YIELD RESPONSE OF *FUSARIUM* INFECTED MAIZE SEED TREATED WITH BIOLOGICAL CONTROL AGENT FORMULATIONS

by

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submitted in accordance with the requirements

for the degree of

MASTER OF SCIENCE

in the subject

AGRICULTURE

at the

UNIVERSITY OF SOUTH AFRICA

SUPERVISOR : PROF D MODISE

November 2010

Student No: 767-461-9

DECLARATION

I declare that YIELD RESPONSE OF *FUSARIUM* INFECTED MAIZE SEED TREATED WITH BIOLOGICAL CONTROL AGENT FORMULATIONS is my own work and that all the sources that I have quoted have been indicated and acknowledged by means of complete references.

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(Mr B.J. Gerber)

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ABSTRACT

Potential vegetative and reproductive increases in yield, as well as the biological efficacy against *Fusarium verticillioides* and *F. proliferatum* causing ear and stem rot in maize crops of commercially-formulated micro-organism formulation T-Gro (*Trichoderma harzianum* isolate DB103 WP) combined with Spartacus (*Beauveria bassiana* isolate DB 105 WP), T-Gro combined with Armenius (*Bacillus subtilis* isolate DB 109 WP), T-Gro combined with Maximus (*Bacillus subtilis* isolate DB 108 WP), T-Gro combined with Shelter (*Bacillus subtilis* isolate DB 101), T-Gro combined with Bismarck (*Microbacterium maritypicum* isolate DB 107 WP), as well as individual treatments of T-Gro, Armenius, Bismarck, Maximus and Shelter, were investigated when applied to maize seed and soil under field conditions. All the micro-organism treatments were compared with Thiram 750WP (750g/kg thiram WP) and an untreated control.

The micro-organism treatments showed an increase in vegetative as well as reproductive yields when compared to the reference product Thiram 750 WP and the untreated control. There were no observations of adverse effects on the germination of maize seed in all the treatments that were applied. The three isolates *B. subtilis*, *T. harzianum*, and *M. maritypicum*, showed a significant reduction in vascular tissue discolouration of the main and ear stems, indicating a potential to be used in the reduction and control of diseases caused by *Fusarium* spp. Results also showed poor to very good increases of stem and foliage biomass as well as cob yield per plant produced by the micro-organism treatments when compared to the untreated control. The highest cob yield per plant that differed significantly from the untreated control was produced by T-Gro and Shelter.

No phytotoxicity of any kind was observed with the application of the micro-organism formulations and they could therefore be deemed suitable to be used for the treatment of maize seed. The micro-organism formulations containing fungal and bacterial biological control agents have the potential to be used in commercial maize production to increase vegetative and reproductive yields and reduce the severity of ear and stem rot in maize.

KEY WORDS

Biological control agents, maize, crop protection, maize diseases, *Fusarium verticillioides*, yields, micro-organisms, growth-promoting agents, fumonisins.

CHAPTER 1

INTRODUCTION

1.1 Background

Agriculture is under huge pressure to provide sufficient food for billions worldwide and growers are highly dependent on crop protection products to sustain or increase such production (Chincholkar & Mukerji, 2007). Yields have increased substantially due to improved pest control with mainly synthetic pesticides, synthetically produced and natural fertilisers and jointly with plant varieties developed that have higher yield capacities and pest resistance (Chincholkar & Mukerji, 2007). The excessive use and abuse of many pesticides and fertilisers have contributed to the pollution of the environment (Hasan, 2010) and pest resistance against some synthetic pesticides is evident in most cases (Richardson, 2005).

There is no doubt that chemical pesticide use has improved the quality and quantity of food production in the World for the last fifty years. We have seen an increase in concern for the environment and non-target organisms with the increase in pesticide use. Due to more strict regulatory processes (Holm *et al.*, 2005; Stark, 2008) we have seen a large number of formulations being withdrawn from the agricultural market as crop protection products (Chincholkar & Mukerji, 2007; EPA, 2010a; European Commission, 2010). This has created many problems where there are not sufficient or effective products to challenge pest problems (Richardson, 2005) and new formulations have to be registered for use in crop protection (EPA, 2010b).

The review of all active substances used in plant protection products within the European Union by the European Commission (2010) started in 1993 and was finalised in March 2009. Each substance was evaluated whether it could be used safely with respect to human health (consumers, farmers, local residents and passers-by) and the environment, particularly groundwater and non-target organism such as birds, mammals, earthworms, beneficial insects, and so forth. It provides assurances that the substances currently on the market are acceptable for human health and the environment. Of the more or less 1000 substances (tens of thousands of products containing the substances), about 250 passed the harmonised European Union safety assessment. The majority of substances were eliminated because

dossiers were not submitted, incomplete or withdrawn by the industry. Around 70 substances failed the review and were removed from the market because the evaluation that was carried out did not show safe and judicial use with respect to human health and the environment.

It is therefore essential that new technologies and methodologies are developed to ensure sustained food production for an increasing world population. The increasing demand for better or equal quality and at the same time safeguarding of the environment from production to the final product for consumption by humans and animals put emphasis on the importance of more environmentally-conscious research (Gliessman, 2001; Chincholkar & Mukerji, 2007; Khan *et al.*, 2008; Walters, 2009; Hasan, 2010; EPA, 2010b).

1.1.1 Plant and soil health management

i) Introduction

Hasan (2010) investigated various disease management strategies and concluded that protection rather than curative action was the best strategy. Several other researchers such as Walters (2009), Gliesman (2009) and Khan *et al.* (2008) also agreed with the above findings. In addition, they also remarked that soil management was very crucial and should be integrated with all disease management strategies in order for disease control to be effective. Some of the above researchers as well as Chincholkar & Mukerji (2007) advanced the idea of incorporation of biological formulations in the soil to enhance biomass populations.
The most common method is still the use of chemicals for fungal and bacterial disease control and referred to as fungicides and bactericides respectively. Fumigants are used to control various soil-borne pathogens but are also known for their toxicity to most living organisms in the soil environment (Hasan, 2010).

One of the alternatives to chemical control is biological control that involves the use of antagonistic micro-organisms before or after infection occurs. These organisms can be applied to the soil, seed or plant as a whole. Another approach to control, which is closely integrated with chemical or biological control methods, is choice of physical and cultural practices to assist in managing diseases in crops (Walters, 2009). The application and use of organic matter and practices which enhance the micro-organism activity in the soil environment can lead to the suppression of pathogenic micro-organisms by competing for nutrients (Ghorbani, 2008; Walters, 2009). The management of the soil environmental

conditions plays an integral part in the disease control strategy which can make a significant contribution to sustainable and environmentally sound agriculture production (Gliessman, 2001; Khan *et al.*, 2008; Walters, 2009).

ii) Disease management

The pathogenic fungus *Fusarium* has been identified as one of the main causes of reduced grain production in crops like wheat, barley and maize (Schisler *et al.*, 2002). The species most likely to cause huge losses in grain yield and quality is *Fusarium verticillioides* (Fandohan *et al.*, 2003) and also the most likely to be found among most common species isolated worldwide from diseased maize (Munkvold & Desjardins, 1997). Even though information is limited in Africa, some reports and surveys done showed that *F. verticillioides* is the most likely to be found on maize in certain African countries (Marasas *et al.*, 1988; Allah, 1998; Kedera *et al.*, 1999; Fandohan *et al.*, 2003). *F. verticillioides* causes diseases commonly known as ear and kernel rot in maize.

The main concern lies in the fact that *F. verticillioides* produces fumonisins that are associated with activities that can promote cancer (Gelderblom *et al.*, 1988), and also animal disease syndromes (Nayaka *et al.*, 2008). Disease symptoms caused by *F. verticillioides* infection in maize can range from asymptomatic plants to severe rotting and wilting. Plants grown in *F. verticillioides*-infested soil are also smaller and show signs of chlorosis (Oren *et al.*, 2003). Being an endophyte, it establishes a long-term relationship with the maize plant (Pitt & Hocking, 1999). Infection can occur via the silk channel or wounds, or infected seeds that result in grain rot during the pre- and post-harvest periods of production (Munkvold & Desjardins, 1997).

Chemical control has been the most common method used for the preventative or curative control of most diseases on crops. Products registered for the control of diseases caused by pathogenic *Fusarium* species on maize and other crops including wheat are very limited and prove to vary in their efficacy against diseases caused by them when used as seed treatments prior to planting. There is one *Trichoderma* formulation registered in South Africa for the control of *Fusarium* spp. in maize (Pesticide Act 36 of 1947, 2010a,b). In South Africa, grain crops are mainly protected against *Fusarium* and other soil-borne diseases with synthetic fungicides used as seed treatments. The active ingredients used in formulations to protect

grain crops against *Fusarium* and other soil borne pathogens include thiram, captab, and fludioxonil/mefenoxam (Nel *et al.*, 2003).

Various physical and cultural methods used to control diseases do not function on their own but are closely integrated with one another as well as with a well structured disease management programme which further includes chemical and/or biological controls and soil health management. Physical and cultural methods that can be used include quarantine regulation by governments, sanitation, disease-free propagating material, eradication of diseased plants, development of disease resistant cultivars, intercropping, mulching and added organic material, flooding for eradication of weeds, proper water management and correct fertiliser use (Walters, 2009; Hasan, 2010).

The science of biological control as an academic discipline started mainly during the 1970's and has matured as a science to date. Beneficial micro-organisms used as biological controls in crop protection can play an important role in lowering the use of synthetic pesticides as well as the reduction of fertiliser inputs (Chincholkar & Mukerji, 2007). The use of biological control agent formulations is far more complex than using synthetic agrochemicals and there are still many questions to be answered before such formulations become a dominant player in the pesticide market (Hasan, 2010).

Biological control has become one of the most important alternatives to chemical control of disease causing pathogens like *Fusarium*, *Pythium* and *Rhizoctonia* (Gliessman, 2001; Chincholkar & Mukerji, 2007; Khan *et al.*, 2008; Walters, 2009; Hasan, 2010). In general, control of pathogenic micro-organisms is due to antagonism between the pathogen and biological control agent. Different mechanisms of antagonisms occur; but the most common one is direct antagonism where there is physical contact between the biological control agent and the pathogen and/or a high degree of selectivity for the pathogen by the biological control agent (Chincholkar & Mukerji, 2007; Hasan, 2010).

Most of the research undertaken about biological control has used a single biocontrol agent as the antagonist against a single pathogen. It is unlikely that a single biocontrol agent will be responsible for action against all pathogens of plant diseases. Some studies have been conducted with mixtures of biological agents where different fungi are combined (Budge *et al.*, 1995; De Boer *et al.*, 1997), or fungi and bacteria (Janisiewicz, 1988; Duffy & Weller,

1995; Hasan *et al.*, 1997; Hasan, 2010) and mixtures of bacteria (Pierson & Weller, 1994; Janisiewicz & Bors, 1995; Mazzola *et al.*, 1995; Raaijmakers *et al.*, 1995; De Boer *et al.*, 1997; Raupach & Kloepper, 1998).

The future of micro-organism crop protection lies in the formulations' ability to be more consequent in its biological efficacy against the target pest when used under various climatic conditions (high versus lower temperatures, humidity, soil types, and so forth) and last but not least to be economically viable to be used (Hasan, 2010).

Biological control of plant pests can increase the ecological and economical sustainability of farming systems by reducing the risk of crop losses and risk to human health if used correctly and conducted in a proper way (Khan *et al.*, 2008; Hasan, 2010).

iii) Soil management

With mechanisation of agricultural practices and increased use of chemicals came many positive aspects and reduced risks in agronomic practices, but unfortunately also some significant problems. The most prominent problems are topsoil depletion, disturbances in micro-organism biodiversity and soil fertility, contamination of soil with chemical pollutants and the increasing cost of agricultural production to sustain or increase production for a growing world population. In a sustainable agricultural production system the health of soils determines largely the health and optimum growth and development of plants, resulting in those that are less susceptible to pathogen infection. The soil is therefore seen as a living and fragile medium that needs protection to ensure future productivity and stability and is the centre to any sustainable farming system where reliance on synthetic fertilisers and pesticides is minimised (Khan *et al.*, 2008; Walters, 2009).

Soils high in organic matter support huge populations of diverse mirco-organisms and because plant diseases may be suppressed by the activities of plant-associated micro-organisms, researchers have studied the soil environment for organisms involved in biological control (Mukerji *et al.*, 2006; Rosas, 2007; Saravanan *et al.*, 2008). Not only will one eliminate or reduce the use of potentially hazardous chemicals to humans and the environment but in time the soil will improve with the addition of organic matter which will

improve the endogenous levels of general disease suppression (Chincholkar & Mukerji, 2007; Hasan, 2010).

Organic amendments to the soil environment include the addition of animal manures, composts and solid wastes. Increased micro-organism activity, a reduction in the aggressiveness and infestation of plant pathogens, an increase in viral resistance, as well as a reduction in soil toxicity are advantages associated with the incorporation of organic matter in the soil environment (Ghorbani *et al.*, 2008). Organic matter further improves the physical and chemical properties and characteristics of soils, which results in more vigorous growth of plants with an increase in resistance to pathogens from the uptake of compounds with antibiotic effects.

Considerable strain has been placed by current strategies to maintain agricultural productivity via high input practices that contribute to a deterioration of soil health and subsequently an adverse effect on crop productivity. Beneficial micro-organisms are being well researched as an alternative to chemical fertilisers for sustainable agriculture production to maintain the overall health of the soil environment (Khan *et al.*, 2008). Included in these beneficial micro-organisms are plant growth promoting micro-organisms which include phosphate solubilising micro-organisms, symbiotic nitrogen fixing bacteria, as well as endophytic bacteria and fungi (Khan *et al.*, 2008). Fertiliser production is dependent on fossil energy sources and the cost to produce them is becoming increasingly higher and thus has brought forward the subject of mineral phosphate solubilisation. Phosphate solubilising micro-organisms are an economically sound alternative to more expensive superphosphates and this system has the ability to make an appreciable amount of nutrients available from the natural reservoir in the soil environment with further enrichment with scarce nutrients. Soluble phosphates become increasingly more available and can therefore enhance plant growth by increasing the effect of biological nitrogen fixation or the availability of micro-nutrients including iron and zinc as well as plant growth promoting regulators being produced (Sattar & Gaur, 1987; Kucey *et al.*, 1989; Ponmurugan & Gopi, 1997; Khan *et al.*, 2008). The genera *Bacillus* and *Pseudomonas* (Illmer & Schinner, 1992) are very important bacterial phosphate solubilisers; where *Aspergillus* and *Penicillium* are important fungal genera (Motsara *et al.*, 1995).

Endophytic bacteria and fungi reside inside plants without causing harm to the host plant. They form an association with plants that supply them with nutrients like nitrogen in return

for shelter. Various compounds are produced by them that play a role as plant growth promoters in relevant direct mechanisms including nitrogen fixation, growth modulating enzyme production, solubilisation of phosphate and other minerals, and phytohormone production (Saravanan *et al.*, 2008).

The following is a short list of some of the endophytic bacteria that occur naturally on maize and sorghum:
Microbacterium testaceum and *Microbacterium* a*rborescens* (Zinniel *et al.* 2002); *Burkholderia unamae* (Caballero-Mellado *et al.*, 2005) and *Burkholderia tropica* (Reis *et al.*, 2004).

The focus of disease and soil management should therefore be on feeding the soil with beneficial micro-organisms, increasing organic matter and carbon content, as well as the enhancing and safeguarding of natural pest controls (Gerber, 2007).

1.1.2 Maize production

Maize (*Zea mays* L.) is the most important source of carbohydrates and the largest field crop produced in the Southern African Development Community (SADC) for human and animal use (Agricultural Statistics, 2010). Just more than 8000 commercial farmers produce the largest quantity of maize and the smaller portion is produced by thousands of small-scale farmers. Statistical information from the South African Department of Agriculture, Forestry and Fisheries on agricultural maize production for the period 2001/2002 and 2008/9 in South Africa is shown in Table 1.1 and includes total maize area planted, total production, as well as production of maize per province (Agricultural Statistics, 2010).

Currently, maize is one of the most important crops worldwide with an annual cultivation area of more than 150 million hectares and an annual harvest more than 800 million tons of grain (FAO Statistics, 2010).

Table 1.1 Maize: area planted, total production, and production per province for 2001/02 and 2008/9 seasons in South Africa (Agricultural Statistics, 2010).

Maize production year	Area planted (x1000 Ha)	Total production (x1000 ton)	Total production per province* (x 1000 ton)								
			WC	EC	NC	FS	KZN	L	M	G	NW
2001/02	3533	9732	14	45	511	3217	402	106	2068	484	2885
2008/09	2896	12050	50	92	634	4527	521	247	2870	534	2575

*Key to abbreviations of provinces : WC (Western Cape) ; EC (Eastern Cape); NC (Northern Cape); FS (Free State); KZN (KwaZulu-Natal); L (Limpopo); M (Mpumalanga); G (Gauteng) and NW (North West)

The International Grains Council report in August 2010 contained the following production figures for three of the most important staple foods in the world today.

Table 1.2 World estimates for maize, wheat and rice production.

Crops	Estimated production of maize, wheat and rice in million tons				
	2006/7	**2007/8**	**2008/9**	**2009/10**	**Forecast for 2010/11**
Maize	710	795	798	809	829
Wheat	598	609	686	677	644
Rice	420	433	448	441	456

Maize production has increased dramatically over the last 40 years worldwide and has become the number one grain cereal crop before wheat and rice (Table 1.2).

The increase in production of maize from 809 to 829 million tons forecast for 2010/2011 is mainly due to improved production prospects from the United States and Africa (International Grains Council Grain Market, 2010).

For millions of people in Africa maize plays a very important role in their diet due to the ease of cultivation, agro-ecological adaptability, many uses, storage capabilities and as a cereal crop the potential for higher yields per hectare than many other crops (Asiedu, 1989**;** Fandohan *et al.*, 2003). Around 200 million people in developing countries consume maize

directly as their staple food. Maize is also used in a processed form for the production of ethanol and starch (Du Plessis, 2003).

Many studies on maize production conducted in African countries like Kenya (Onyango, 2010), Nigeria (Oluleye & Akinrinde, 2009), and Ethiopia (Negassa *et al.*, 2005) are aimed to optimise yields with more economical use of fertilisers for sustained or increased maize production (Negassa *et al.*, 2005; Xu *et al.*, 2006; Oluleye & Akinrinde, 2009; Onyango, 2010). The cost of fertilisers for small-scale farmers in the rural areas increases quite drastically the greater the distances between them and provincial centres, mainly due to transport cost (Xu *et al.* 2006). Maize production is more profitable for farmers near such centres and alternative methods of better utilization of inorganic fertilisers at lower application rates can ensure future production for those that have difficult access to commercial fertilisers.

Maize is grown under diverse conditions. Rainfall between 450 and 600 mm of water per season is sufficient for dry land production. The main nutrient requirements for optimum production are nitrogen, phosphorous and potassium. It is estimated that for each ton of grain produced, 15.0 to 18.0 kg of nitrogen, 2.5 to 3.0 kg of phosphorous and 3.0 to 4.0 kg of potassium is removed from the soil (Du Plessis, 2003). It is here where the use of fertilisers plays such an important role to sustain or increase yields, with an attendant unfortunate increase in cost of production. As a warm weather crop, maize is grown in areas where the daily temperature is not less than 19°C and the mean temperature of the summer months around 23°C. Emergence of maize at 20°C is within five to six days and 120 to 140 frost free days are normally needed for the growth period. The most suitable soil for maize production is deep, well drained, and with optimal moisture retention (Du Plessis, 2003).

The total maize yield potential not only depends on climate and soil but also the choice of cultivar, cultivation, crop protection and fertilising practices followed (Du Plessis, 2003). Many cultivars differ in their susceptibility to diseases such as ear rot, maize streak virus disease, cob-and-tassel smut, stem rot, and root rots.

Various micro-organisms applied to the soil with the incorporation of organic matter and practices will contribute to a healthier soil environment and the suppression of pathogenic micro-organisms by competing for nutrients (Ghorbani, 2008; Khan *et al.* 2008). Soil micro-organism biomass plays a very important role in the recycling of nitrogen, phosphorous and sulphur, which are very important nutrients for maize production (Ghorbani, 2008).

As with many other crops the main cause of grain loss in maize is often due to insect pest damage in the field or in storage. Pathogenic fungi are ranked second after insect damage to be the cause of grain loss and it is estimated that up to 80% of damage to maize during storage is as a result of the development of these fungi under favourable conditions (Kossou & Aho, 1993). *Fusarium*, *Aspergillus* and *Penicillium* are the most important genera of plant disease causing fungi in Africa (Samson, 1991; Orsi *et al.*, 2000).

Various plant-parasitic nematode species are present in South African maize production soils and can cause progressive losses over a number of seasons. The high cost of nematicides makes it uneconomical to use for many farmers (Du Plessis, 2003). This is an area where the incorporation of micro-organism formulations in the soil can play an important role in the reduction of plant-parasitic nematodes and the increase of non-parasitic nematodes to create balance in the soil biomass (Dagutat Science, 2010; Biological Control Products, 2010).

1.2 Justification for the study

The total area of maize planted in South Africa has decreased from 2001/02 season to the 2008/09 season, however, with an increase in total maize production (Table 1.1). Maize production has increased dramatically in all provinces except for North West Province that showed a slight decrease. The decrease in the total area planted could be an indication that new methods and technologies to sustain or increase maize production per hectare have to be researched.

In the year 2000, South Africa became a signatory to the United Nations Global Compact (UNGC) that promotes friendlier alternatives of crop protection for use in agricultural production to safeguard humans and the environment (United Nations Global Compact, 2010 http://www.unglobalcompact.org/AboutTheGC/index.html.). Ten years later South Africa is still allowing the use of many of the most hazardous pesticides on crops, including maize (Directorate: Food Safety and Quality Assurance South Africa, 2007; Pesticide Act 36 of 1947, 2010a,b). Micro-organism formulations that have been well investigated can potentially be the replacements of hazardous pesticides in time.

The removal of many crop protection products in the European Union (European Commission, 2010), and other countries (EPA, 2010a), which are hazardous to human health

and the environment, necessitated wider research on micro-organism formulations as replacements. These micro-organism formulations that have been well researched, can replace hazardous pesticides within time. South African farmers still use some crop protection products known to be among the most hazardous to humans, animals and the environment. This study aimed to evaluate micro-organism formulations that can replace many synthetic pesticides, which might have adverse effects on humans, animals and the environment.

This study examines not only the biological efficacy of various micro-organism formulations against *Fusarium* spp. causing ear and stem rot, but also their potential as plant-growth promoting micro-organisms to increase vegetative biomass and reproductive yields which can play a huge role in future reduction of fertiliser input and a subsequent reduction in production costs of small-scale farmers in Africa.

Millions of people in Africa consume maize and maize-related products contaminated with mycotoxins (fumonisins) daily and are not even aware of the potential health hazard that can be caused by it (Shephard *et al.*, 1996; Doko *et al.*, 1995; Hell *et al.*, 1995; Kedera *et al.*, 1999; Kpodo *et al.*, 2000; Gamanya & Sibanda, 2001; Ngoko *et al.*, 2001). In the Transkei some areas have a high rate of oesophageal cancer that is correlated with *Fusarium verticillioides* isolated from home grown maize that produces fumonisins (Marasas *et al.*, 1988). These health hazards alone provide adequate impetus to focus on maize research in order to sustain or increased production of maize that is safe for consumption by humans and animals and at the same time producing sufficient food by following environmentally sound practices that are sustainable.

The simplicity, safety, and the ease of application of most of the micro-organism formulations used for biological control of plant diseases make them very accessible to all farmers, including those farming large distances from major centres of supply and have the potential to reduce input costs in maize production (Khan *et al.*, 2008; Hasan, 2010; Dagutat Science, 2010). The actual use and application of the micro-organism formulations in this study cannot be simpler and could play a very important role in future treatments of maize seed and other grain, legume and oilseed crops.

1.3 Study aims and objectives

1.3.1 Aims

- This research aims to compare different fungal and bacterial micro-organism biological control agents with one another regarding their effects individually or combined on vegetative as well as reproductive yield of *Fusarium* infected maize.
- This research aims further to evaluate the biological efficacy and field stability of commercially formulated wettable powder fungal and bacterial micro-organism biological control formulations against *Fusarium* spp. causing stem and ear rot in maize.

1.3.2 Objectives

- To determine the vegetative as well as reproductive yield of *Fusarium* infected maize plant treated with micro-organism biological control agent formulations.
- To investigate the biological efficacy and phytotoxicity effects of micro-organism biological control agent formulations on *Fusarium* infected maize.

CHAPTER 2

LITERATURE REVIEW

2.1 Maize pathogens

Fusarium verticillioides, *Fusarium proliferatum*, *Fusarium graminearum* and *Fusarium anthophilum* are just some of the many phytopathogenic *Fusarium* species found associated with maize crops. Other strains include *F. oxysporum and F. pallidoroseum* that are known causal agents of seedling blight and *Fusarium* stalk rot of maize (Danielsen & Jensen, 1999; Andres-Ares *et al.*, 2004; Saunders & Kohn, 2008).

Factors that can influence and contribute to the infection of maize with *Fusarium* species producing fumonisins as contaminants include environmental conditions like climate, temperature and humidity, interactions among fungi in maize, damage caused by insects prior and post harvesting, agricultural practices, maize characteristics and post harvest operations (Fandohan *et al.*, 2003).

Environmental factors like drastic variations in rainfall and relative humidity during production prior to harvesting can cause physiological stress, which is likely to contribute to, increased fumonisins production (Visconti, 1996). It has been suggested that dry weather just before pollination of maize might play an important role in fumonisins production (Shelby *et al.*, 1994). Temperature and moisture during the production growth period of maize as well as during storage of grain play a role in the infection by *Fusarium* spp. as well as fumonisins production prior to harvesting. Water available for fungal growth is essential for increased infection by *Fusarium* spp. It has also been found under *in vitro* experiments that fungal competition of for example *F. verticilliodes*, is higher at temperatures round 25°C compared to 15°C and that activity further increases with increased water (Velluti *et al.*, 2000). This study was conducted and planned for the main growth period during the higher rainfall months (January, February and March) of the summer rainfall region in Gauteng and when temperature and moisture were normal and general conditions favourable during the pollination process. Irrigation water lines were used to overcome potential stress conditions during prolonged days of no rain. Temperature statistics for the trial site chosen for the period from January to March were also very favourable for *Fusarium* spp.

Yates *et al.* (2005) conducted maize trials where the growth response of maize plants was evaluated between *F. verticillioides* inoculated seed and uninoculated seed. Their findings showed that *F. verticillioides* did not necessarily cause a reduction in plant biomass as well as the cob yield, and that favourable growing conditions are of great importance prior to considering methods of control. One thing Yates *et al.* (2005) agreed on with other authors like Gelderblom *et al* (1988) and Nayaka *et al.* (2008) is the fact that *F. verticillioides* holds a health threat to humans and animals due to the production of fumonisins. This study investigated the use of micro-organism biological control agents not just for the control of *Fusarium* spp. but also for the characteristics as plant-growth promoters.

Maize harvested in tropical regions contain spores and mycelia of various fungal species of which *Fusarium*, *Aspergillus* and *Penicillium* are the most prominent. All these pathogens compete for food and can cause the reduced presence of one another and with subsequent reduced fumonisins production. For example, the presence of *F. verticilloides* and *F. proliferatum* (two of the most important producers of fumonisins) may be reduced significantly by the presence of *F. graminearum* (Velluti *et al.*, 2000). On the other hand *F. verticillioides* and *F. proliferatum* can be highly competitive against *Aspergillus flavus* as well as *Penicillium* species (Marin *et al.*, 1998). This study included *F. verticillioides* and *F. proliferatum* as inoculum of the maize seed prior to treatment with micro-organism biological control agents. The combined effect of the two pathogens guaranteed sufficient levels of crop loss for evaluation of significant differences between the biological control agent treatments, chemically treated and untreated control.

Parasitic nematodes in the soil and insects acting as vectors for spreading fungi as well as those causing physical damage to plants contribute to increased inoculums of fungal infection in plants (Dowd, 1998). Some Lepidopteran stem and cob borers, thrips and sap beetles (Flett & Van Rensburg, 1992; Munkvold & Desjardins, 1997; Cardwell *et al.*, 2000; Ako *et al.*, 2003) are known to be some of the causal agents to increase infection with *Fusarium* spp. (Fandohan *et al.*, 2003). It has been suggested by Schulthess *et al.* (2002) that by keeping plants free of fungus infection the insect damage to grain as well as to stems may be reduced. On the other hand, Fandohan *et al.* (2003) proposed that by keeping the plants free of insect infestation, infection by pathogens such as *F. verticillioides* may be reduced.

Agricultural practices play an important role in the potential infection of maize by *Fusarium* spp. Late plantings of maize which results in harvesting during wet conditions favours disease development caused by *F. verticillioides* (Bilgrami & Choudhary, 1998). Al-Heeti (1987) confirmed that wet weather late in the season increases the presence of this fungus in maize plants. Bilgrami & Choudhary (1998) further intimated that fungal infection is further enhanced by repeatedly planting maize and other cereals in the same or nearby fields. When using wheat, Lipps & Deep (1991), alluded to the fact that higher levels of *F. graminearum* occur with an increase in conservation tillage production in a double cropping system. As with the management of many other diseases on other crops it would be ideal to rotate maize with a non-host crop of *Fusarium* which may also be found on weeds and therefore the eradication of weeds may assist in the reduction of inoculum (Bilgrami & Choudhary, 1998).

Fungal infection and subsequent fumonisin production may also be influenced by the type of maize cultivar and grain characteristics such as colour, chemical composition and stage of development as well as endosperm type. *Fusarium* disease susceptibility is higher with late-maturing maize cultivars where there is a slow reduction of grain moisture content below 30% (Manninger, 1979). Infection is also more likely for maize cultivars with more upright cobs and tight husks (Emerson & Hunter, 1980) and thin grain pericarp (Riley & Norred, 1999). The role that colour plays on fumonisins contamination is not clear even though significantly higher levels have been reported in yellow maize compared to white in some years and the reverse situation found again in other years (Shephard *et al.*, 1996). The age of the grain may influence fumonisin production in maize and it has been found that levels are higher during the dent stage and much lower during the immature stage that again indicated the production of this mycotoxin during the early stages of cob development with an increase towards physiological maturity (Warfield & Gilchrist, 1999). It has also been reported that fumonisins contamination reached increased levels after physiological maturity (Chulze *et al.*, 1996).

Fungal infection and fumonisin production may be affected negatively or positively by postharvest handling and processing like washing, milling, cooking, fermentation, sorting and dehulling. Fungal spores may enter maize cobs and grain during mechanical handling (Fandohan *et al.*, 2003). No post harvest evaluations were done in this study due to harvesting at hard dough stage.

2.2 Micro-organisms associated with biological control of plant diseases

There are many biological control products already available and registered abroad against the control of *Fusarium* species on various crops but only one biocontrol product has been registered in South Africa for *Fusarium* control, namely, Tri-Cure (*Trichoderma harzianum* isolate MIT04), registered by MBF International cc (Information obtained from Pesticide Act 36 of 1947, 2010b). Many biocontrol formulations are undergoing registration field trials at present in South Africa, including some of the formulations used in this research project (Gerber, 2008; Gerber, 2010; Dagutat Science, 2010; Biological Control Products, 2010; Plant Health Products, 2010). All the micro-organism formulations under investigation may be applied as damp and dry inoculation of seed, soil drench via irrigation and spray. The potential is huge for the control of *Fusarium* on maize with biological agent formulations registered for use in crops like wheat, barley, oats, soybean and other legumes (Hasan, 2010).

As with chemical pesticides the majority of micro-organism field research is done by registration holding companies and kept on file with regulatory bodies and not necessarily published (Dagutat Science, 2010; Biological Control Products, 2010; Plant Health Products, 2010). Some companies publish field trial results on their websites (Plant Health Products, 2010). The only time micro-organism crop protection research is officially published is when studies are conducted with the main purpose of post graduate studies at tertiary institutions (Dagutat Science, 2010; Biological Control Products, 2010; Plant Health Products, 2010). Table 2.1 contains formulated micro-organism formulations registered as a result of efficacy evaluation of these formulations conducted by private companies, independent research consultants and field researchers, as well as studies conducted by academic institutions (Scala *et al.*, 2007; Whipps & McQuilken, 2009; EPA, 2010c; Pesticide Act 36 of 1947, 2010b).

There are many formulations already registered for use on a wide selection of crops in other countries (Table 2.1). This study not only looked at the individual performance of a formulation but also it's combined effect with another on maize yield response.

Trichoderma harzianum

The natural occurring fungus *Trichoderma harzianum* is just one of the many antagonists identified that are found as free-living organisms in the soil and environment. These antagonists normally release various compounds that induce localised or systemic resistance responses and plants are then protected from numerous plant pathogens. Root colonisation by

organisms like *Trichoderma* spp. enhances root development and crop production, reduces stress and increases nutrient uptake. Plants are protected against various pathogens by responses that are similar to systemic acquired resistance and rhizobacteria-induced systemic resistance (Harman *et al.*, 2004).

Many *Trichoderma* isolates have been found that are of great use in biological control of a wide spectrum of pathogens including *Fusarium* spp., *Pythium* spp., *Phytopthora* spp., *Rhizoctonia solani*, *Botrytis cinerea*, *Sclerotium rolfsii* and others (Harman *et al.*, 2004; Scala *et al.*, 2007). There are many commercial formulations registered as well as unregistered formulations pending and in the process of registration in South Africa and countries abroad (Scala *et al.*, 2007; Gerber, 2008; Gerber, 2010; Pesticide Act 36 of 1947, 2010b). Many products are marketed prior to registration and their availability and distribution is much wider than realised; especially since *Trichoderma* is listed in the European Union and the United States as an approved substance for use in organic farming (Scala *et al.*, 2007). Table 2.1 contains many *Trichoderma* formulations marketed worldwide, most registered for use on crops in countries including New Zealand, United States, South Africa, India, and Sweden.

The possible mechanisms involved for *Trichoderma* species antagonism are (1) the production of volatile or non-volatile antibiotics (2) competition for space, source of energy and nutrients (Sivan & Chet, 1989) and (3) direct mycoparasitism where the pathogenic fungus cell wall is degraded by the lytic enzymes secreted by *Trichoderma* spp. (Chet *et al.*, 1996). Studies have been conducted by various authors to evaluate the use of combined micro-organisms including different fungi (Budge *et al.*, 1995; De Boer *et al.*, 1997), or fungi and bacteria (Janisiewicz, 1988; Duffy & Weller, 1995; Hasan *et al.*, 1997; Hasan, 2010). None of the studies evaluated the compatibility of *Trichoderma harzianum* with *Microbacterium maritypicum or Bacillus subtilis* isolates and therefore the inclusion of such combinations in the present study.

Bacillus subtilis

The research done with *Trichoderma* spp. in biological control against disease causing pathogenic fungi is followed closely by studies done on the biological effect of *Bacillus* spp. on a wide range of crops in terms of available literature (Nehl *et al.*, 2006; Kapooria, 2007; Taguchi & Hyakumachi, 2007; Zhou *et al.*, 2007; Lee & Lee, 2007; Saravanan *et al.*, 2008). *Bacillus subtilis* species are known for their hardiness in terms of their resistance to heat, drying and other destructive environmental factors in comparison to vegetative cells and are

highly suitable for commercial use due to their ease of genetic manipulation and cultivation. *Bacillus subtilis* and other isolates of the genera *Bacillus* are used not only in the fermentation process of human foods but also for industrial and medicinal purposes. Some of these strains like for example *B. subtilis* isolate RRC101 have also shown strong plant growth-enhancing characteristics, as well as antagonistic characteristics as an endophyte against pathogenic micro-organisms in maize (Hinton & Bacon, 1995; Bacon *et al.*, 2001). It is therefore of importance that as many as possible *Bacillus subtilis* isolates were included for evaluation in this study. Potentially these isolates have dual functions; biological control of the *Fusarium* spp. used as inoculums of the maize seed as well as their potential to promote vegetative growth as well as increased reproductive yield.

Microbacterium maritypicum

Microbacterium maritypicum is a beneficial bacterial inoculant used for biocontrol of bacterial pathogens (Hygrotech Sustainable Solutions, 2010; Dagutat Science, 2010). It out competes other bacteria and fungi through the production of antibiotics. *M. maritypicum* was isolated in South Africa by Dagutat Science and have been used for biocontrol with success on a commercial level for *Pectobacterium* bacterial disease on potatoes (Data not published). It is is not a well known biocontrol agent like *Bacillus* or *Trichoderma*. No data on the biocontrol characteristics of *Microbacterium* is available as yet. The American Type Culture Collection (ATCC) is a private biological resource center in the United States. No *Microbacterium* cultures were deposited in the ATCC culture collection; however a *Microbacterium maritypicum* culture was deposited in the Belgian Coordinated Collections of Micro-organisms (BCCM) bacteria collection at University of Gent in Belgium (ATCC, 2010; BCCM/LMG, 2010). This study is very likely the first to evaluate the effect of *M. maritypicum* against *Fusarium* spp. in maize as well as it's potential as a plant-growth promoting rhizobacterium.

Beauveria bassiana

The entomopathogenic fungus *Beauveria bassiana* has been studied for many years with focus during the 1970's on the biological control of insects (Ferron, 1981). East European countries studied the effect of *B. bassian*a to be used for the control of insects like the Colorado beetle as early as the 1950's (Roberts & Yendol, 1971).

In more recent years dual biological control of insects as well as plant disease pathogens have been reported. There is evidence that *Beauveria bassiana* reduces diseases caused by pathogenic fungi like *Fusarium* spp. (Orole & Adejumo, 2009). More research is needed to understand how *B. bassiana* and other fungi as epiphytes and endophytes are involved in the suppression of plant disease. Many strains have been registered for use as bioinsecticides in crop protection and the use as a biofungicide thus offering opportunities for a lot of research work to be done (Vega *et al.*, 2009). The *B. bassiana* isolate used in this study has been successfully used against the reduction of parasitic nematodes and is registered for use on carrots in South Africa. This biological control agent was used in the present study in combination with *T. harzianum* to evaluate their compatibility and possible synergistic effect in the reduction and control of *Fusarium* spp and contribution to increased plant biomass and yield.

Table 2.1 List of bacterial and fungal biological control agent formulations commercially produced and registered worldwide, target disease or activity and crop registered for (Scala *et al.*, 2007; Whipps & McQuilken, 2009; EPA, 2010c; Pesticide Act 36 of 1947, 2010b).

Biological control agent	**Product name and Country of registration**	**Target pathogen / disease name and growth activity**	**Crops**
Agrobacterium radiobacter	Galltrol-A, Norback 84-C, Nogall, Diegall, Dygall - Australia, United States, and New Zealand	Crown gall caused by *Agrobacterium tumefaciens*	Fruit, nuts, ornamental nursery stock
Ampelomyces quisqualis isolate M-10	AQ10 - USA	Powdery mildew	Cucurbits, apples, grapes, ornamentals, strawberries, tomatoes
Bacillus cereus BP01	Mepplus – United States	Plant-growth promotion	Cotton
Bacillus pumillus GB34	YieldShield concentrate, GB34 Biological Fungicide – United States	Soilborne fungal pathogens causing root diseases	Soybean
Bacillus subtilis	Cillus, Green-all G – Korea	Pythium damping-off	Tomato
Bacillus subtilis	Kodiak, Epic, Concentrate, Kodiak HB, Quantum 4000, System 3- USA	*Rhizoctonia solani*, *Fusarium*, *Alternaria*, and *Aspergillus* spp.	Root rots and seedling diseases in general
Bacillus subtilis	Phytovit WG- Germany	*Fusarium*, *Verticillium*, *Pythium* spp. and *Rhizoctonia solani*	Various vegetable and field crops

Bacillus subtilis GB03	Companion – United States	*Fusarium* spp., *Rhizoctonia solani, Pythium spp, Phytopthora spp.*	Ornamentals, turf, dry and snap beans, cotton, peanuts, soybean, wheat, barley
Bacillus subtilis isolate DB101	Maximus- South Africa (Registration pending in SA; still undergoing field trials)	Suppression of pathogenic fungi causing wilts. Increased plant biomass and grain yields.	Grain crops, soybean, tomato
Bacillus subtilis isolate DB109	Armenius (Registration pending in SA; still undergoing field trials)	*Xanthomonas, Pseudomonas, Erwinia* + *Fusarium* spp. Increased plant biomass and grain yields	Grain crops, potato + tomato, soybean
Bacillus subtilis isolateDB108	Shelter- South Africa (Registered in SA for Botrytis and powdery mildew)	Botrytis and powdery mildew. Increased plant biomass and grain yields.	Registered on table grapes. Grain crops, and soybean
Bacillus subtilis MB1600	HiStick N/T, Pro-mix, Subtilex HB – United States and Mexico	*Fusarium, Aspergillus, Rhizoctonia, Alternaria, Rhizoctonia solani*	Soybean, peanuts, alfalfa and dry/snap beans, field crops, turf, cotton
Bacillus subtilis QST716	Serenade- USA	Powdery and downy mildew, cercospora leaf spot, early and late blight, brown rot and fire blight, etc.	Grapes, hops, cucurbits, vegetables, peanuts, stone and pome fruit plus others
Beauveria bassiana isolate DB105	Spartacus- South Africa (Undergoing efficacy field trials on many other crops in SA)	Registered in SA against parasitic nematodes. Increased plant biomass and yields	Carrots. Grain crops, tomato + potato
Burkholderia cepacia type Wisconsin	Deny- USA	*Rhizoctonia solani, Pythium,* and *Fusarium* spp.	Alfalfa, barley, beans, clover, cotton, peas, grain sorghum, vegetables, wheat
Burkholderia cepacia type Wisconsin	Intercept - USA	*Rhizoctonia solani, Pythium,* and *Fusarium* spp.	Maize, vegetables, cotton
Coniothyrium minitans	Koni - Hungary	*Sclerotinia sclerotiorum* and *S. minor*	Glasshouse crops and amenity areas
Gliocladium catenulatum	Primastop, Prestop Mix, Prestop WP - Finland	Seed, root and stem rot plus wilt caused by soil-borne pathogens	Damping-off of vegetables, herbs, and ornamentals plus numerous other plants
Gliocladium virens	SoilGard 12G - USA	*Rhizoctonia solani* and *Pythium* spp. plus other damping-off and root pathogens	Damping-off of bedding plants, greenhouse crops and ornamentals
Microbacterium maritypicum isolate DB 107	Bismarck- South Africa (Registration pending in SA; still undergoing efficacy field trials)	*Xanthomonas, Pseudomonas,* and *Erwinia* .Increased plant biomass and grain yields	Maize, tomato, potato
Pseudomonas syringae	Bio-save 10LP,110 - USA	*Botrytis cinerea, Mucor pyroformis, Penicillium* spp.	Pome fruit, citrus, cherries, potato

Pseudomonas aureofaciens	Bioject Spot-Less - USA	Anthracnose, dollar spot, *Pythium aphanidermatum*, Michrochium patch	Turf and others
Pseudomonas fluorescens	Blightban A506 - USA	Frost damage, *Erwinia amylovora* as well as russet-inducing bacteria	Apple, almond, apricot, cherry, blueberry, peach, pear, potato, tomato, and strawberry
Streptomyces griseoviridus	Mycostop- Finland	*Fusarium, Botrytis, Pythium*, and *Phytopthora* spp. + *Alternaria brassicola*, that cause seed, root and wilt diseases	Field, vegetable and ornamental crops
Streptomyces lydicus	Actinovate - USA	Soil-borne diseases	Greenhouse and nursery crops, and turf
Trichoderma harzianum	Tri-Cure - South Africa	*Fusarium* spp., *Rhizoctonia spp.* Stem canker, Black scurf	Maize, wheat, drybeans, peanuts, soybean, vegetable seedlings.
Trichoderma harzianum	T-Gro- South Africa (Registered for Botrytis + powdery mildew on table grapes)	*Fusarium* spp., *Rhizoctonia solani, Botrytis cinererea*, + *Pythium* spp., and powdery mildew	Table grapes, maize, wheat and other field crops, soybean, sunflower, and vegetables
Trichoderma harzianum	Eco-T - South Africa	*Pythium* and *Rhizoctonia solani* seedling diseases	Numerous crops
Trichoderma harzianum	Supresivit – Czech Republic	Various fungi	Legumes and leaf vegetables
Trichoderma harzianum	Trichoplus- South Africa	Various fungi	Various seedlings
Trichoderma harzianum + *Trichoderma polysporum*	BINAB-TW- Sweden	Various root-infecting fungi	Glasshouse crops
Trichoderma harzianum + *Trichoderma viride*	Trichodry, Trichoflow, Trichogrow, Trichopel R, Trichopel – New Zealand	*Rhizoctonia solani, Pythium* spp., *Phytophthora* spp. and *Fusarium* spp.	Field crops, vegetables, ornamentals and turf
Trichoderma harzianum Rifai strain KRL-AG2	Plantshield HC, T-22 Planterbox, T22 HC, Rootshield-22 , Turf Shield- Netherlands	*Pythium and Fusarium spp* + *Rhizoctonia solani* + *Thielaviopsis spp., Sclerotinia homeocarpa*	Range of crops, ornamentals and turf
Trichoderma harzianum T35+ *Trichoderma harzianum* TH315	Root Pro, Root-Potato - Israel	*Rhizoctonia solani, Pythium* spp., *Sclerotium rolfsii* and *Fusarium* spp.	Seedling diseases on range of crops and potato
Trichoderma harzianum GBF-0208	Green-all T WP - Korea	Numerous pathogens	Vegetables, bulbs and turf
Trichoderma viride	Ecoderma - India	*Rhizoctonia solani, Pythium* spp., and *Fusarium* spp.	Damping-off, root rots and collar rots of various plants

Table 2.2. Type of formulations and their application methods commonly used depending on the target area of treatment (soil, seed, foliage, fruit, stem, as well as roots).

Type of Formulation	**Application Method**
Granules	Granules mixed with soil
Liquid	Drench with seeding or transplanting, or spray
Peat-based dried biomass from solid fermentation; aqueous suspension	Applied to seed with sticking agent; Aqueous suspension via drip irrigation
Petri plates with pure culture grown on agar	Suspension of bacterial mass in water applied to seeds, seedlings, cuttings, and roots as a soil drench
Dry powder	Dry application (dusting)
Water dispersible granule	Spray
Wettable powder	Damp, or dry inoculation of seed, spray and/or soil drench via irrigation

2.3 Biological efficacy studies against *Fusarium* spp. in maize

Biological control of *Fusarium* spp. in maize holds considerable promise and includes the treatment of seed, soil, foliage and heads with antagonists to reduce pathogen inoculum. Many studies have been conducted with bacterium strains, including *Bacillus* isolates, being antagonistic to *Fusarium* spp. (Gliessman, 2001; Chincholkar & Mukerji, 2007; Khan *et al.*, 2008; Walters, 2009; Hasan, 2010).

Table 2.1 shows the many studies that have been conducted to investigate suitable micro-organism agents for biological control of *Fusarium* spp. on certain crops as well as many other plant pathogens on a wide variety of crops (Gliessman, 2001; Chincholkar & Mukerji, 2007; Khan *et al.*, 2008; Walters, 2009; Hasan, 2010). It is clear that not many products have been registered for use in maize and it is very likely that many unpublished studies are still being completed for registration in specific countries, as might be the case with many formulations currently in South Africa (Dagutat Science, 2010; Plant Health Products, 2010).

Biological agents *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* applied to inoculated maize seeds with the pathogenic strain *Fusarium fluorescens,* showed a significant reduction in the population count of *F. fluorescens* at the inner tissue of the roots (Pereira *et al.*, 2009). In this study *Microbacterium maritypicum* isolate DB 107 is evaluated for its potential to reduce *Fusarium* spp. inoculum as well as its potential to increase vegetative and reproductive yields in maize. The research conducted by Pereira *et al.* (2009) showed that

species from the genera *Microbaterium* have potential to be used in crop protection against *Fusarium* spp.

Green house trials in Argentina conducted with multiple strains of *B. subtilis* obtained from the rhizoplane of maize showed strong inhibition of *F. verticillioides* and could therefore be a candidate as a biological control agent at root level (Cavaglieri *et al.*, 2005). The findings in this study confirm the importance of *B. subtilis* isolates to be evaluated against *F. verticillioides* and thus offers a strong argument and justification for its inclusion in this study. Most research work on biological control agents is conducted under greenhouse conditions. The present study is conducted under field conditions and results should be considered as more representative of what may be expected under larger commercial conditions of maize production.

During *in vitro* trials in India, a pure culture of *P. fluorescens* was used as maize seed and foliage spray treatments against *F. verticillioides* resulting in increased plant growth as well as a reduction in the levels of fumonisins produced. This clearly indicated that this strain of *P. fluorescens* strain has great potential to be used as a biological control agent in formulations to manage ear rot disease in maize production (Nayaka *et al.*, 2008).

In a study conducted by Orole and Adejumo in 2009, the antagonistic potential of endophytes *Trichoderma koningii, Acremonium strictum, Alternaria alternata, Phoma* spp. and *Beauveria bassiana* has been examined under greenhouse conditions as a potential method to manage wilt and rot diseases of maize caused by *F. oxysporum, F. pallidoroseum, F. verticillioides* and *Cladosporium herbarum. A. alternata* and *T. koningii* showed the highest antagonism against these wilt causing pathogens. *Acremonium strictum* and the *Phoma* spp. showed no significant differences in the ability to suppress wilting and yellowing of leaves of maize seedlings under greenhouse conditions. The performance of *B. bassiana* was between that of the highest and lowest antagonists, but not significant. These results under greenhouse conditions do not necessarily mean that the same results will be obtained under field conditions and need further investigation.

The application of fungicides can reduce the horizontal infection spread of the facultative fungal endophyte *Fusarium verticillioides* (= *F. moniliforme*) that is the manner by which this fungus is spread contagiously and through which infection occurs from the outside. Transmission of the endophytic phase of the fungus occurs vertically and cannot be

controlled by seed applications of chemical fungicides. An endophytic bacterium, *B. subtilis*, has been used in a biological control system that showed great potential for the reduction of mycotoxin accumulation during the endophytic (vertical transmission) growth stage. Another system has been developed with an isolate of *Trichoderma* spp. that showed potential in the postharvest control of growth and toxin accumulation of *F. verticilliodes* on maize in storage (Bacon *et al.*, 2001).

2.4 Vegetative and reproductive yields

Yates *et al*., 2005 investigated the dual roles of *F. verticillioides* in producing harmful mycotoxins and at the same time stimulate growth of other plants within the Graminae family. The possibility of the fungus to exist in a symbiotic, mutualistic relationship with the maize plant until external abiotic and/or biotic factors influence the stability of such relationship was also investigated. The study was the first documentation of the field performance of maize grown from *F. verticillioides* inoculated seed in comparison with uninoculated seed. Results showed that *F. verticillioides* inoculated maize seed produced plants that equalled or exceeded the yield performance of plants grown from the uninoculated seed. It was also found that vegetative mass decreased as seed weight increased. They speculated that the fungus *F. verticillioides* mobilised nutrients from the vegetative tissue for kernel filling during environmental stress conditions.

From these results they further proposed that controls of *F. verticillioides* should be focused on external factors that can minimise the adverse effects of the fungus on the performance of maize plants. In addition they proposed that proper growth conditions as well as storage conditions of maize products be maintained to prevent disease and mycotoxin production in the host/fungal relationship.

Wu *et al.*, 2005 conducted a study on maize under greenhouse conditions to evaluate the effects of four biofertilisers which consisted of arbuscular mychorrhizal fungi (*Glomus mosseae* or *G. intraradices*) with or without a Nitrogen fixer (*Azotobacter chroococcum*), a phosphate solubiliser (*Bacillus megaterium*), and a potassium solubiliser (*Bacillus mucilaginous*). The micro-organism inoculants increased the growth and nutritional assimilation (total nitrogen, phosphate and potassium) of the maize plant with an additional increase in soil properties. These results confirmed the importance of the inclusion of the

genera *Bacillus* in this study to identify potential micro-organisms with phosphate or potassium solubilising characteristics. This study did not include any abuscular mychorrhizal fungi or *Azotobacter* spp. in the treatments and the *Bacillus* species differed from that of the ones used by Wu *et al.* (2005).

CHAPTER 3

METHODOLOGY

3.1 Research site

This study was conducted in the field. The field trial site was situated on a private research farm positioned next to Roodeplaat Nature Reserve, in Leeuwfontein, Dinokeng, Gauteng Province, South Africa (co-ordinates: S 025° 39.662¹ E 028° 28.388¹). Only field trials for efficacy evaluation of micro-organism crop protection formulations were done at these facilities. This site is positioned in the summer rainfall area of South Africa with warm to hot summers and an average rainfall around 400 to 500 mm annually.

3.2 Application treatments

The treatments in this study included various fungal and bacterial biological control micro-organisms commercially formulated as well as a reference product and an untreated control.

3.2.1 Micro-organisms (test products)

Biological control agent formulations containing fungal and bacterial strains were supplied by Dagutat Science, the company that is the formulator, manufacturer and registration holder of the test products. Total production of formulations was done by Dr. Helga Dagutat a PhD holder in Microbiology and Nita Peacock a holder of an MSc Plant Physiology and both are employed by Dagutat Science in Pretoria, Republic of South Africa.

The biological control micro-organism formulations used in this study were as follows:

Table 3.1. Fungal strains used in study

Product name	Formulation type	Fungal strain
T-Gro WP	Wettable powder	1 x 10^9 active spores/g *Trichoderma harzianum* isolate DB103
Spartacus WP	Wettable powder	1.5 x 10^9 colony forming units/g *Beauveria bassiana* isolate DB 105

Table 3.2. Bacterial strains used in study

Product name	Formulation type	Bacterial strain
Maximus WP	Wettable powder	3 x 10^7 colony forming units/g *Bacillus subtilis* isolate DB 108
Shelter WP	Wettable powder	5 x 10^7 colony forming units/g *Bacillus subtilis* isolate DB 101
Armenius WP	Wettable powder	5 x 10^7 colony forming units/g *Bacillus subtilis* isolate DB 109
Bismarck WP	Wettable powder	6 x 10^8 colony forming units/g *Microbacterium maritypicum* isolate DB 107

3.2.2 Reference product

The reference product selected for the purpose of this study was Thiram 750WP (Act 36 of 1947 Reg. no L7097), a wettable powder formulation from Villa Crop Protection, South Africa and contains 750g/kg thiram as active ingredient for seed treatment prior to planting in soil. Date of manufacture April 2008; Batch no: 0804NB; Expiry date: April 2010.

3.2.3 Untreated control

The untreated control plots were planted with *Fusarium* inoculated maize seed as for all the other treatments. Plots received no other plant protection products of any kind for the duration of the trial. All treatments, including the untreated plots received the same quantity of fertiliser as well as water and all cultivation practises applied. The main purpose of the

inclusion of an untreated control as a treatment was to be able to compare the efficacy of the micro-organism treatments as well as the reference product treatment against the *Fusarium* spp. causing stem and ear rot in maize, as well as to determine differences in vegetative and reproductive yields.

3.2.4 Mode of application

(i) Type of application and equipment

a) Seed treatment application

A 1.5 L plastic container with lid was used to mix the maize seed with the micro-organism formulations and the reference product (adapted from EPPO Standards PP1, 2004f). For big agricultural quantities the use of a concrete mixer is recommended. The seed was mixed in a container for thorough mixing by measuring the recommended quantity of prepared suspension concentrate mixture per weight seed for the micro-organism formulations (Refer to Table 3.3) and for the reference product.

b) Soil drench application

A fine rose watering can with a total water capacity of 10L was used for the drench of the micro-organism formulations per micro-plot. Under commercial productions these products are applied through the pivot irrigation system (Dagutat Science, 2010).

(ii) Time and frequency of application

Micro-organism biological control formulations were applied as a soil drench on day of planting seed, a second application between two and three weeks thereafter, and a third application between five and six weeks post date of planting maize seed. Micro-organism formulations were applied early morning. The reference product Thiram was only applied once to the seed prior to planting.

(iii) Doses and volumes

Dagutat Science micro-organism formulations were formulated and packed in 450 g units of wettable powder for ease of use per hectare on commercial field crops and for micro trial plots, to ensure even distribution of fungal spores and bacteria cells (Dagutat Science, 2010).

To ensure the effective distribution of micro-organism spores and cells when measuring small quantities of the biological formulated products, a special mixture was prepared and recommended dosages measured for seed treatment and soil applications from the mixture below was used. The mixture was prepared as follows:

1. One packet (450g) Dagutat Science product was mixed with 1ℓ good quality water in a container with a lid so that the mixture could be shaken. Nu-Film P at a dosage rate of 1 mℓ was added as a sticker to every 1-litre water, pre-mixed with micro-organism formulations prior to measurement of smaller quantities for the seed treatments. No sticker was added to mixtures for the soil drench applications.
2. The water pH was between 6.0 and 7.5.
3. The container was shaken well.
4. The suspension concentrate mixture was then ready for use.

Table 3.3. Dosage rates of prepared micro-organism mixture used for measuring smaller quantities.

Number of 450g packets per Hectare	Dosage in grams of 450g wettable powder packet per hectare	Dosage in grams of prepared mixture of 1450g (450g + 1 ℓ water) per hectare	Dosage in grams of prepared mixture of 1450g (450g + 1ℓ water) per 10m² soil area	Dosage in grams of 450g packet wettable powder per 10m² soil area
1	450	1450	1.45	1.74
2	900	2900	2.90	3.48
3	1350	4350	4.35	5.22
4	1800	5800	5.80	6.96
5	2250	7250	7.25	8.7
6	2700	8700	8.70	10.44

Steps taken in the mixing and application for soil drenching:

1. The recommended dosage per micro-organism treatment was taken from well shaken prepared suspension mixture as per dosage rate for the treatment of 10m² soil area in Table 3.4.

2. The measured dosage was then mixed with a total of 10 ℓ of water in a watering can.

3. A total volume of 10ℓ mixture/10m^2 was applied as a soil drench with a watering can to simulate a pivot application. Products were mixed together in the 10ℓ of water as a single application. The volume of water applied as a drench was sufficient to cover the total area of the plot but was deemed not sufficient to wash the micro-organisms into the root zone area.

All soil drench applications were watered well directly after application to minimise exposure of micro-organisms to harmful ultra violet rays.

a) Dosage rates for seed treatment

Table 3.4. Micro-organism formulation and reference product treatments and their dosage rates per kg seed (adapted from EPPO Standards PP1, 2004c)

Product	**Formulation**	**Application rate per product per 100 kg seed or as indicated**
1.Untreated control	-	-
2.T-Gro	*Trichoderma harzianum* isolate DB 103	900g T-Gro / 100kg seed mixed with 1ℓ water
3.Bismarck	*Microbacterium maritypicum* isolate DB 107	900g Bismarck / 100kg seed mixed with 1ℓ water
4. Shelter	*Bacillus subtilis* isolate DB 108	900g Shelter / 100kg seed mixed with 1ℓ water
5.Maximus	*Bacillus subtilis* isolate DB 101	900g Maximus/ 100kg seed mixed with 1ℓ water
6.Armenius	*Bacillus subtilis* isolate DB 109	900g Armenius / 100kg seed mixed with 1ℓ water
7.T-Gro+Shelter	*Trichoderma harzianum* isolate DB 103+ *Bacillus subtilis* isolate DB 108	1 x 450g T-Gro and 450g Shelter/ 100kg seed mixed with 1ℓ water
8.T-Gro+ Maximus	*Trichoderma harzianum* isolate DB 103 + *Bacillus subtilis* isolate DB 101	450g T-Gro and 450g Maximus / 100kg seed mixed with 1ℓ water
9.T-Gro +Bismarck	*Trichoderma harzianum* isolate DB 103+ *Microbacterium maritypicum* isolate DB 107	450g T-Gro and 450g Bismarck / 100kg seed mixed with 1ℓ water
10. T-Gro + Armenius	*Trichoderma harzianum* isolate DB 103 + *Bacillus subtilis* isolate DB 109	450g T-Gro and 450g Armenius/ 100kg seed mixed with 1ℓ water
11.T-Gro +Spartacus	*Trichoderma harzianum* isolate DB 103 + *Beauveria bassiana* isolate DB 105	450g T-Gro and 450g Spartacus/ 100kg seed mixed with 1ℓ water
12. Standard Thiram	750g/kg WP thiram	Mix 180g per 100kg maize seed.

b) Dosage rates for soil drench treatments

Table 3.5. Micro-organism formulation and reference product treatments and their dosage rates per hectare or 10m² soil drench applications post planting of seed (adapted from EPPO Standards PP1, 2004c).

Product	**Formulation**	**Frequency of soil applications with micro-organism formulations plus the dosage rate of products per hectare or 10m² trial plot**	
		Frequency of applications and product in kg wettable powder per hectare field via irrigation	**Frequency of applications and product in g of 1450 g prepared mixture in 10 ℓ water per 10m² plot**
1.Untreated control	-	-	-
2.T-Gro	*Trichoderma harzianum* isolate DB 103	2.7kg/Ha on day 1 (directly after planting), day16, and day 38	8.7g per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards
3.Bismarck	*Microbacterium maritypicum* isolate DB 107	2.7kg/Ha on day 1 (directly after planting), day16, and day 38	8.7g per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards
4. Shelter	*Bacillus subtilis* isolate DB 108	2.7kg/Ha on day 1 (directly after planting), day16, and day 38	8.7g per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards
5.Maximus	*Bacillus subtilis* isolate DB 101	2.7kg/Ha on day 1 (directly after planting), day16, and day 38	8.7g per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards
6.Armenius	*Bacillus subtilis* isolate DB 109	2.7kg/Ha on day 1 (directly after planting), day16, and day 38	8.7g per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards
7.T-Gro + Shelter	*Trichoderma harzianum* isolate DB 103+ *Bacillus subtilis* isolate DB 108	2.7kg/Ha of both products on day 1 (directly after planting), day16, and day 38	8.7g of both per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards.
8.T-Gro + Maximus	*Trichoderma harzianum* isolate DB 103 + *Bacillus subtilis* isolate DB 101	2.7kg/Ha of both products on day 1 (directly after planting), day16, and day 38	8.7g of both per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards.
9.T-Gro + Bismarck	*Trichoderma harzianum* isolate DB 103+ *Microbacterium maritypicum* isolate DB 107	2.7kg/Ha of both products on day 1 (directly after planting), day16, and day 38	8.7g of both per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards.
10. T-Gro + Armenius	*Trichoderma harzianum* isolate DB 103 + *Bacillus subtilis* isolate DB 109	2.7kg/Ha of both products on day 1 (directly after planting), day16, and day 38	8.7g of both per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards.
11.T-Gro + Spartacus	*Trichoderma harzianum* isolate DB 103 + *Beauveria bassiana* isolate DB 105	2.7kg/Ha of both products on day 1 (directly after planting), day16, and day 38	8.7g of both per 10m² / 10ℓ water on day 1(directly after planting), day16, and day 38. Water well afterwards.
12. Reference Thiram	750g/kg WP thiram	No soil applications	No soil or foliage applications after planting of seed.

3.3 Soil preparation and cultivation

The area selected for the study was planted with *Fusarium verticillioides* and *Fusarium proliferatum* inoculated wheat seed in April 2009. Wheat was harvested early October 2009 and the maize experiment was then conducted on the same site to ensure the presence of *Fusarium* spp. in the soil environment. The field trial site was prepared for planting maize under irrigation during the last week in December 2009. Soil samples were taken from the site prior to fertilising and planting and analyzed by ECO Analytica, North West University, Potchefstroom, South Africa (see Appendix 3).

Experimental plots were fertilised on day of planting with maize seed. Seed was inoculated with *Fusarium* spp. one day prior to treatment with reference product Thiram and the micro-organism formulations. Seed was then planted by hand the next morning after seed treatments with the micro-organism formulations and the reference product. One kilogram of seed was treated for each treatment as per recommended dosage and one hundred treated seeds were then planted per plot. The untreated plots received only maize seed inoculated with *Fusarium* spp. Watering was carried out via a micro-mist irrigation system every second day when no rain occurred to the point of saturation of the top 10 cm of the soil. Rainfall was recorded for the duration of the trial (see Appendix 2). All applications as well as evaluations were done by the author.

Trial plots in this study were weeded by hand and the practice of zero-tillage applied. The additional application of the biological control agent treatments post planting as a soil drench and washed into the rhizophere of the maize plants was intended to minimise potential horizontal transmission of the *Fusarium* spp. from debris and the soil to healthy maize plants. The main purpose of seed treatment with the biological control agents in this study was to ensure protection of the seed from day one of planting and through the germination process to evaluate their effect in the reduction of vertical transmission of the endophytic *Fusarium* spp.

No fungicides or insecticides of any kind were applied to the soil or foliage for the duration of the trial; only treatments to the soil environment were applied as per Table 3.5.

3.4 Seed inoculation

3.4.1 Seed material

Untreated, certified seed of the cultivar Pioneer 32D96B was obtained from Pioneer Du Pont in Rosslyn, South Africa. Pioneer 32D96B is a stable Bt yellow maize hybrid, widely adapted with sound agronomic characteristics. Genetically it contains the YieldGard® resistance gene against stalk borer. It also has reliable drought resistance; good stalk strength and quick dry-down. The cultivar is an excellent irrigation hybrid, especially with early plantings (Pioneer Du Pont South Africa, 2010).

3.4.2 Preparation of *Fusarium verticillioides* and *Fusarium proliferatum* inoculums

A number of ten (10) *Fusarium* infected Pioneer Seed maize cobs were sampled from KwaZulu-Natal (Fig 3.3a and 3.3b). Agar plates containing peptone pentachloronitrobenzene (PCNB), and antibiotics for inhibition of bacterial growth: benzylpenicillin, pendistrep and chloramphenicol pure were used in the preparation following a protocol previously utilised by Van der Walt *et al*. (2007). Water agar plates and carnation leaf agar (CLA) were used for the purification and preparation of single spore cultures of the *Fusarium* isolates. As for the identification of species single-spore cultures were transferred to potato dextrose agar to observe colony morphology, and to CLA plates and synthetic nutrient agar (SNA) to examine microscopic structure (Van der Walt *et al*., 2007).

3.4.3 Inoculation of maize seed with prepared *Fusarium* culture

A 200mℓ *Fusarium* spp. inoculum with a count of 10^4 conidia / mℓ was prepared by Dagutat Science company. The 200mℓ *Fusarium* spp. inoculum was mixed with 1kg seed/ treatment. Seed was inoculated with *Fusarium* spp. 1 day prior to treatment with reference product Thiram and the micro-organism formulations. Seed was then planted by hand the next morning after treatment with the micro-organism formulations and the reference product. One (1) Kg seed was treated for each treatment as per recommended dosage and 100 treated seeds were then planted per plot. The untreated plots received only maize seed inoculated with *Fusarium* spp. Seed was planted by hand with equal spacing of 10cm in the row and 50 cm in between rows.

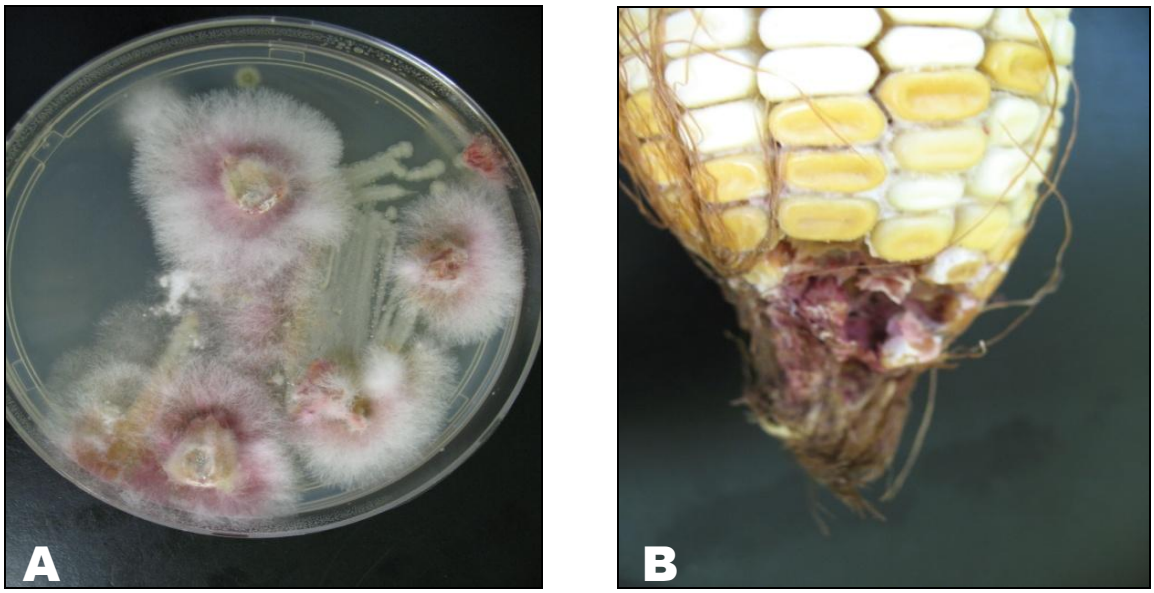

Fig. 3.1a. *Fusarium* growth on agar from inoculum prepared. **3.1b.** Infected maize from KwaZulu-Natal.

3.5 Experimental design and analysis

Test products, reference product and untreated control were arranged in a randomised block design as according to the European and Mediterranean Plant Protection Organization Standards (EPPO Standards PP1, 2004a). The standard for the efficacy evaluation of seed-borne cereal fungi (EPPO Standards PP1, 2004b) was also adapted for the evaluation of maize treated with various micro-organism biological control formulations to evaluate the micro-organism formulations' biological efficacy against *Fusarium* spp. For the evaluation of the effects of the micro-organism formulations on vegetative and reproductive yields the standard for phytotoxicity assessment was adapted (EPPO Standards PP1, 2004f). The rating scales used for the assessment of disease symptoms as well as tasselling and silking development were adapted from various standards as described in the European and Mediterranean Plant Protection Organization Standards for the efficacy evaluation of fungicides and bactericides (EPPO Standards PP1, 2004d,e). The trial consisted of twelve treatments with four replicates for every treatment. The means of the data were subjected to analysis of variance (ANOVA) using MSTAT 5.3.1 software (MSTAT, 2010). Mean values among treatments were compared by the Least Significant Difference (LSD) test and multiple comparisons with Student's T test at 5% level of significance.

3.6 Trial layout

3.6.1 Arrangement of plots

There were a total of 48 plots. The plots of each treatment within blocks were placed to ensure that they were equally affected by environmental variables (e.g. gradient down the field) as well as for example fertilising, watering, and possible foliage applications to control insect pests. Two guard rows of untreated maize were planted on the outside of the trial site (North and South side) in order to eliminate external influence on the experimental plants (See Fig. 3.2).

Two x Outside rows of untreated maize planted							
0.5 m-wide space from treated plots							
BLOCK 1	**3**	**2**	**9**	**4**	**8**	**7**	
	0.5 m-wide row as furrow to divert run-offwater						
	6	**1**	**11**	**5**	**10**	**12**	
	0.5 m-wide spacing as furrow to divert run-offwater						
BLOCK 2	**11**	**7**	**5**	**12**	**1**	**6**	**N**
	0.5 m-wide spacing as furrow to divert run-offwater						
	9	**4**	**8**	**10**	**3**	**2**	
	0.5 m-wide spacing as furrow to divert run-offwater						
BLOCK 3	**5**	**6**	**11**	**7**	**2**	**10**	
	0.5 m-wide spacing as furrow to divert run-offwater						**S**
	3	**9**	**1**	**4**	**12**	**8**	
	0.5 m-wide spacing as furrow to divert run-offwater						
BLOCK 4	**2**	**4**	**12**	**8**	**9**	**5**	
	0.5 m-wide spacing as furrow to divert run-offwater						
	1	**3**	**6**	**10**	**11**	**7**	
0.5 m-wide space from treated plots							
Two x Outside rows of untreated maize planted							

← Direction of run-off water flow (from east to west)

Fig. 3.2. Schematic arrangement of treatment plots in randomised blocks (adapted from EPPO Standards PP1, 2004a).

3.6.2 Plot size

a) Length of individual plot = 6m

b) Width of individual plot = 1.8m

c) Total soil area of plot for soil drench applications = 10m²

d) Width between rows = 50 cm

e) Spacing between plants in row = 10 cm

f) Number of rows 6 m length = 2

g) Number of plants per row = 50

h) Total number of plants per plot = 100

A furrow was dug between plots to divert run-off water as a preventative measure for cross contamination with micro-organisms after irrigation or rain (See Fig. 3.3). Each block had two waterlines that were able to water all treatments in the specific block equally. This ensured that all the treatments received the same quantity of water in that block.

Fig. 3.3. Spacing of maize plants planted in rows, as well as the spaces between rows and individual plots within the trial site.

In the present study, maize was planted late in the season (end of December 2009) and a decision was made to harvest cobs at hard dough stage to minimise bird damage that could have increased from the hard dough to the physiological mature stages. The study site was plagued during previous maize trials conducted by various bird species that tend to remove kernels round the tip of the ear. The damage combined with potential wet conditions would also have increased ear rot that could have resulted in a larger error for determining

reproductive yield. In this study fungal infection was enhanced due to previous *Fusarium* infected wheat crop planted on the same trial site.

3.7 Data collection

3.7.1 Efficacy evaluation of vegetative and reproductive yields

a) Fresh plant biomass

Ten (10) plants were selected from both rows (5 per row) for determining the fresh weight (biomass) of the roots, as well as the aerial parts of the plants (foliage and stem). Five (5) plants were taken from each row by selecting every second plant from the centre of the row towards the end of the row in both directions. No plant samples were taken at the end of the rows. This selection for vegetative yield evaluation left a sufficient number of plants to be selected for cob yield evaluation.

Plants selected were carefully removed with a standard garden spade to ensure maximum roots were lifted with more or less equal soil per plant as determined by the size of the spade. Soil was washed off roots, excess water shaken off roots and whole plants weighed to determine the mean fresh weight per total plant. Roots were carefully removed at the first stem internode and weighed separately from the stem and foliage growth (adapted from EPPO Standards PP1, 2004e,f). The means for the fresh weight of roots, stem and foliage, as well as total fresh weight were calculated and subjected to statistical analysis to determine if there were any significant differences between all the treatments.

b) Fresh cob yield per plant

The reproductive process of the maize plant starts with the development of auxiliary buds that develop into ear shoots where the most upper one has the greatest growth (Duncan, 1975). All the maize plants of this specific cultivar produced only one ear per plant. Fully developed cobs were harvested from a total of 20 plants selected at random from the centre of the two middle rows of each plot. Husks and all silk were removed from cobs (adapted from EPPO Standards PP1, 2004f). The same cobs were also used for evaluating the percentage grain loss per cob prior to weighing which is also a criterion for evaluating the effect of

Fusarium spp. on grain fill. The mean cob weight per maize plant was calculated and the means then subjected to statistical analysis to determine significant differences in yield between the treatments.

c) Rate of ear and silking development

The rate at which the silk developed with the elongation of the ear (adapted from EPPO Standards PP1, 2004f) was evaluated on a scale of 0 to 5 where:

0 = no sign of ear /silk

1 = length of ear < 5cm

2 = length of ear < 10 cm

3 = length of ear < 15 cm

4 = length of ear < 20 cm

5 = length of ear > 15 cm.

A total of 10 plants selected at random from the centre rows were evaluated on 01 March 2010. The mean rating of ear and silk development was subjected to statistical analysis to determine if there were any significant differences between all the treatments in ear elongation and silk development

Rating = 1

Rating = 3

Rating = 5

Fig. 3.4. Some stages of cob elongation for rating scale to evaluate silking

d) Tasselling development rate

The rate at which the tassel developed as it emerged from the leaf was evaluated on a scale of 0 to 5 where (adapted from EPPO Standards PP1, 2004f):

0 = no sign of tassel (still inside leaf whorl)

1 = tassel just emerging from leaf whorl

2 = 25 % of tassel emerged;

3 = 50% of tassel emerged

4 = 75% of tassel emerged

5 = tassel 100% emerged and developed.

A total of 10 plants selected at random from the centre rows were evaluated on 01 March 2010. The mean rating of tassel development was subjected to statistical analysis to determine if there are any significant differences between all the treatments in the development of the tassel.

Rating = 1

Rating = 3

Rating = 5

Fig. 3.5. Some stages of tassel development for rating scale to evaluate progress

e) Plant height

The mean length (height) of plants was also determined on 27 January 2010 (5 days prior to third soil drench application) and again on 15 February 2010 (14 days post soil drench application 3). The difference between these two evaluations was calculated to determine the mean stem growth rate for the period of 18 days. The mean height per maize plant was determined during leaf stage seven on 26 January 2010, two days prior to the evaluation of fresh biomass on 27 January 2010. A second evaluation of height was done on 15 February

2010 during leaf stage eleven, to determine the rate of stem growth in height between the two evaluations. Ten (10) plants were selected at random from the centre of both rows and their heights measured from the soil at the base of the stem to the tip of the youngest leaf fully emerged (adapted from EPPO Standards PP1, 2004f). The means of the plant heights as well as the means of the growth rate were subjected to statistical analysis to determine if there were any significant differences between the treatments in the development of the maize plants.

3.7.2 Efficacy evaluations of micro-organism formulations against diseases

After the seed was inoculated with *Fusarium* species (i.e. two days prior to planting and allowed to dry under room temperature), micro-organism formulations and reference product were applied to *Fusarium* inoculated seed the next day, i.e. one day prior to planting in soil.

The following criteria were used to evaluate the biological efficacy of the different treatments against *Fusarium* spp.:

a) Percentage seedlings emerged

The total number of seedlings emerged was counted for each plot and the percentage germination calculated out of a total of 100 seeds planted per plot (adapted from EPPO Standards PP1, 2004f). The means of the percentage seedlings emerged were subjected to statistical analysis to determine if there were any significant differences between the treatments.

Rating = 0 0-5% loss | Rating = 3 25-30% loss | Rating = 5 45-50% loss | Rating = 7 65-70% loss | Rating = 10 95-100% loss

Fig. 3.6. Examples of rating for percentage grain (kernel) loss per ear/cob

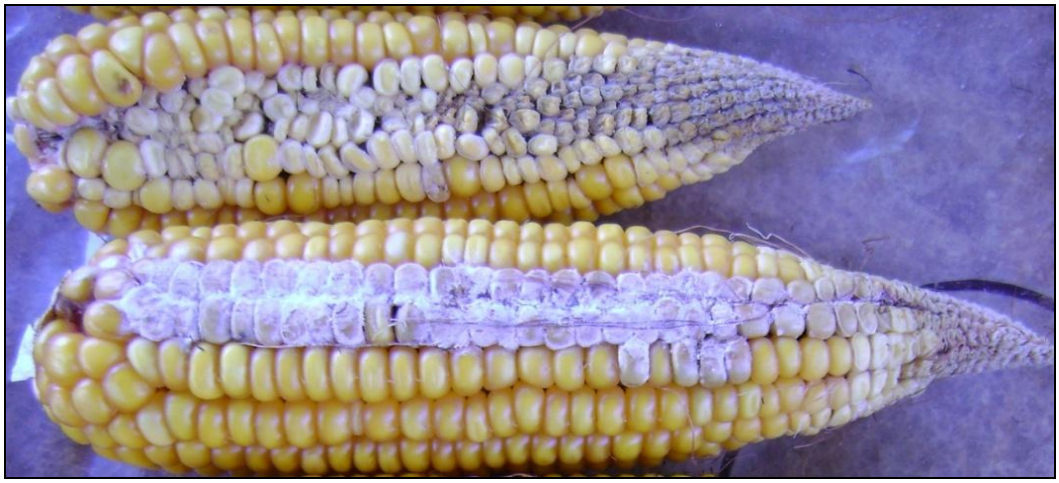

Fig. 3.7. *Fusarium* ear rot

b) Percentage grain loss per cob

Refer to Fig. 3.6. To assess the mean percentage grain loss per maize cob, the following rating scale was used as adapted from EPPO Standards PP1 (2004e):

0 = 0% kernel loss

1 = 10% grain loss

2 = 20% grain loss

3 = 30% kernel loss

4 = 40% kernel loss

5 = 50% kernel loss

6 = 60% kernel loss

7 = 70% kernel loss

8 = 80% kernel loss;

9 = 90 % kernel loss

10 = 100% kernel loss.

All cobs harvested were evaluated to determine the mean percentage grain loss. The means of the percentage grain loss per cob were subjected to statistical analysis to determine if there were any significant differences between the treatments

c) Discolouration of vascular stem tissue at the first internode above soil level.

To assess the discolouration of vascular stem tissue at the first internode, a cross-cut was made at the first internode of the stem of the 20 plants that were selected at random for harvesting of ears/cobs from the two centre rows at hard dough stage. The severity of vascular stem discolouration (adapted from EPPO Standards PP1, 2004d) and disintegration of tissue was evaluated on a rating scale of 0 to 10, where:

0 = clean/clear tissue;
5 = 50% of tissue disintegrated and dark coloured
10 = stem completely hollow from tissue that disintegrated as well as dark coloured.

The means of the disease severity rating causing discolouration were subjected to statistical analysis to determine if there were any significant differences between all the treatments.

Rating = 0 Rating = 2 Rating = 8 Rating = 10

Fig. 3.8. Some examples of the rating scale of vascular stem tissue discolouration and disintegration

d) Severity of vascular cob/ear stem tissue discolouration

Discolouration of vascular stem tissue of the ear stem at point where attached to ear. To assess the discolouration of vascular ear stem tissue, a cross-cut was made at the point where

the ear is attached to the stem of the twenty plants that were selected at random for harvesting of ears/cobs from the two centre rows at hard dough stage.

The severity of vascular stem discolouration (adapted from EPPO Standards PP1, 2004d) and disintegration of tissue was evaluated on a scale of 1 to 7, where:

1 = clean/clear tissue;

4 = moderately discoloured

7 = tissue severely discoloured starting to rot.

The means of the disease severity rating causing discolouration were subjected to statistical analysis to determine if there were any significant differences between the treatments.

Rating = 1 Rating = 4 Rating = 7

Fig. 3.9. Some examples of the severity rating of vascular cob stem tissue discolouration

3.7.3 Phytotoxicity assessment

Phytotoxicity effects were observed at emergence, during growth and at harvest. The criteria for the assessment of phytotoxicity as per EPPO Standards PP1 (2004f) of crop protection formulations when applied to maize seed and plants include:

- delay in emergence and plant growth
- delay in tasselling, silking and grain ripening
- reduction in number of plants tasselling, total fresh weight of cobs without husks, as well as total grain yield, fresh and dry weight of forage
- signs of plant deformation and discolouration
- signs of necrotic tissue

The above effects were assessed by comparison of the treated plots with the untreated plots. The methods used to assess the individual symptoms of phytotoxicity were the same methods used as for the evaluation of vegetative and reproductive yields (EPPO Standards PP1, 2004f).

3.7.4 Meteorological and edaphic data

Meteorological and edaphic data as per EPPO Standards PP1 (2004b). Weather conditions at the time of applications were measured: temperature, relative humidity, wind, soil moisture, cloud cover and rainfall recorded over the trial period (see Appendix 1).

3.7.5 Effects on non-target organisms

Any adverse effects of the treatments on natural occurring organisms as for example earthworms, spiders, pollinators and other natural insect predators and parasites were observed and recorded (EPPO Standards PP1, 2004b).

CHAPTER 4

RESULTS

4.1 Introduction

Inoculation of maize seed with *Fusarium verticilliodes* and *F. proliferatum* prior to treatment with biological control agent formulations and the reference product used in the field trial was to ensure that maize seed was infected. The *Fusarium* inoculum would be further enhanced as a result of the previous crop planted in the same field to be evaluated for biological efficacy of various biological control agent formulations against *Fusarium* spp. in wheat. The maize seed as well as soil infected with *Fusarium* inoculum may have increased disease pressure and allowed better chances of vertical as well as horizontal transmission of the pathogens from infected seed and debris in the soil to maize plants. This could contribute to even more significant results where the biological control agent treatments showed significant differences when compared to the untreated control as well as the reference product.

4.2 Experimental information

4.2.1 Planting Date

Maize seed was planted on 29 December 2009.

4.2.2 Soil drench application dates

Application 1 (Day 1) direct after planting of seed: 29 December 2009. Drenched total planted soil area of plots with a watering can at 10L mixture/10m² and watered in directly after application.

Application 2 (Day 16): 13 January 2010 (5-6 leaf stage). Drenched over maize plants to cover total planted soil area of plots with a watering can at 10L mixture/10m² and watered well to wash micro-organisms into soil directly after application.

Application 3 (Day 38): 04 February 2010 (8-9 leaf stage). Drenched over maize plants to cover total planted soil area of plots with a watering can at 10L mixture/10m² and watered well to wash micro-organisms into the root zone area direct after application.

4.2.3 Assessment dates

a) Percentage seedlings emerged : 04 January 2010 (2 true leaf stage)
b) Root length, fresh weight of root, stem and foliage: 27 January 2010 (7-8 leaf stage)
c) Height of plants : 27 January 2010 (7-8 leaf stage); 15 February 2010 (10-11 leaf stage)
d) Silking and tasselling evaluation : 01 March 2010 (12-13 leaf stage)
e) Cob yield, grain fill, as well as vascular stem tissue : Hard dough stage on 03 May 2010

4.2.4 Soil fertilisation

A complete soil analysis was done prior to planting of the seed in the field (see Appendix 3). Based on the soil test results all plots received 100g Rapid Razor pelleted chicken manure plus 20 mℓ Phlolime (980g/ℓ calcium carbonate) per 10m² plot one day prior to planting of the seed (i.e. 29 December 2009) in soil, and a second application of 100g Rapid Razor a day before the third micro-organism soil drench application on the 03 February 2010. No fungicides or insecticides of any kind were applied to the soil or foliage for the duration of the trial; only treatments to the soil environment were applied as per Table 3.5.

4.2.5 Meteorological and edaphic data

Meteorological data

Cooler conditions prevailed as from 02 January 2010 to 06 January 2010. Warm conditions started as from 09 January 2010 and continued for the duration of January. The relative humidity was high for most of the month of January 2010, especially during the last week. Very hot conditions were found during the first week of February 2010 with the relative humidity between 53-63%. Nights and mornings became cooler (14°C) as from 10 February 2010 with days very hot (up to 34°C), until 16 February after which temperatures returned to between 18-20°C minimum to 26-30°C maximum for the duration of the month. Dry, hot days as from the second week in March (16-34°C) prevailed for the duration of the month with the relative humidity between 47 and 51%. Cloudy with lower temperatures started from

29 March 2010 and continued into April. First heavy dew with cooler mornings and warm days started around 21 April 2010 with relative humidity between 51 and 58% and lasted until day of harvest on 03 May.

Refer to Appendix 1 for information on weather conditions at time of applications.

Edaphic data

Refer to Appendix 3 for soil analysis data conducted by ECO Analytica.

4.3 Seedling emergence

Maize seedlings started to emerge on 02 January 2010. Germination success rate of the maize seed planted varied between 94% and 97.50% (Table 4.1) that was very good for all the treatments. The untreated control gave the lowest germination percentage and treatments 6 (Armenius) and 9 (T-Gro combined with Bismarck) the highest. There were no significant differences between all the biological control agent treatments and the reference product. All the treatments differed significantly from the untreated control, except for treatment 7 (T-Gro combined with Shelter).

Table 4.1. The effect of the micro-organism formulations and the reference product on the percentage seedling emergence of maize under field conditions.

Treatments	**Dosage rate in g or mℓ per kg seed and/or 10m² soil area**	**Mean percentage seedling emergence evaluated on 09 January 2010** LSD = 1.916 ; P < 0.5
1.Untreated control	-	94.0 a
2.T-Gro	Seed: 90g/kg Soil: 8.7g/10m²	96.8 b
3.Bismarck	Seed: 90g/kg Soil: 8.7g/10m²	96.3 b
4.Shelter	Seed: 90g/kg Soil: 8.7g/10m²	97.0 b
5.Maximus	Seed: 90g/kg Soil: 8.7g/10m²	97.3 b
6.Armenius	Seed: 90g/kg Soil: 8.7g/10m²	97.5 b
7.T-Gro + Shelter	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	95.8 ab
8.T-Gro + Maximus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	97.3 b
9.T-Gro + Bismarck	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	97.5 b
10. T-Gro + Armenius	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	96.5 b
11.T-Gro + Spartacus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	96.5 b
12. Reference product	Seed: 1.8 g per kg maize seed	96.5 b

Means with the same letter do not differ significantly at the 5% level according to the least significant difference (LSD)

4.4 Root, stem, foliage, cob and total plant biomass

Increases in vegetative yield (Table 4.2) obtained by the biological control agents under investigation were determined by evaluating differences in root, stem and foliage, as well as total plant biomass between the biological control agent treatments, the untreated control and reference product. Measuring the length of the roots at the time of the first biomass evaluation served as a further measurement for vegetative yield, as well as biological efficacy against the *Fusarium* spp. Evaluation of plant height at two different growth stages gave a very good indication of stem growth in length versus biomass of the roots, stem and foliage and the total maize plant. Differences in growth of all the treatments were a very good measurement of the potential growth stimulating effect of the biological control agent formulations on the maize plants. Vegetative yield factors contributed either to an increase in

reproductive yield or a reduction in grain loss, which is the most important when it comes to production.

The main objective of evaluating the use of biological control agent formulations in maize production was to see an increase in grain yield which was achieved with most of the biological agent control treatments, combined with the reduction in mycotoxins produced by *Fusarium* spp. The level of mycotoxins in the plants was not measured. Increased and vigorous vegetative growth of maize plants is the main objective when produced for fodder (e.g. silage), and many of the treatments succeeded in that respect. Biological efficacy of all the biological control agent formulations was further measured by evaluating the percentage grain loss per cob as a possible result of adverse affects of the *Fusarium* spp., either via vertical or horizontal transmission of the pathogens, and all showed a reduction in grain loss per cob/ear compared to the untreated control. The rate at which elongation of the ears took place was used as a parameter to evaluate possible delays or enhancements in the physiological maturing process of the plants. In addition to the rating of silking was the rating of tassel development to evaluate possible delayed or enhanced physiological maturity.

Root length, as well as the fresh root, stem and foliage, as well as total plant biomass was evaluated at leaf stage eight, almost 1 month after the planting of seed (Table 4.2). Plants of all the treatments showed no signs of chlorosis, necrosis, or any form of distorted growth. There were no significant differences in the length of roots amongst all treatments, including the untreated control. The shortest roots were recorded for treatments 8 (T-Gro combined with Maximus) and 12 (reference product Thiram), and the longest roots for treatments 3 (Bismarck) and 9 (T-Gro combined with Bismarck) (Table 4.2). This shows that the length of the roots of the biological control agents under investigation is not so much an indicator of the growth-enhancing characteristics that was expected from the biological control agent formulations as contrary to the findings in the literature review.

Table 4.2. The effect of the micro-organism formulations and the reference product on the vegetative as well as reproductive yield of maize under field conditions**.**

Treatment	Dosage rate in g per kg seed for 10m² soil area	Mean length and fresh weight of roots, fresh weight of stem and leaves, and total weight per maize plant in grams (g) evaluated 30 days after date of planting seed as well as the mean cob weight per plant at hard dough stage in grams					
		Mean root length per maize plant on 27-01-10 LSD = 3.273 P < 0.5	Mean fresh weights per maize plant on 27-01-10			Mean cob yield per plant on 03-05-2010	
			Roots LSD = 3.0 P < 0.2	Stem +foliage LSD = 19.70 P < 0.05	Total plant LSD = 21.76 P < 0.05	Weight LSD = 30.25 P < 0.2	% gain or loss
1.Untreated control	-	20.7 ab	7.3 a	43.3 a	50.5 a	209.6 a	-
2.T-Gro	Seed: 90g/kg Soil: 8.7g/10m²	20.8 ab	11.3 cd	85.0 ef	98.1 de	244.5 c	16.7%
3.Bismarck	Seed: 90g/kg Soil: 8.7g/10m²	22.6 ab	14.6 e	102.3 f	116.9 e	225.0 abc	7.3%
4.Shelter	Seed: 90g/kg Soil: 8.7g/10m²	22.3 ab	11.2 cd	84.1 ef d	95.3 dec	243.0 bc	15.9%
5.Maximus	Seed: 90g/kg Soil: 8.7g/10m²	21.0 ab	10.4 bcd	67.5 ebcd	77.8 dbc	225.5 abc	7.6%
6.Armenius	Seed: 90g/kg Soil: 8.7g/10m²	20.5 ab	13.1 e d	76.6 e cd	89.8 d c	215.6 abc	2.9%
7.T-Gro + Shelter	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	20.3 ab	11.9 e cd	77.4 e d	89.1 d c	213.5 ab	1.9%
8.T-Gro + Maximus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	19.8 a	10.3 abcd	67.5 ebcd	77.8 dbc	217.1 abc	3.6%
9.T-Gro + Bismarck	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	23.3 b	9.0 abc	57.1 ab	66.1 ab	236.8 abc	13.0%
10. T-Gro + Armenius	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	22.2 ab	9.5 abc	65.3 bcd	74.6 bc	214.7 abc	2.4%
11.T-Gro + Spartacus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	22.3 ab	10.1 abc	68.1 ebcd	78.3 dbc	242.6 bc	16%
12. Reference Thiram	Seed: 1.8g per kg maize seed	19.8 a	7.9 ab	52.9 ab	60.8 ab	222.5 abc	2%

Means with the same letter do not differ significantly at the 5% level according to the least significant difference (LSD)

The highest root biomass per maize plant was produced by treatments 3 (Bismarck), followed by 6 (Armenius), 7 (T-Gro combined with Shelter), 2 (T-Gro), 4 (Shelter), and 5 (Maximus) with no significant differences among them (Table 4.2). They all differed significantly from the untreated control and reference product that produced the lowest root biomass. All the biological control agent treatments produced roots higher in biomass than that of the untreated control and have therefore no adverse effects on root development when used as seed treatments as well as soil drench applications.

Untreated

Shelter

Bismarck

Reference product Thiram

Fig. 4.1. Roots of maize plant treatments Bismarck, Shelter, reference product and untreated control as evaluated on 27 January 2010

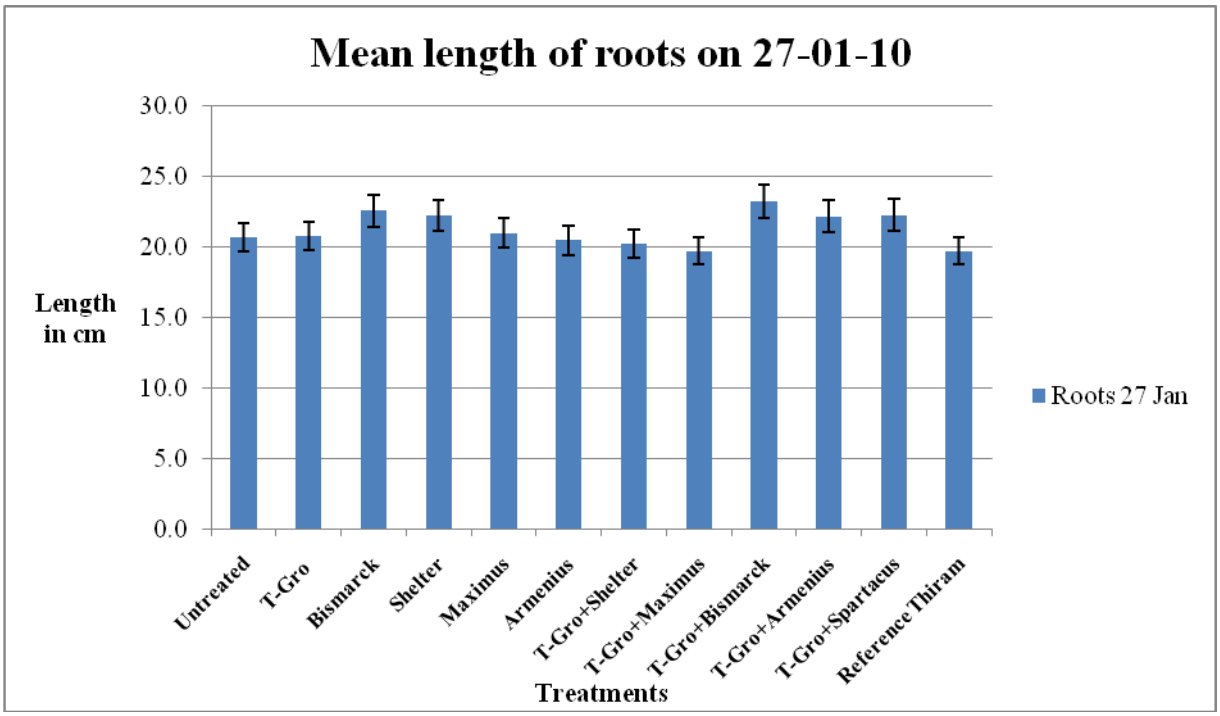

Fig. 4.2. Length of roots in cm as on 27 January 2010

Results of the treatments for the total fresh plant biomass produced identical results to that of the fresh stem and foliage biomass (Table 4.2). In the order of highest to lower fresh stem and foliage biomass as well as total plant biomass produced were treatments 3 (Bismarck), 2 (T-Gro), 4 (Shelter), 7 (T-Gro combined with Shelter), and 6 (Armenius). There were no significant differences in fresh stem and foliage biomass as well as total plant biomass among them, but they all differed significantly from the untreated control and reference product Thiram. All the other biological control agent treatments showed an increase in fresh stem and foliage biomass as well as total plant biomass compared to the untreated control and reference product (Table 4.2). The lowest fresh stem and foliage biomass as well as total plant biomass produced by biological control agent formulations were with treatments 9 (T-Gro combined with Bismarck), and 10 (T-Gro combined with Armenius).

Results from the same table above showed no significant differences in fresh stem and foliage biomass as well as total plant biomass among biological control agent treatments 5 (Maximus), 8 (T-Gro combined with Maximus), and 11 (T-Gro combined with Spartacus), which were in between the highest and lowest weights. They differed significantly from the untreated control and reference product. Fresh stem and foliage biomass as well as total plant biomass of treatments 5 (Maximus), 8 (T-Gro combined with Maximus) and 11 (T-Gro combined with Spartacus) were lower than that of treatments 3 (Bismarck), 2 (T-Gro), 4 (Shelter), 7 (T-Gro combined with Shelter), 6 (Armenius) and differed only significantly from treatment 3 (Bismarck). Bismarck (*Microbacterium maritypicum* isolate DB107), T-Gro (*Trichoderma harzianum* isolate DB103) and Shelter (*Bacillus subtilis* isolate DB108) were the three top perfomers in stimulating stem and foliage growth of maize plants when used as seed and soil treatments (Table 4.2).

4.5 Plant Height

Plant height is an indicator of growth-enhancing properties as well as the possible biological efficacy of the biological control agents against *Fusarium* infection that may cause stunted growth in maize plants (Table 4.3). The height of the maize plants was measured one week before the second and last fertilising with Rapid Razor pelleted chicken manure and again 12 days after fertilising. Differences in growth for this period between the two evaluations were

a good indicator of healthy plant growth development prior to the reproductive stages of silking and tasselling that started almost two weeks after the last plant height evaluation.

Plant height in order of highest to lowest was treatments 2 (T-Gro), 3 (Bismarck), 7 (T-Gro combined with Shelter), 11 (T-Gro combined with Spartacus), 4 (Shelter) and 5 (Maximus) at the time of evaluating the fresh root, stem and foliage biomass with no significant difference in height among them (Table 4.3)

Table 4.3. The effect of the micro-organism formulations and the reference product at recommended dosage rates on the height of maize plants under field conditions.

Treatments	**Dosage rate in g or mℓ per kg seed and/or m² soil area**	**Mean height in cm per maize plant evaluated 30 days and 48 days after date of planting**		
		Mean height 30 days post date of planting seed evaluated on 27-01-2010 LSD = 6.916 P < 0.05	**Mean height 48 days post date of planting seed evaluated on 15-02-2010** LSD = 24.19 P < 0.8	**Mean growth between 27 January and 15 February 2010** LSD = 20.30 P < 0.3
1.Untreated control	-	59.3 ebcd	137.8 abcd	78.5 abc
2.T-Gro	Seed: 90g/kg Soil: 8.7g/10m²	69.5 f	164.8 f e	95.3 e cd
3.Bismarck	Seed: 90g/kg Soil: 8.7g/10m²	65.3 e f	175.0 f	109.8 e
4.Shelter	Seed: 90g/kg Soil: 8.7g/10m²	63.3 e cdf	165.5 f e	102.3 e d
5.Maximus	Seed: 90g/kg Soil: 8.7g/10m²	62.0 ebcd	150.8 cde	88.8 bcd
6.Armenius	Seed: 90g/kg Soil: 8.7g/10m²	56.5 abc	146.0 bcde	89.5 ebcd
7.T-Gro + Shelter	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	65.0 e f	157.5 f de	92.5 e cd
8.T-Gro + Maximus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	61.8 ebcd	157.3 f cde	95.5 e cd
9.T-Gro + Bismarck	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	49.8 a	119.0 a	69.3 ab
10. T-Gro + Armenius	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	57.6 bcd	133.3 abc	75.8 abc
11.T-Gro + Spartacus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	63.8 e df	142.3 abcde	78.5 abc
12. Reference product	Seed: 1.8 g per kg maize seed	56.3 ab	123.3 ab	66.8 a

Means with the same letter do not differ significantly at the 5% level according to the least significant difference (LSD)

09 January 2010

11 February 2010

10 March 2010

Fig. 4.3. Vegetative growth of maize plants from seedling stage 09 January 2010 (top left) to cobbing 10 March 2010 (right).

Untreated T-Gro Shelter Maximus Armenius Reference

Fig. 4.4. Maize plant growth for the single formulation treatments at the end of the rows of selected plots as on 11 February 2010

Difference in height of only treatment 2 (T-Gro) was significantly higher than that of the untreated control (Table 4.3) and did not differ significantly from the biological control agent treatments 3 (Bismarck), 4 (Shelter), 7 (T-Gro combined with Shelter) and 11 (T-Gro combined with Spartacus).

The reference product, biological control agent treatments 9 (T-Gro combined with Bismarck), 6 (Armenius) and 10 (T-Gro combined with Armenius) produced plants lower in height than that of the untreated control. Fresh stem and foliage biomass of treatments 9 (T-Gro combined with Bismarck), and 10 (T-Gro combined with Armenius) were slightly higher than the untreated control, and treatment 6 (Armenius) produced significantly higher biomass than the untreated control and the two biological control agent treatments, which indicates that treatment 6 (Armenius) might have produced a much larger leaf canopy and/or thicker stems of maize plants. Only maize plants of treatment 9 (T-Gro combined with Bismarck) were significantly lower in height than the untreated control, followed by the reference product (Thiram). Stem and foliage biomass of treatment 9 (T-Gro combined with Bismarck) was slightly higher than that of the untreated control and showed the poorest performance when evaluating height and biomass compared to the untreated control of all the biological control agent treatments.

The top performing treatments for the second evaluation of height, from highest to lowest were 3 (Bismarck), 4 (Shelter), 2 (T-Gro), 7 (T-Gro combined with Shelter), 8 (T-Gro combined with Maximus) and 5 (Maximus). Poorest performers in height were treatments 9 (T-Gro combined with Bismarck), 12 (reference product Thiram), 10 (T-Gro combined with Armenius), followed by the untreated control (Table 4.3). Treatment 9 (T-Gro combined with Bismarck) differed significantly from the untreated control but not from the other two lowest performers in height of the maize plants. Other treatments including 11 (T-Gro combined with Spartacus), 6 (Armenius) and 5 (Maximus) produced plants greater in height than that of the untreated control but did not differ significantly.

Best performing treatments compared to the untreated control in calculating the difference in growth of the maize plants between the two different plant height evaluations, were treatments 2 (T-Gro), 3 (Bismarck), 7 (T-Gro combined with Shelter), 11 (T-Gro combined with Spartacus), 4 (Shelter), 5 (Maximus) and 8 (T-Gro combined with Maximus). Only treatment 2 (T-Gro) produced more growth in length during that period that differed significantly from the untreated control (Table 4.3). All the other biological control agent

treatments that produced more growth in length than the untreated control during that period did not differ significantly from the untreated control.

Poorest growth for the period between the two plant height evaluations was with treatments nine (T-Gro combined with Bismarck), twelve (reference product Thiram), six (Armenius) and ten (T-Gro combined with Armenius). Only treatment nine differed significantly from the untreated control.

4.6 Silking and Tasselling

First signs of silking and tasselling processes started to show on maize from 28 February 2010. When it came to evaluating the effect of the various biological control agent treatments on the silking and tasselling physiological growth processes, treatments 2 (T-Gro), 3 (Bismarck), 4 (Shelter), 5 (Maximus) and 7 (T-Gro combined with Shelter) increased the rate of silk and tassel development compared to the untreated control where development was much slower (Fig. 4.5). Treatments 2 (T-Gro), 3 (Bismarck) and 4 (Shelter) differed significantly in the rate of ear elongation and silk development compared to the untreated control, with no significant differences among themselves. Results of the rate at which tassel development took place also differed significantly from the untreated control for treatments 2 (T-Gro), 3 (Bismarck), 4 (Shelter), 5 (Maximus) and 7 (T-Gro combined with Shelter). Tassel development in the order of slowest to fastest rate was with treatments 12 (reference product Thiram), 1 (untreated control), 9 (T-Gro combined with Bismarck), 10 (T-Gro combined with Armenius), 6 (Armenius), 11 (T-Gro combined with Spartacus) and 8 (T-Gro combined with Maximus). There were no significant differences between all these biological control agent treatments and the reference product compared to the untreated control (Fig. 4.5). From these results is it possible to assume that treatments 9 (T-Gro combined with Bismarck), 10 (T-Gro combined with Armenius) and 12 (reference product Thiram) slowed down the rate at which the tassel developed when compared to all the other biological control agent treatments. All the other biological control agent treatments increased the rate of tassel development compared to the untreated control plants.

It appears further that treatments 12 (reference product Thiram), 1 (untreated control), 9 (T-Gro combined with Bismarck), 10 (T-Gro combined with Armenius), 6

(Armenius) and 8 (T-Gro combined with Maximus) resulted in the slowest rate of ear elongation and silk development, with no significant differences among all the treatments.

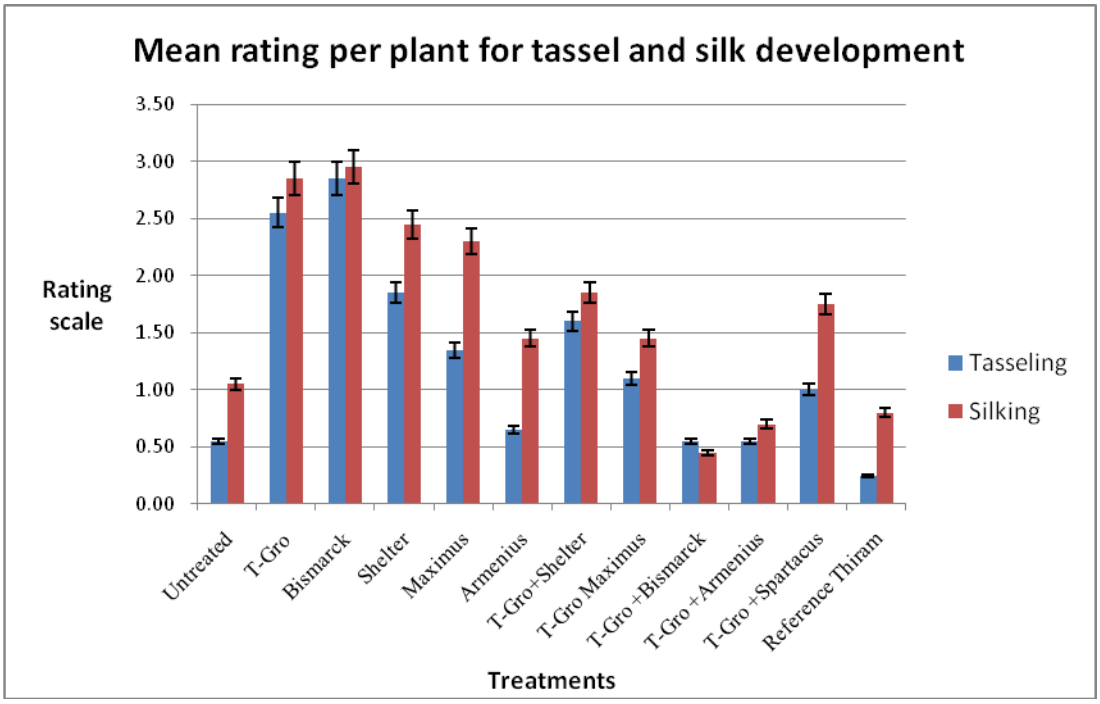

Fig. 4.5. Mean rating of tassel and silk development per plant as on 01 March 2010.

The higher the rating the more were the cobs/ears developed in length and tassels that emerged from the leaf whorl.

4.7 Kernel loss per cob

Calculated percentage kernel loss per cob for each treatment can be an indication of the adverse effect of the *Fusarium* spp on the grain-fill process. Percentage grain loss per cob can also be related to the final cob yield per maize plant. Grain loss in the order of lowest to highest for the best four treatments was produced by treatments 4 (Shelter), 2 (T-Gro), 3 (Bismarck) and 5 (Maximus). All these biological control agent treatments differed significantly from the untreated control, with no significant difference among themselves (Fig. 4.6 and Table 4.4).

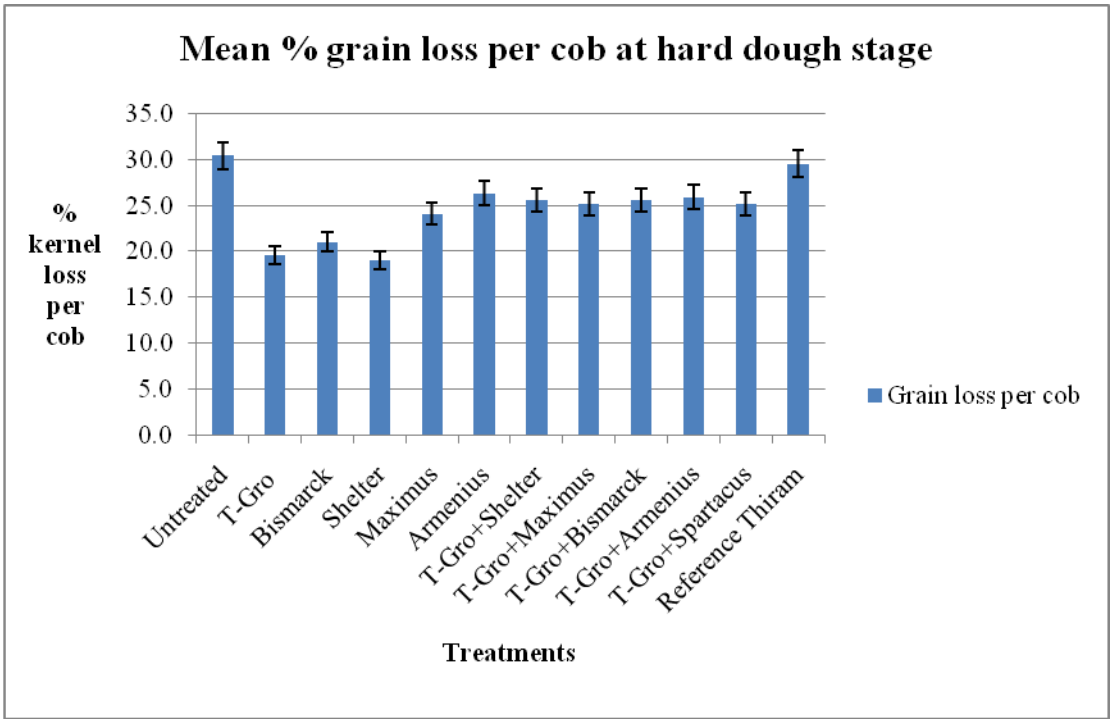

Fig. 4.6. Mean percentage grain loss per cob

The highest percentage loss of kernels came from the untreated control, followed by treatments 12 (reference product Thiram), 6 (Armenius), 10 (T-Gro combined with Armenius), 9 (T-Gro combined with Bismarck), 7 (T-Gro combined with Shelter), 11 (T-Gro combined with Spartacus) and 8 (T-Gro combined with Maximus) with no significant differences between these treatments and the untreated control (Fig. 4.6 and Table 4.4).

Biological efficacy against *Fusarium* spp. causing stem and ear rot involves evaluation of discolouration of vascular tissue of the main and ear stem resulting in rot.

Table 4.4. The effect of the micro-organism formulation treatments and reference product at recommended dosage rates on the reduction of disease severity of vascular stem tissue as well as kernel (grain) loss of maize plants under field conditions.

Treatments	**Dosage rate in g or mℓ per kg seed and/or m² soil area**	**Mean percentage kernel loss per ear as well as mean rating of vascular main stem and ear stem tissue discolouration (indicating disease severity) per maize plant at hard dough stage on 03 May 2010**		
		Percentage kernel loss per ear/cob LSD = 6.870 P < 0.2	**Main stem tissue discolouration at 2nd internode** LSD = 0.3104 P < 0.05	**Severity of vascular cob stem tissue discolouration** LSD = 0.2793 P < 0.05
1.Untreated control	-	30.4 e	2.4 g	3.7 c
2.T-Gro	Seed: 90g/kg Soil: 8.7g/10m²	19.6 ab	1.8 f	3.2 b
3.Bismarck	Seed: 90g/kg Soil: 8.7g/10m²	21.0 abc	1.8 ef	2.6 a
4.Shelter	Seed: 90g/kg Soil: 8.7g/10m²	19.0 a	1.6 cdef	3.2 b
5.Maximus	Seed: 90g/kg Soil: 8.7g/10m²	24.1 abcd	1.7 def	3.4 b
6.Armenius	Seed: 90g/kg Soil: 8.7g/10m²	26.3 cde	1.5 bcdef	2.7 a
7.T-Gro + Shelter	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	25.6 bcde	1.2 ab	3.2 b
8.T-Gro + Maximus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	25.1 abcde	1.3 ab	2.7 a
9.T-Gro + Bismarck	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	25.6 bcde	1.2 a	2.6 a
10. T-Gro + Armenius	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	25.9 cde	1.3 abc	2.7 a
11.T-Gro + Spartacus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	25.1 abcde	1.4 abcd	2.8 a
12. Reference product	Seed: 1.8 g per kg maize seed	29.5 de	1.5 abcde	3.3 b

Means with the same letter do not differ significantly at the 5% level according to the Least Significant Difference (LSD)

4.8 Vascular main and ear stem tissue discolouration

Vascular main stem tissue discolouration was the most severe in the untreated control plants which differed significantly from all the other biological control agents as well as the

reference product (Table 4.4 and Table 4.5). The least discolouration was with maize treated with T-Gro combined with Bismarck, T-Gro combined with Shelter, T-Gro combined with Maximus, T-Gro combined with Armenius, and T-Gro combined with Spartacus. The single application treatments T-Gro, Bismarck, Maximus, Shelter and Armenius showed more main vascular tissue discolouration than the combined applications of the four bacteria with T-Gro, with no significant differences among them. The least discolouration of the vascular tissue of the ear stems was produced by treatments Bismarck, T-Gro combined with Bismarck, Armenius, T-Gro combined with Maximus, T-Gro combined with Armenius, and T-Gro combined with Spartacus, with no significant differences among all the treatments, which differed significantly from the untreated control and the reference product Thiram.

Table 4.5. Percentage reduction or increase in grain loss, discolouration of vascular main and ear stem tissue, as well as cob yield per plant of micro-organism formulation treatments and the reference product compared to the untreated control at hard dough stage on 03 May 2010.

Treatments	**Dosage rate in g or mℓ per kg seed and/or m² soil area**	**Reduced/increased percentage grain loss per cob, vascular stem tissue discolouration(indicating disease severity), and cob yield per plant**			
		% Grain loss per ear reduced compared to untreated control	**% ear stem tissue discolouration reduced compared to untreated control**	**% main stem tissue discolouration reduced compared to untreated control**	**% cob yield per plant increase compared to untreated control**
1.Untreated control	-	-	-	-	-
2.T-Gro	Seed: 90g/kg Soil: 8.7g/10m²	35.5%	12.3%	24.2%	16.7%
3.Bismarck	Seed: 90g/kg Soil: 8.7g/10m²	30.9%	27.9%	25.3%	7.3%
4.Shelter	Seed: 90g/kg Soil: 8.7g/10m²	37.5%	11.5%	31.6%	15.9%
5.Maximus	Seed: 90g/kg Soil: 8.7g/10m²	20.7%	8.2%	30.5%	7.6%
6.Armenius	Seed: 90g/kg Soil: 8.7g/10m²	13.5%	26.6%	36.8%	2.9%
7.T-Gro + Shelter	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	15.8%	12.9%	49.5%	1.9%
8.T-Gro + Maximus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	17.4%	25.2%	45.3%	3.6%
9.T-Gro + Bismarck	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	15.8%	27.9%	50.5%	13.0%
10. T-Gro + Armenius	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	14.8%	25.2%	44.2%	2.4%
11.T-Gro + Spartacus	Seed: 45g + 45g /kg Soil: 8.7g + 8.7g/10m²	17.4%	23.8%	41.1%	16%
12. Reference product	Seed: 1.8 g per kg maize seed	3.0%	10.1%	37.9%	2%

All treatments showed less vascular tissue discolouration of the ear stems compared to the untreated control, with significant differences between all the treatments and the untreated control (Table 4.5). These results might indicate the biological control agent's characteristics to be similar to that of endophytic bacteria and fungi as explained by Illmer & Schinner (1992) and Saravanan, *et al.* (2008).

4.9 Cob yield per plant

As it is with all crop production objectives the final yield per plant obtained from the different treatments compared to the untreated control and reference product is the most important factor. Cob yield per plant was thus measured to determine effect of the treatments on harvestable products. Without sustained or rather increased grain yield is it of no use to apply any of the biological control agent formulations that would merely add to unwanted production costs. The cob yield per maize plant ranked from highest to lowest for the treatments were 2 (T-Gro), 4 (Shelter), 11 (T-Gro combined with Spartacus), 9 (T-Gro combined with Bismarck), 5 (Maximus), 3 (Bismarck), 12 (reference product Thiram), 8 (T-Gro combined with Maximus), 6 (Armenius), 10 (T-Gro combined with Armenius, 7 (T-Gro combined with Shelter) and last the untreated control which did not differ significantly from all the treatments with the exception of the three top performing biological control agent treatments T-Gro, Shelter and T-Gro combined with Spartacus. The increase in cob/ear yield in all the treatments compared to the untreated control varied from the lowest of 2% (reference product Thiram) to the highest 16.7% (T-Gro). Increased cob yields of 12% or more are significant for most grain productions and treatments T-Gro, Shelter, T-Gro combined with Spartacus, and T-Gro combined with Bismarck, achieved just that (Table 4.5).

With the exception of the maize plants treated with Bismarck that produced the highest fresh stem and foliage biomass; the second and third highest stem and foliage fresh biomass produced by T-Gro and Shelter as single applications to maize seed and soil, produced also the highest cob yield per plant. Bismarck showed an increase in cob yield per maize plant of 7.3% which is still very much an acceptable increase in grain yield compared to the untreated control (Table 4.5).

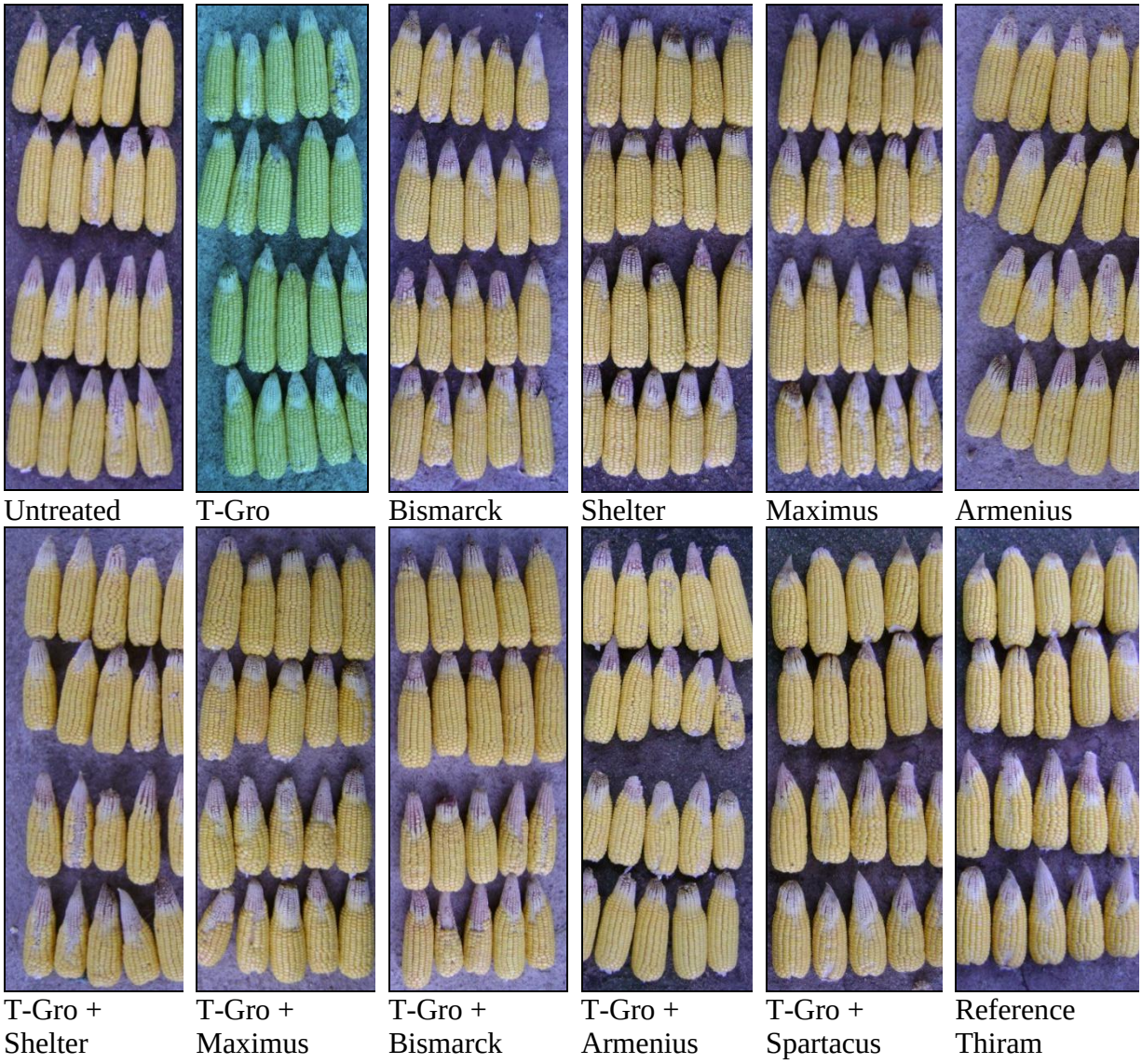

Fig. 4.7. Maize cobs evaluated per treatment to determine the mean percentage grain loss per cob

4.10 Phytotoxicity

The micro-organism formulations showed no signs of phytotoxicity symptoms as per EPPO Standards PP1 (2004f), including chlorosis, necrosis, and any other abnormalities in growth visible for the duration of the study. The stem growth of the treatments T-Gro combined with Bismarck, T-Gro combined with Armenius as well as the reference product Thiram, were slightly slower or almost equal to that of the untreated control for the growth period 27 January to 15 February 2010. The same treatments showed a delay in silking when compared

to the untreated control but still produced higher cob yield per maize plant. There were no significant differences in the stem growth as well as silking rate between these treatments. The reference product Thiram showed a serious delay in the rate of tasselling compared to the untreated control and the use as seed treatment might have caused a phytotoxic effect. All the micro-organism treatments showed better root, stem and foliage growth, as well as cob yield per plant compared to the untreated control. See Tables 4.2 and 4.3 that show the fresh weight as well as height of plants from various treatments.

4.11 Effects on non-target organisms

No adverse effects on beneficial organisms were observed. Earthworm activity remained very high for the duration of the experiment. No official counts were done but plants were actively visited by predatory spotted ladybirds feeding on aphids, as well as healthy spider populations (mainly wolfspiders) on the soil. Many antlion pits were also visible on the soil surface.

CHAPTER 5

DISCUSSION

Maize seed inoculated with *Fusarium verticillioides* and *Fusarium proliferatum* and treated with commercially formulated micro-organism formulation T-Gro (*Trichoderma harzianum* isolate DB103 WP) combined with Spartacus (*Beauveria bassiana* isolate DB 105 WP), T-Gro combined with Armenius (*Bacillus subtilis* isolate DB 109 WP), T-Gro combined with Maximus (*Bacillus subtilis* isolate DB 108 WP), T-Gro combined with Shelter (*Bacillus subtilis* isolate DB 101), T-Gro combined with Bismarck (*Microbacterium maritypicum* isolate DB 107 WP), as well as individual treatments of T-Gro, Armenius, Bismarck, Maximus and Shelter showed poor to very good reduction of *Fusarium* inoculum causing stem and ear rot symptoms as well as average to very good increases in vegetative biomass as well as reproductive yield of the maize plants grown under field conditions. Plants treated with the micro-organism formulations performed in most cases significantly better than the plants treated with the chemical reference product Thiram 750WP and when compared to the untreated control plants. The results with the above formulations confirmed various findings by Scala *et al.* (2007) and Vega *et al.* (2009) of studies conducted with micro-organism biological control formulations used to reduce or control pathogenic fungi like *Fusarium* spp. worldwide for the purpose of registration in other countries under their regulatory bodies. It further confirms results obtained by Wu *et al.* (2005) regarding their potential to be used as biofertilisers with the ability to increase the growth and nutrient assimilation with subsequent increases in yield.

This experimental field study was conducted during the peak summer months in Gauteng, South Africa, where the environmental conditions were favourable not only for the development of stem and ear rot caused by *F. verticiliioides* and *F. proliferatum* but also for vegetative and reproductive growth of the maize cultivar planted. The maximum daily temperatures exceeded 25°C for the duration of the plant growth and development as from germination to silking and grain fill which are very much ideal conditions for *Fusarium* spp infection as described by *Velluti et al.* (2000). Yates *et al.* (2005) conducted very important studies where they indicated that *F. verticillioides* has a stimulating effect on vegetative biomass as well as reproductive yield. They further indicated the importance of climatic conditions to be considered very carefully when designing control programmes. Micro-

organism biological control agents should be selected that can survive the same conditions as that of the pathogenic fungi or they would not succeed in competing against the pathogens. Most of the micro-organism biological control agents used in this field trial showed that they functioned well under such warm conditions. There is a possibility that the poorer performers among the micro-organism formulations tested could have been adversely affected by the high temperatures that prevailed for most of the summer into autumn period of the experiment. It is important to take into account that the soil conditions in terms of sufficient moisture and favourable alkalinity at time of planting and drench applications were favourable for both pathogens and micro-organisms applied. The ideal pH of the soil (Dagutat Science, 2010) for most of these micro-organism formulations should preferably be between 6.0 and 7.5. The pH for this study, according to the soil analysis done prior to planting was 7.25 which would not have affected the micro-organisms adversely.

The importance of organic material in the form of farmyard manures to increase yields in soil low in fertility has been reported by Onyango (2010). In this investigation, a commercially-available pelleted chicken manure was used and applied broadcast at the same recommended dosage rate for all the treatments. This contributed to the organic content of the soil that created a more favourable environment for micro-organisms applied to the soil environment. Even though no additional treatments with reduced fertiliser applications were included in the experiment, the growth response of micro-organism treated plots was significantly higher than that of the untreated and chemically treated plots. The results of increased vegetative biomass and yield of maize produced by most of the micro-organism formulations evaluated in this study indicated the potential of these formulations to be used with fertilisers to increase or sustain yield with a subsequent reduction in the quantity of fertiliser applied as was found with studies conducted by Adesemoye *et al.* (2009) on greenhouse tomatoes.

The pathogenic fungi *F. verticillioides* and *F. proliferatum* are known to be highly competitive against many other pathogens as was found by Marin *et al.* (1998). This also means that it was possible that the presence of both increased disease pressure and antagonism of these pathogens towards the non-pathogenic fungi and bacteria used in this maize trial. This concludes that environmental conditions can play an important role in the performance of biological control agents to reduce inoculums of pathogenic *Fusarium* and other species in maize.

No nematode population counts were done to evaluate the actual effect they might have had on vegetative as well as reproductive maize yield. This was due to the fact that this experiment was conducted on a site with a history of crops planted for the evaluation of crop protection micro-organism formulations against nematode species, and thus results of increased growth are even more significant. The *T. harzianum* as well as the *B. bassiana* isolate used in this study have been evaluated for nematode control on other crops with significant reduction of parasitic populations and an increase in populations of non-parasitic nematodes (Dagutat Science, 2010). Dowd (1998) investigated the effect of nematode populations on the spread of pathogenic fungi and this should to be taken into consideration when evaluating yield loss. The good results obtained with T-Gro combined with Spartacus confirms the possibility that they might have reduced the parasitic nematode populations which resulted in increased yields compared to some of the other treatments.

Insect pests, including stalk borer may contribute to increased *Fusarium* inoculum as reported by various authors such as Flett & Van Rensburg (1992), Munkvold & Desjardin (1997), Cardwell *et al.* (2000) and Ako *et al.* (2003). The use of a Bt maize hybrid in this study did not exclude the plants from damage caused by stalk borer on the leaves. The main advantage of using this hybrid with this built-in gene resistant to stalk borer was that the ears/cobs were completely unaffected and that a more accurate cob yield per plant was produced with no loss due to borer damage on the stems or cobs which could have contributed to increasing the rotting of tissue. No application of any insecticide was necessary to control insect pests on this hybrid maize cultivar used in the trial. Aphid populations started to build up during early tasselling but the natural predators in the trial site environment dealt with their populations very successfully. This suggested that the micro-organism formulations had no adverse effect on the predatory ladybird populations and is very unlikely to have any adverse effect on predatory as well as parasitic insects when used as seed and/or as soil treatments. Healthy earthworm as well as ant lion activity in the soil was another indicator of no adverse effect on their populations with the use of all micro-organisms as seed and soil treatments as well as the chemical reference product used as a seed treatment.

The percentage of seedlings that emerged in all the treatments gave a good indication of the seed treatments effect against damping-off disease, as well as potential phytotoxic effects of the maize seed treatments that usually cause poor germination. Small losses occurred during germination due to damping-off, which confirms the findings by Yates *et al.* (2005) as well as Nayaka *et al.* (2008) that maize seedlings are often not adversely affected by the *Fusarium*

species during the early stage of seedling growth but by other diseases. The results thus indicated that all the treatments had no or very little adverse effects on the germination of the seed and are therefore suitable for use as seed treatments at the dosage rate tested.

The significant increases in root, stem and foliage as well as total plant biomass produced by the *B. subtilis, T. harzianum* and *M. maritypicum* isolates compared to the untreated control and reference product Thiram, showed typical characteristics of growth-enhancing endophyte bacteria and fungi as described by Saravanan, *et al.* (2008) and Zinniel, *et al.* (2002). It is therefore evident that these biological control agents, either applied as single or combined applications to maize seed and the soil, contributed to general growth of roots, stems, and foliage in a positive manner. All the biological control agent treatments produced roots higher in biomass than that of the untreated control and have therefore no adverse effects on root development when used as seed treatments as well as soil drench applications. Root length of biological control agent treatments was not significantly different from the untreated control plants and this points at possible increased secondary and fine surface root development as a result of increased root biomass as proposed by Khan *et al.* (2008). This illustrates that the length of the roots is not so much an indicator of the growth-enhancing characteristics that were expected from the biological control agent formulations, but more the actual increases in root weight (Kucey *et al.*, 1889; Khan *et al.*, 2008). These biological control agent formulations used in the study may play a very important role in future seed protection against many problem soil- and seed-borne pathogens causing huge losses in crop production, and simultaneously increasing the fresh root biomass to ensure healthy seedling development.

All the *B. subtilis* isolates, as well as *T. harzianum* and *M. maritypicum* isolates showed levels of biological efficacy against the *Fusarium* spp. that usually cause stem and ear rot in maize by reducing the discolouration of vascular tissue of the main and ear stem which can result in rot. Similar findings were made by Nehl *et al.* (2006), Kapoori (2007), and Saravanan *et al. (*2008) when using *B. subtilis* strains on a wide variety of crops. They observed the biological effect of *B. subtilis* strains in reducing the inoculum of various pathogenic fungi, including *Fusarium* spp. This shows that they all have the potential to be used in programmes for the reduction of diseases caused by *Fusarium* spp. It is thus evident that these reductions in disease inoculum may have contributed to increased performance in vegetative as well as reproductive yields of the maize crop.

The reduction in disease development and the increase in vegetative biomass as well as reproductive yield of the maize crop in this study confirmed the findings by Harman *et al.* (2004) and Bacon *et al.* (2001) that some strains of *T. harzianum* and *B. subtilis* have the ability to reduce inoculum of various pathogenic fungi as well as enhancing root development and nutrient uptake. The dual function of these micro-organisms has therefore the potential to reduce or control pathogenic fungi (including *Fusarium* spp.) and promote an increase in vegetative and reproductive yields.

At present, no research published on *M. maritypicum* could be found in available literature and the results of this study suggest a great potential of this marine bacterium to be used in the reduction and control of *Fusarium* spp. while simultaneously increasing vegetative as well as reproductive yields. Studies conducted by Pereira *et al.* (2009) with *M. oleovorans* when applied to maize seed inoculated with *F. fluorescens* showed a significant reduction in pathogen count. The reduction in discolouration of vascular stem tissue in maize treated with *M. maritypicum* confirms the potential of species from the genera *Microbacterium* to be used in the control of *Fusarium* spp.

It can be deduced that the biological control agents that were tested in this study, showed the same characteristics as endophytic bacteria and fungi that enhance growth but also reduce plant diseases caused by *Fusarium* spp. and other plant disease pathogens. The results were also confirmed in previous studies carried out by Illmer and Schinner (1992) as well as Saravanan, *et al.* (2008). From these results of the micro-organism formulations, it appears that the best performance in reducing grain loss was with single biological control agent applications including T-Gro, Bismarck, Shelter and Maximus, followed by combination applications of T-Gro combined with Maximus and T-Gro combined with Spartacus. Many studies have been conducted with *B. subtilis* spp. as well as *Trichoderma* spp. where their effects against pathogenic fungi including *Fusarium* spp. have been reported by many including Harman, *et al.* (2004), Nehl *et al.* (2006), and Scala, *et al.* (2007). Interestingly, the findings of all the researchers mentioned above are in agreement with the present ones from this study. There is a general consensus that *Bacillus* and *Trichoderma* tend to result in plants higher in vegetative biomass as well as reduced pathogen inoculums. The only point of departure is that these studies were carried out mostly under greenhouse conditions (for example greenhouse trials in Argentina by Cavaglieri *et al.* (2005) with multiple strains of *B. subtilis* against *F. verticillioides* in maize at root level) and that this study was conducted under field conditions.

Results showed that the silking and tasselling development rate increased with seed and soil treatments of biological control agents T-Gro, Shelter, Bismarck and Maximus. Ears were also the most developed when treated with T-Gro, Shelter or Bismarck, compared to the untreated and chemically treated plants. None of the studies conducted with micro-organism biological control formulations reviewed for the purpose of this study used the rate at which the development of silking or tasselling took place as criteria to evaluate the performance of maize plants when treated with micro-organism biological agent inoculums or formulated products. From this study it is therefore evident that these biological control agents have potential to speed-up physiological maturity towards grain fill and needs further investigation. T-Gro combined with Bismarck, as well as T-Gro combined with Armenius had the opposite effect on the rate of silk and tassel development of the maize plants treated and may be an indication of possible antagonism between the biological agents combined. Ear elongation as well as tassel development was slower than the single applications of T-Gro, Shelter, Bismarck or Maximus and more studies should be conducted to evaluate the compatibility of these biological control agent formulations when used as combinations and their effects on physiological growth.

Most research has been conducted with the application of a single biological control agent as was the case in this study with T-Gro, Armenius, Bismarck, Maximus and Shelter. Fungal combined with bacterial biological control agents evaluated in field trials as indicated by Janisiewicz (1988), as well as by Duffy & Weller (1994), demonstrate the importance of studies evaluating the compatibility of micro-organism biological control agents when used as seed and or soil treatments. In this present study the combined applications still performed better than the untreated control but if not better than the single applications, the use of both treatments when applied simulateously, should be questioned. The use of combined formulations from different genera is justifiable if the mixture needs to reduce or control a wider spectrum of pathogens that might include fungal as well as bacterial pathogens.

Treatments T-Gro, Shelter, T-Gro combined with Spartacus, and T-Gro combined with Bismarck produced significant increases in cob yield per plant when used as maize seed treatments plus post-planting soil drench applications, and further research to optimise yields with these biological control agents should to be done. These results confirmed similar findings of increased yields with the treatment of maize with micro-organism formulations as suggested by Hinton & Bacon (1995) as well as by Bacon *et al.* (2001). With the exception of the maize plants treated with Bismarck that produced the highest fresh stem and foliage

biomass; the second and third highest stem and foliage fresh biomass produced by T-Gro and Shelter as single applications to maize seed and soil, produced also the highest cob yield per plant. The Bismarck treatment exhibited an increase in cob yield per maize plant compared to the untreated control. No obvious phytotoxicity symptoms caused by the treatments of any kind were observed for the duration of the field trial, therefore formulations are safe to be used at the recommended dosage rates for seed as well as soil applications.

The potential exhibited by *B. subtilis* and *Trichoderma* isolates used in the above studies is in line with findings by Bacon *et al.* (2001), that they can reduce growth and toxin accumulation of mycotoxins. These isolates could thus be used as seed treatments with the potential to prevent vertical transmission of *F. verticillioides* during the endophytic growth stage.

The findings by Yates *et al*. (2005) also demonstrates that the use of micro-organism biological control agents has the potential to reduce *Fusarium* inoculums in the plant with subsequent reduction in fumonisin production and also the potential to enhance general root, stem and leaf growth and cause an increase in reproductive yield. The reduction of mycotoxins combined with the improvement of general growth conditions could optimize vegetative and reproductive yields. In this study micro-organism biological control agents applied to seed inoculated with *F. verticillioides* as well as *F. proliferatum* were investigated as it is unlikely that only one pathogenic fungal strain can be the cause of yield loss and that it is normally a group of pathogens that can cause loss under field conditions.

Adesemoye *et al.* (2009) conducted greenhouse studies similar to that of Wu *et al.* (2005) as mentioned above. They conducted studies on tomatoes to determine if reduced rates of inorganic fertiliser coupled with microbial inoculants would produce plant growth and nutrient uptake levels equal to those which received full rates of fertiliser and to investigate the minimum level to which the fertiliser could be reduced when inoculants were used. The microbial inoculants used were a mixture of plant growth promoting rhizobacteria (PGPR) strains *Bacillus amyloliquefaciens* IN937 and *Bacillus pumilus* T4, a commercial PGPR formulation which consisted of many *Bacillus* strains, and an arbuscular mychorrhiza *Glomus intraradices*. Results showed that supplementing 75% of the recommended fertiliser rate with inoculants, the growth, yield and nutrient uptake were equal to that of the full fertiliser use without inoculants. Results were inconsistent when fertiliser was reduced below 75%. Without inoculants plant growth, nutrient uptake and yield were much lower with reduced dosage rate of fertilisers compared to the recommended higher dosage rate. These results on

tomatoes concluded that the same effects can occur on crops like maize with the correct selection of PGPR strains. This study did not include lower fertiliser applications combined with PGBR inoculums but the potential differences in growth and yield among the various treatments that received the same dose of pelleted chicken manure could be used to select future PGBR strains for further research to reduce fertiliser use in maize.

Negassa *et al.* (2005) reported major benefits in combining farmyard manure with nitrogen and phosphorous fertilisers at lower rates to be used for maize production in Ethiopia. This method contributed to an increase in organic content of the soil that resulted in an increase in soil fertility and maintenance of the soil biological activity. It further reduced the cost of inorganic fertiliser input and showed the importance of locally available organic fertilisers that should be used on a continuous basis for the recovering of degraded soils (Negassa *et al.*, 2005). The addition of micro-organisms to the seed prior to planting could have increased biological activity with subsequent increases in plant biomass and reproductive yield as investigated in this field study.

Soil fertility is a problem in the maize production areas of Kenya and Onyango (2010) experimented with various fertiliser treatments to increase maize yields. Maize yield has been declining over the years in Kenya (Onyango *et al.*, 2000) and soil fertility, continuous cropping, inappropriate production technologies were some of the reasons for this decline (Kamidi *et al.* 2000). In field experiments conducted by Onyango (2010) various fertiliser options were used on a number of maize cultivars. The options were cow dung farmyard manure (FYM), single superphosphate (SSP), and two different application rates of diammonium phosphate (DAP). Onyango found that there were significant differences among the fertiliser treatments and among the varieties of maize used and came to the conclusion that organic manure is just as good as any of the inorganic fertilisers and that the practice could save small farmers the high cost of inorganic fertilisers. This is in agreement with the findings of Negassa *et al.* (2005) as well as with the Ethiopian and Kenyan experiments alluded to above, that there is a potential to incorporate micro-organism formulations that can assist in the uitilisation of organic nutrients available as well as the control or reduction of diseases like ear rot caused by *Fusarium* spp. It appears that one of the major causes of yield loss in the experiment conducted in Kenya was due to rotten ears, which is very likely ear rot as described by Onyango (2010) and as explained in the present study.

The biological control agent formulations used in this study on maize that performed best in most of the evaluations namely: T-Gro, Shelter, Bismarck and Maximus (single applications to maize seed) as well as combined applications (T-Gro with Spartacus, T-Gro with Bismarck, as well as T-Gro combined with Maximus), therefore present new information that has not been published yet. All these formulations justify further research to ensure consistency in increased reduction of grain loss, which should subsequently result in increased maize yields of good quality.

CONCLUSION

Poor to very good increases in root, stem and foliage fresh biomass was produced by the *Bacillus subtilis*, *Trichoderma harzianum* and *Microbacterium maritypicum* isolates used in the commercial formulations compared to the untreated control and reference product Thiram. The micro-organism treatments showed poor to good reductions of grain loss as well as the severity of ear and stem rot symptoms compared to the untreated control and reference product Thiram 750WP. Silking and tasselling occurred at an equal to much faster rate of development when treated with micro-organism formulations in comparison to the untreated control and reference product Thiram 750WP.

Increases in cob yield varied from poor to very good for the micro-organism treatments when compared to the untreated control. The highest cob yields per plant that differed significantly from the untreated control were produced by T-Gro (*T. harzianum* isolate DB 103) and Shelter (*B. subtilis* isolate DB 101). This may be contributed to their strong growth-promoting properties combined with a reduction in *Fusarium* inoculum as endophytic fungus and bacterium.

The *T. harzianum* and *B. subtilis* isolates used in the micro-organism formulations evaluated in this study showed potential to be registered for use on maize against *Fusarium* spp causing stem and ear rot as well as growth stimulants for enhanced vegetative and reproductive yields due to their similar characteristics of most of the formulations already registerd worldwide on other crops for the control of soil-borne fungal pathogens, including *Fusarium* spp. The formulations T-Gro, Shelter, Maximus and Armenius can be associated with registered products in other countries abroad including Plantshield HC, Trichodry and Trichopel

containing *T. harzianum* and Companion, Kodiak HP, Epic, and Phytovit which all contain strains of *B. subtilis* as indicated by Scala *et al.* (2007).

No phytotoxicity of any kind was observed with the application of the micro-organism formulations and they are therefore suitable to be used for the treatment of maize seed as well as soil environment. The micro-organism formulations containing fungal and bacterial biological control agents have the potential to be used in commercial maize production to increase vegetative and reproductive yields and reduce the severity of ear and stem rot in maize. The use of these formulations in crop protection is very unlikely to have any adverse effects on humans, animals and the environment and can play a very important role in the future use of crop protection products that are safe.

RECOMMENDATIONS FOR FUTURE RESEARCH

Further research is justifiable to ensure consistency (repeated significant results) in increased vegeatative as well as reproduction yields under commercial field conditions with the selection of the best performers. The isolate *Microbacterium maritypicum* needs more attention due to the fact that research done worldwide with this genus is very limited to date.

The potential of the micro-organism formulations used in seed and soil treatment programmes is promising to reduce the input of fertilisers and still sustain or increase yields in maize and other crops. This will enable production to be more cost effective, sustainable and environmentally friendly.

The reduction of fumonisins (mycotoxins) produced by the *Fusarium* spp. should be quantified when treated with the micro-organism formulations and their biological efficacy should be evaluated for the potential control of other problem pathogens in maize, especially bacteria from the genera *Erwinia* which are quite often secondary to *Fusarium* infections.

ACKNOWLEDGEMENTS

Prof David Modise (UNISA), my supervisor, for his guidance and commitment in the completion of this project.

Nita Peacock (Dagutat Science), thank you for your time and valuable assistance with the proof reading.

Dr Helga Dagutat (Dagutat Science), thank you for your support and allowing me access to your company's micro-organism formulations for use in this research project.

Leslie Adriaanse (UNISA librarian), thank you for your assistance with the literature review.

Joan Hettema, special word of thanks for the final editing.

DISCLAIMER

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APPENDICES

Appendix 1: Weather conditions at time of applications

Applications	1	2	3	4
Date	29-12-09	13-01-10	04-02-10	02-03-10
Time	09h00	09h00	09h30	10h00
Temperature (°C)	Min : 16°C Max : 29°C	Min : 18°C Max : 30°C	Min : 14°C Max : 28°C	Min : 16°C Max : 30°C
Relative humidity (%)	51%	47%	53%	43%
Wind according to Bedford scale	None	None	1	2
Soil moisture	Wet, near saturation	Wet, near saturation	Wet, near saturation	Wet, near saturation
Cloud cover	50%	80%	30%	20%

Minimum temperatures vary between 18°C and 20°C , and maximum temperatures between 28°C and 34°C for the months of December 2009 , January 2010 and February 2010.

Meteorological and edaphic data as per EPPO Standards PP1 Volume 2, 2004.

Appendix 2: Rainfall recorded

Month	**Dates plus mm rain**	**Total Rain / Month**
December 2009	02/12(15mm); 06/12(12mm); 09/12(15mm); 10/12(7mm);13-14/12(11.5mm); 20/12(22mm);27/12(18mm); 29/12 (1.5mm)	102mm
January 2010	02/01(30mm); 04/01(15mm); 07/01(30mm); 19/01(2mm); 20-21/01(19mm); 25-26/01(40mm)	136mm
February 2010	06/02(9mm); 16/02(25mm); 16/02(8mm); 25/02(8mm)	51mm
March 2010	17/03(17mm); 25/02(5mm); 31/03(1.5mm)	24.5mm
April 2010	01/04(15mm); 02/04(15mm); 04/04(20mm); 05/04(20mm); 18/04(8mm); 24/04(45mm); 26/04(5mm); 27/04(6mm)	132mm
May 2010	No rain until final day of harvest on 03 May 2010 at hard dough stage.	0mm

Appendix 3: Results of soil analysis for the trial site by ECO Analytica.

Macro-nutrients										
Site sample	Ca	Mg	K	Na	PO_4	SO_4	NO_3	NH_4	Cl	HCO_3
	Millimol per litre									
A	0.63	0.26	0.38	0.44	0.01	0.15	1.70	O.04	0.07	0.60

Micro-nutrients								P-BRAY 1 (*ppm*)	Al *mg/ℓ*
Site sample	Fe	Mn	Cu	Zn	B	pH	EC (*mS/cm*)		
	Micromol per litre								
A	27.90	0.61	0.19	0.36	27	7.25	0.27	3.97	18

Soil particle size distribution of trial site:

Particles > 2mm = 2.2 % ; Particles < 2mm = 58.7% sand; 15.6% silt; 25.7% clay