	9.1524	0.1173		
Total	119	99.4330			

Tables of means
Grand mean: 7.277

Isolate	A1080	Bb21	Bk41	F16	F327	F447	F672
	7.089	8.547	8.562	7.153	6.425	6.573	6.779

Isolate	F99
	7.086

Day	3	4	6	7	8
	6.946	6.990	7.316	7.667	7.466

Isolate	Day	3	4	6	7	8
A1080		7.067	6.742	7.086	7.440	7.112
Bb21		8.227	8.278	8.740	8.946	8.543

Bk41		8.249	8.295	8.755	8.946	8.566
F16		7.139	7.028	7.123	7.157	7.319
F327		5.729	5.865	6.624	6.982	6.926
F447		6.013	6.156	6.676	7.028	6.991
F672		6.480	6.628	6.398	7.349	7.043
F99		6.663	6.928	7.125	7.486	7.227
Year	2015	2016	2017			
	7.248	7.539	7.043			

Least significant differences of means (5% level)

Table	Isolate	Day	Isolate Day	Year
rep.	15	24	3	40
d.f.	78	78	78	78
l.s.d.	0.2490	0.1969	0.5568	0.1525

Table A.76 Analysis of variance microsclerotia production during liquid fermentation of entomopathogenic fungi

Variate: Log MS/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	7	86.03592	12.29085	130.93	<.001
Day	4	26.68690	6.67173	71.07	<.001
Isolate.Day	28	18.46152	0.65934	7.02	<.001
Year	2	2.33266	1.16633	12.42	<.001
Residual	78	7.32185	0.09387		
Total	119	140.83885			

Tables of means

Grand mean: 3.789

Isolate	A1080	Bb21	Bk41	F16	F327	F447	F672
	4.268	4.452	2.356	3.962	4.672	4.686	3.053
Isolate	F99						
	2.863						
Day	3	4	6	7	8		
	3.005	3.478	4.129	4.161	4.172		

Isolate	Day	3	4	6	7	8
A1080		4.122	3.907	4.538	4.270	4.504
Bb21		2.954	4.180	4.952	4.967	5.207
Bk41		1.854	2.013	2.835	2.602	2.473
F16		2.846	3.543	4.708	4.823	3.890
F327		4.362	4.655	4.985	4.616	4.740
F447		2.962	4.654	5.190	5.265	5.356
F672		2.072	2.295	2.810	3.854	4.235
F99		2.867	2.579	3.011	2.887	2.971
Year	2015	2016	2017			
	3.803	3.952	3.611			

Least significant differences of means (5% level)

Table	Isolate	Day	Isolate Day	Year
rep.	15	24	3	40
d.f.	78	78	78	78
l.s.d.	0.2227	0.1761	0.4980	0.1364

Section 4.3.2 Microsclerotia production and formulation for seed coating

Table A.77 Analysis of variance biomass during liquid fermentation of entomopathogenic fungi for microsclerotia production

Variate: Biomass (mg/mL)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	25.24	12.62	0.82	0.461
Isolate	2	688.53	344.26	22.46	<.001
Residual	13	199.29	15.33		
Total	17	913.06			

Tables of means

Grand mean: 88.42

Trial	1	2	3
	90.03	87.22	88.03

Isolate	A1080	F447	F672
	81.37	96.43	87.47

Least significant differences of means (5% level)

Table	Trial	Isolate
rep.	6	6
d.f.	13	13
l.s.d.	4.884	4.884

Table A.78 Analysis of variance microsclerotia production during liquid fermentation of entomopathogenic fungi

Variate: Log MS/mL

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	0.039777	0.019889	2.50	0.121
Isolate	2	0.079960	0.039980	5.02	0.024
Residual	13	0.103600	0.007969		
Total	17	0.223337			

Tables of means

Grand mean: 5.054

Trial	1	2	3
	5.027	5.014	5.120

Isolate	A1080	F447	F672
	5.004	5.148	5.009

Least significant differences of means (5% level)

Table	Trial	Isolate
rep.	6	6
d.f.	13	13
l.s.d.	0.1113	0.1113

Table A.79 Analysis of variance of germination of formulated microsclerotia

Variate: MS Germination (%)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	42.11	21.06	0.96	0.408
Isolate	2	69.44	34.72	1.59	0.242
Residual	13	284.56	21.89		
Total	17	396.11			

Tables of means
Grand mean: 93.8

Trial	1	2	3
	95.8	92.2	93.3

Isolate	A1080	F447	F672
	95.2	91.0	95.2

Least significant differences of means (5% level)

Table	Trial	Isolate
rep.	6	6
d.f.	13	13
l.s.d.	5.84	5.84

Table A.80 Analysis of variance of production of conidia per gram of MS-DE

Variate: conidia/gr MS-DE

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Isolate	2	1.386E+20	6.929E+19	32.75	<.001
Residual	15	3.173E+19	2.116E+18		
Total	17	1.703E+20			

Tables of means
Grand mean: 5 E+09

Isolate	A1080	F447	F672
	2.E+09	4.E+09	9.E+09

Least significant differences of means (5% level)

Table	Isolate
rep.	6
d.f.	15
l.s.d.	1.790E+09

Table A.81 Analysis of variance quantification of MS-DE coated to maize seeds

Variate: Conidia/g MS coated seeds

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	2	7.434E+13	3.717E+13	8.93	0.033
Treatment	2	5.045E+13	2.523E+13	6.06	0.062
Residual	4	1.664E+13	4.161E+12		
Total	8	1.414E+14			

Tables of means

Grand mean: 4917244.

Trial	1	2	3
	871549.	7278651.	6601532.

Treatment	A1080	F 672	F477
	4875944.	7837446.	2038342.

Least significant differences of means (5% level)

Table	Trial	Treatment
rep.	3	3
d.f.	4	4
l.s.d.	4624091.1	4624091.1

Section 4.3.3 Evaluation of maize plants after microsclerotia coating and growth in presence of *F. graminearum*

Table A.82 Analysis of variance total length of maize plants seed coated with microsclerotia in the presence of *Fusarium graminearum*

Variate: Log Total Length (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	3	0.001421	0.000474	0.14	0.935
Genera	3	0.052932	0.017644	5.30	0.002
Residual	153	0.509687	0.003331		
Total	159	0.564040			

Tables of means

Grand mean: 2.7879

Trial	1	2	3	4
	2.7843	2.7904	2.7913	2.7857

Genera	CS	CSfg	F672	F447
	2.8150	2.7691	2.7938	2.7738

Least significant differences of means (5% level)

Table	Trial	Genera
rep.	40	40
d.f.	153	153
l.s.d.	0.02550	0.02550

Table A.83 Analysis of variance root length of maize plants seed coated with microsclerotia in the presence of *Fusarium graminearum*

Variate: Log Root Length (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	3	0.003817	0.001272	0.26	0.857
Genera	3	0.032296	0.010765	2.16	0.095
Residual	153	0.761774	0.004979		
Total	159	0.797887			

Tables of means
Grand mean: 2.5960

Trial	1	2	3	4
	2.5886	2.6000	2.6008	2.5947

Genera	CS	CSfg	M. anisopliae	M. robertsii
	2.6148	2.5824	2.6047	2.5822

Least significant differences of means (5% level)

Table	Trial	Genera
rep.	40	40
d.f.	153	153
l.s.d.	0.03117	0.03117

Table A.84 Analysis of variance shoot length of maize plants seed coated with microsclerotia in the presence of *Fusarium graminearum*

Variate: Log Soot Length (cm)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	3	0.000796	0.000265	0.08	0.970
Genera	3	0.110015	0.036672	11.28	<.001
Residual	153	0.497373	0.003251		
Total	159	0.608184			

Tables of means
Grand mean: 2.3375

Trial	1	2	3	4
	2.3399	2.3369	2.3390	2.3341

Genera	CS	CSfg	*M. anisopliae*	*M. robertsii*
	2.3787	2.3081	2.3391	2.3240

Least significant differences of means (5% level)

Table	Trial	Genera
rep.	40	40
d.f.	153	153
l.s.d.	0.02519	0.02519

Table A.85 Analysis of variance total weight of maize plants seed coated with microsclerotia in the presence of *Fusarium graminearum*

Variate: Log Total weight (mg)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	3	0.007085	0.002362	0.54	0.657
Genera	3	1.917417	0.639139	145.58	<.001
Residual	153	0.671698	0.004390		
Total	159	2.596199			

Tables of means
Grand mean: 2.4178

Trial	1	2	3	4
	2.4262	2.4155	2.4212	2.4084

Genera	CS	CSfg	F672	F447
	2.5566	2.2566	2.4639	2.3941

Least significant differences of means (5% level)

Table	Trial	Genera
rep.	40	40
d.f.	153	153
l.s.d.	0.02927	0.02927

Table A.86 Analysis of variance root weight of maize plants seed coated with microsclerotia in the presence of *Fusarium graminearum*

Variate: Log Root weight (mg)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	3	0.023865	0.007955	1.37	0.253
Genera	3	1.397751	0.465917	80.52	<.001
Residual	153	0.885291	0.005786		
Total	159	2.306907			

Tables of means
Grand mean: 2.1909

Trial	1	2	3	4
	2.2061	2.1861	2.1976	2.1736

Genera	CS	CSfg	F672	F447
	2.3045	2.0541	2.2417	2.1632

Least significant differences of means (5% level)

Table	Trial	Genera
rep.	40	40
d.f.	153	153
l.s.d.	0.03360	0.03360

Table A.87 Analysis of variance shoot weight of maize plants seed coated with microsclerotia in the presence of *Fusarium graminearum*

Variate: Log Shoot Weight (mg)

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Trial	3	0.00069	0.00023	0.02	0.997
Genera	3	3.34946	1.11649	73.45	<.001
Residual	153	2.32580	0.01520		
Total	159	5.67595			

Tables of means
Grand mean: 2.0126

Trial	1	2	3	4
	2.0117	2.0100	2.0157	2.0131

Genera	CS	CSfg	F672	F447
	2.1963	1.7948	2.0612	1.9983

Least significant differences of means (5% level)

Table	Trial	Genera
rep.	40	40
d.f.	153	153
l.s.d.	0.05447	0.05447

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