STUDIES ON THE BIOCONTROL OF SEEDLING DISEASES CAUSED BY *RHIZOCTONIA SOLANI* AND *PYTHIUM* SP. ON SORGHUM AND TEF

by

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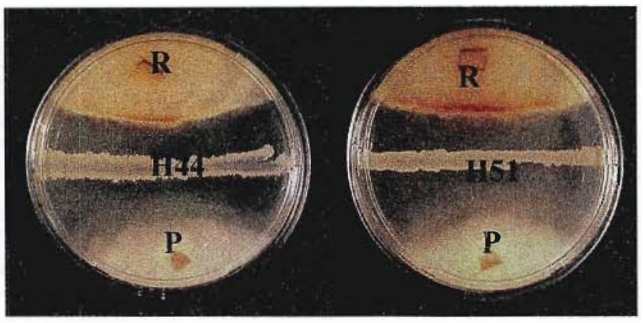
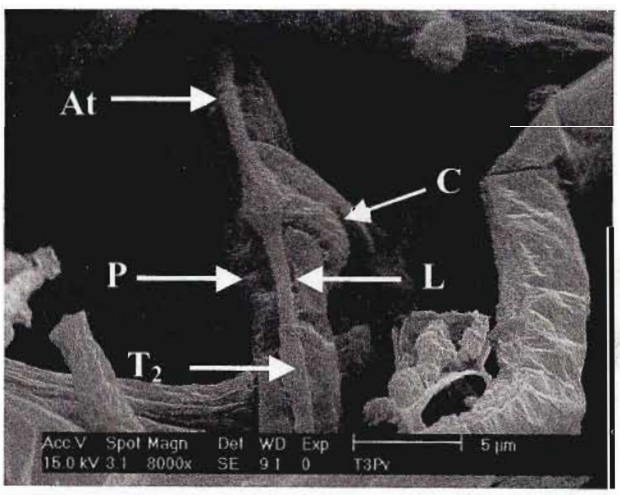
FRONTISPIECE - BIOCONTROL OF *RHIZOCTONIA SOLANI* AND *PYTHIUM* SP.

Comparison of biocontrol treatment on sorghum seedlings inoculated with *Pythium* sp. (far left), control-*Pythium* only (middle) and control (Nil)- water only (far right).



Plates showing antibiotic activities *Bacillus* spp. H44 (left) and H51 (right) on *Rhizoctonia solani* (R) and *Pythium* sp. (P)

Dual culture test showing hyphal interactions between *Trichoderma harzianum* Eco-T (T_1) and *Pythium* sp. (P). Formation of inhibition zone (I) and mycoparastitic action (M) of *Trichoderma* on *Pythium*.



Microscopic investigations on hyphal interactions between *Trichoderma* sp. SY3 (T_2) on *Pythium* sp. (P). *Trichoderma* attaches (At) and coils (C) around the hyphae of *Pythium*, resulting in the lysis of the host's cell wall (L).

ABSTRACT

Rhizoctonia solani and *Pythium* spp. are aggressive soil-borne fungal pathogens responsible for seed rot and seedling damping-off of many crops. With increased environmental and public concern over the use of chemicals, biological control of these diseases has been attracting more attention. However, success with this strategy depends on the development of effective antagonists, which requires repeated *in vitro* and *in vivo* tests.

Bacillus spp. were isolated from a soil sample obtained from a field where sorghum and tef had been grown for at least two years. Potential *Bacillus* isolates were screened for their ability to inhibit *in vitro* growth of *R. solani* and *Pythium* sp. Among 80 isolates tested, endospore forming *Bacillus* spp. H44 and H51 gave highest antifungal activity against the two test-pathogens in three consecutive tests. Results demonstrated that both H44 and H51 have potential as biocontrol agents against diseases caused by these two pathogenic fungi.

The interaction between three isolates of *Trichoderma* (*T. harzianum* Eco-T, *Trichoderma* spp. SY3 and SY4) and *Pythium* sp. were investigated using *in vitro* bioassays together with environmental scanning electron microscopy (ESEM). Visual observation on the dual culture tests revealed that hyphal growth of *Pythium* was inhibited by these antagonists soon after contact between the two organisms within 3-4 days of incubation. The ESEM investigations showed that all three isolates of *Trichoderma* grew toward the pathogen, attached firmly, coiled around and penetrated the hyphae of the pathogen, leading to the collapse and disintegration of the host's cell wall. Degradation of the host cell wall was postulated as being due to the production of lytic enzymes. Based on these observations, antibiosis (only by Eco-T) and mycoparasitism (by all three isolates) were the mechanisms of action by which *in vitro* growth of *Pythium* sp. was suppressed by these *Trichoderma* isolates.

The reduction of seedling diseases caused by R. solani and a pythium sp. were evaluated by applying the antagonists as seed coating and drenching antagonistic *Bacillus* spp. (B81, H44 and H51) and *Trichoderma* (*T. harzianum* Eco-T and *Trichoderma* spp. SY3 and SY4). On both crops, *R. solani* and *Pythium* sp. affected stand and growth of seedlings severely. With

the exceptions of H51, applications all of isoltes to seeds reduced damping-off caused by *R. solani* in both crops. Application of Eco-T, H44 and SY3 to sorghum controlled *R. solani* and *Pythium* sp. effectively by yielding similar results to that of Previcur®. On tef, biological treatments with Eco-T and SY4 reduced seedling damping-off caused by *R. solani* and *Pythium* sp., respectively, by providing seedling results similar to the standard fungicides, Benlate® and Previcur®. Most other treatments gave substantial control of the two pathogens on tef. Overall, *Bacillus* sp. H44 and *T. harzianum* Eco-T were the best biocontrol agents from their respective groups in reducing damping-off by the two pathogens. In all instances, effects of application method on performance of biocontrol agents and adhesive on emergence and growth of seedlings were not significant.

A field trial was conducted at Ukulinga Research Farm at the University of Natal, Pietermaritzburg, South Africa, to determine efficacy of biological and chemical treatments on growth promotion and reduction of damping-off incited by *R. solani* and *Pythium* sp., and to evaluate the effects of a seed coating material, carboxymethyl cellulose (CMC), on seedling emergence and disease incidence. Seeds of sorghum and tef were treated with suspensions of antagonistic *Bacillus* H44 or *T. harzianum* Eco-T, or sprayed with fungicides, Benlate® or Previcur®. Application of Benlate® and Previcur® during planting significantly increased the final stand and growth of sorghum seedlings. Seed treatments with both H44 and Eco-T substantially controlled damping-off caused by *Pythium,* resulting in greater dry weights of seedlings than the standard fungicide. However, they had negative effects when they were tested for their growth stimulation and control of *R. solani.* The CMC had no significant effect on germination and disease levels. These results showed that these antagonists can be used as biocontrol agents against *Pythium* sp. However, repeated trials and better understanding of the interactions among the antagonists, the pathogens, the crop and their environment are needed to enhance control efficiency and growth promotion of these antagonists.

Some of these biocontrol agents used in this study have the potential to diseases caused by *R. solani* and *Pythium* sp. However, a thorough understanding of the host, pathogen, the antagonist and the environment and the interactions among each other is needed for successful disease control using these antagonists.

DECLARATION

I, Habtom Butsuamlak Tesfagiorgis, declare that the research reported in this thesis, except where otherwise indicated, is my own original research. The thesis has not been submitted for any degree or examination at any other university.

Habtom Butsuamlak Tesfagiorgis

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PREFACE

The number of students and researchers joining the research team, Biocontrol for Africa, at the Department of Plant Pathology, University of Natal, is increasing each year. As a result, promising progress is being undertaken in investigating beneficial microorganisms for disease control and growth stimulation. The research contained in this thesis reflcts part of the investigations being conducted.

All the research was conducted at the University of Natal, Pietermaritzburg, South Africa. In the first year, isolation and *in vitro* screening using visual and electron microscopic studies and greenhouse trials were conducted. In the second year, a field trial was done at Ukulinga Research Farm, University of Natal, Pietermaritzburg, SA. The main emphasis of the research was to screen and determine efficacy of potential biocontrol agents in controlling seedling diseases caused by *Rhizoctonia solani* and *Pythium* sp.

Application of chemical fungicides have been used for decades in controlling seedling diseases caused by *R. solani* and *Pythium* spp. in a number of crops. However, safety, health and environmental concerns and the evidence of pathogen resistance to these chemicals, have promoted a search for alternative control tactics.

There is increasing interest in the exploitation of microorganisms for disease control and plant growth promotion, as evidenced by the number of publications appearing every year. During the last 30 years, considerable progress has been made in the area of biological control of plant diseases mainly as a result of public and environmental concerns over chemical fungicides. Such intensive investigations have yielded the development and release of some biocontrol products for the market. Most of these products are intended to protect soil-borne diseases, especially damping-off. However, the market price of these bio-products covers very little percentage of the total crop protection cost. The reason for this phenomenon is that the performance of most biocontrol agents has been variable.

Most of our knowledge about the biocontrol activity of beneficial microbes comes from investigations conducted under laboratory conditions. As a result, failure of the technique is not surprising when biocontrol products are tested under natural conditions. In order to develop biocontrol agents for disease suppression, potential isolates were tested against the test-pathogens (*R. solani* and *Pythium* sp.) under laboratory, greenhouse and field conditions.

The aims of this research were to:

- isolate and screen potential biocontrol agents;
- study hyphal interactions of selected isolates of *Trichoderma* and *Pythium;*
- evaluate biocontrol activities of selected biocontrol agents in controlling damping-off under greenhouse and field conditions;
- compare disease control efficacy of biocontrol agents against standard fungicides;
- investigate effects of application methods in biocontrol efficacy of antagonists and
- determine the effect of a sticker on the germination of seeds and disease incidence.

The scope of this thesis is broad, containing six chapters, each chapter presented as a discrete paper, resulting in repetition of some references between chapters.

1. Chapter 1 presents a general review of literature highlights the crop plants, i.e., tef and sorghum, seedling diseases and their causal organisms and biological control of plant diseases with special reference to the antagonists *Bacillus* and *Trichoderma* spp.
2. Chapter 2 reports isolation and *in vitro* screening of *Bacillus* spp. against *R. solani* and *Pythium* sp.
3. Chapter 3 covers *in vitro* and ultrastructure of hyphal interactions between species of *Trichoderma* and *Pythium.*
4. Chapter 4 encompasses evaluation of bacterial and fungal biocontrol agents in controlling seedling diseases of sorghum and tef caused by *R. solani* and *Pythium* sp. under controlled greenhouse conditions.
5. Chapter 5 presents field evaluations of biological and chemical treatments in controlling damping-off and growth stimulation of sorghum and tef.

6. Chapter 6 summarizes the experimental results and concludes with the efficiency of the potential isolates in disease suppression as well as forecasting future needs.

CHAPTER 1

LITERATURE REVIEW ON BIOLOGICAL CONTROL OF SEEDLING AND ROOT DISEASES OF SORGHUM AND TEF

1.1 GENERAL INTRODUCTION

Production of adequate, palatable and safe food for the Third World's ever-increasing population presents a major challenge to agricultural experts. In the last few decades, agricultural production has increased as a result of an increase in cultivated land and the use of agrochemicals for crop production and protection. However, such environmentally unfriendly and unsustainable agricultural systems will not solve the problem of food shortage. Food security and agricultural sustainability require both development of new and appropriate technologies and an understanding of the environment in which they are to be implemented. The alternative is a new type of agriculture and agrochemical industry based on sustainable production without intense use of fertilizers and pesticides, which would need, and could lead, to the development of different biocontrol strategies (Campbell, 1989).

During the last 30-40 years, attitudes toward pest, disease and weed control have changed, partly as a result of pressure from conservationists and consumers and more recently, from organic growers (Finch, 1992). Ministries of the environment and agricultural enterprises want to decrease the use of chemicals, consumers demand products grown with a minimum use of chemicals and farmers are asking for alternative control options (Finch, 1992; Whipps and Lumsden, 2001).

The use of certain bacteria and fungi with the objective of obtaining disease control and growth stimulation was investigated. The genera *Bacillus* and *Trichoderma, represent* soil-inhabiting microbes that have been extensively studied as antagonists against several soil-borne pathogens (Cook and Baker, 1983). For years, these bacteria and fungi have been isolated from soil and tested for their ability to control plant pathogens and promote plant

growth. Significant control of diseases and a consequent increase in plant development and yield have been obtained on a variety of plants both in greenhouse and field trials when seeds were treated with these biocontrol agents (BCAs).

Recently, some of the more promising strains of the genus *Bacillus*, *Pseudomonas*, *Gliocladium* and *Trichoderma* have been further developed and marketed as alternatives to the traditional chemical-based fungicides and growth promoters (Berger *et al.*, 1996; Whipps and Lumsden, 2001). The major problem, however, is the failure to repeat these results consistently on different soils or in different years in naturally contaminated fields and to make biological control of soil-borne pathogens competitive to chemical control (Schippers, 1988). The most important constraints are physical, chemical and biological factors in the soil (Cook and Baker, 1983) and insufficient root colonization by the introduced BCAs (Mahaffee and Backman, 1993).

In 1984, the global market value of biocontrol products was less than 1% of the total expenditure on crop protection and public health. It was estimated to increase by 2-3% each year until at least 2000 (Jutsum, 1988). This slow development in the use of BCAs is largely due to their inconsistent results in the field (Berger *et al.*, 1996). To improve this situation, more fundamental knowledge is needed on the biotic and abiotic factors affecting the population dynamics, survival and antagonistic activity of BCAs in soil (Campbell, 1989; Mao *et al.*, 1998).

The successful development of BCAs is likely to depend on a thorough understanding of the biology and ecology of the pathogens and antagonists included and their interactions with other inhabitants of the soil (Kerry, 1992) as well as the specific crop and its husbandry (Berger *et al.*, 1996). Efficiency of biological control relies on the ability of the introduced antagonist to be established in the soil, which in turn is affected by its formulation, storage and application method (Lewis and Papavizas, 1987). Other biotic and abiotic factors such as dose of the antagonist applied, inoculum density of the pathogen, host genotype and conduciveness of the environment to the disease also affect the survival and activities of BCAs (Landa *et al.*, 2001).

In this chapter, a review of the crop plants, tef and sorghum, is presented paying attention to their ecology and agronomy; seedling diseases of sorghum; causal agents of those diseases and their control; biological control of plant diseases, i.e., required characterstics of BCAs, relationship between BCAs and chemicals, application and mode of action of BCAs and a brief description on *Bacillus* and *Trichoderma* as BCAs. Finally, knowledge on the components of biocontrol systems is presented as strategies for the use of BCAs for the control of plant diseases.

1.2 SORGHUM [*SORGHUM BICOLOR* (L.) MOENCH] AND TEF [*ERAGROSTIS TEF* (ZUCC.) TROTTER]

Sorghum and tef are two cereal crops that originated in Africa (Anonymous, 1996). Both are annual crops belonging to the family Paeceae (Skerman and Riveros, 1990). In different regions of the world, they are produced as food for human or animal consumption depending on the crop variety and economy of the producing country. The one thing they have in common is their ability to grow in suboptimal climatic and soil conditions. There is hope that these two crops may provide a reliable food supply for the fast growing populations of the drier regions of the African continent.

Sorghum is a global crop, placed fifth on the world's cereal production list. Tef is grown as food in limited regions, East Africa, especially Ethiopia and Eritrea. Little attention has been given to grain sorghum in areas where ecological conditions are ideal for maize production. Similarly, tef appears to be less important as a human food in most countries. Perhaps, for this reason and due to its small-sized seeds, researchers have been reluctant to conduct research on this crop. To date, there is no report on damping-off of tef. However, on sorghum, a few studies have been conducted on soil-borne diseases mainly incited by several species of *Pythium*, and their control through the use of resistant cultivars (Forbes *et al.*, 1987) and chemical fungicides (Davis and Bockus, 2001). In addition, McLaren (1987) made an assessment of soil-borne pathogens responsible for damping-off of sorghum disease management options were not given.

1.2.1 Sorghum

1.2.1.1 Sorghum and its importance

Sorghum is the second major staple food in Africa and the land covered by sorghum and millet is bigger than all other food crops combined (Taylor and Belton, 2002). In the United States of America (USA) and Europe, sorghum serves mainly as feed for poultry and livestock but can be processed into many valuable products (Anonymous, 1996).

1.2.1.2 Ecological adaptations of sorghum

Sorghum is adapted to a wide range of ecological conditions and produces substantial yields of grain under conditions unfavourable for most other cereals (Anonymous, 1996). Commonly, it is grown in warm or hot regions with summer rainfall as well as in irrigated areas (Martin et al., 1976). It can tolerate hot and dry conditions, but also has the ability to grow in waterlogged fields (Skerman and Riveros, 1990; Maunder, 2002).

Sorghum grows best at temperatures ranging from 27-30°C and reduces its rate of growth if temperature drops below 16°C (Martin et al., 1976; Metcalfe and Elkins, 1980). The average precipitation requirement of sorghum is 400-750 mm per annum (Skerman and Riveros, 1990). During periods of extreme drought the plant becomes dormant, but does not wither or die, growth being resumed when rain commences (Metcalfe and Elkins, 1980). Compared to maize and most other cereals, sorghum has higher water use efficiency. It has numerous roots and small-sized leaves, covered with a waxy cuticle, which are capable of rolling inward to reduce transpiration (Fageria et al., 1991). The plant can grow well in all types of soil with a pH of 5.5-8.5, and also in soils with high salinity, alkalinity and poor drainage (Doggett, 1988).

Sorghum is a short day plant, but cultivars differ in their sensitivity to photoperiod (Kimber, 2000). According to Fageria *et al.* (1991), effect of day length is realized when certain

minimum temperature requirements are fulfilled. Therefore, time of maturity of the plant is determined by genotype, photoperiod and temperature.

1.2.1.3 Husbandry of sorghum

Land preparation: Sorghum can be planted from zero tillage to moderate ploughing. However, judicious tillage can improve seedling emergence and establishment of sorghum and provide better management of weeds. Land preparation is undertaken using hoes, animal-drawn implements, discs or ploughs, depending on power availability (House *et al.*, 2000).

Planting practices: Sorghum is usually cultivated in rows 0.5-3.0m apart with a seeding depth of 4-5cm depending on the tillering habit of the cultivar (Martin *et al.*, 1976). The amount of seed needed per unit area for a given stand depends on conditions of the seedbed, seed viability, seed size and weather conditions at seeding time. As a general guide, Metcalfe and Elkins (1980) indicated that opimal yield is obtained with seeding rate of 22-45kg ha^{-1} in dry land conditions and 7-11kg ha^{-1} in humid or irrigated regions.

Soil moisture, soil temperature and seeding depth affect seedling emergence. Martin *et al.* (1976) noted that percentage and rate of germination are slightly reduced when seeds are planted deeper than 6.25cm or when soil temperature is below 25°C. It is not advisable to sow seeds in dry soils or in soils that are in the process of drying (Chantereau and Nicou, 1994). In addition, at the time of planting, seeds are subjected to attack by several pathogens and insects, and every effort must be made to reduce related damage (Anonymous, 1996).

Fertilization: In dry land areas, little or no response is obtained by the application of fertilizer. However, where water is available for irrigation, high yields can be obtained by applying nitrogen (N) and phosphorus (P) (Metcalfe and Elkins, 1980). As a general recommendation, 40-120kg ha^{-1} nitrogen plus 45-70kg ha^{-1} for each of phosphate (P_2O_5) and potash (K_2O) are used (Martin *et al.*, 1976). If NPK is preferred as a fertilizer, it is mixed with the soil before ploughing (Chantereau and Nicou, 1994). Since the fertilizer requirement of a crop is significantly influenced by climate and soil type, the above recommendations may not work in all areas/regions. Fertilization must therefore be based on the soil analysis and requirements of the specific cultivar and its yield expectancy.

Yield: Grain yield of sorghum varies from 300-2000kg ha^{-1} in India and Africa under rainfall conditions, to 4500-6500kg ha^{-1} under irrigated areas in the US and Australia (Skerman and Riveros, 1990).

1.2.2 Tef

1.2.2.1 Tef and its importance

Tef is an important crop in limited regions of the world, especially Ethiopia, where it is preferred to any other cereal and comprises about two-thirds of the total protein diet of the population (Anonymous, 1996). It is also used in Eritrea for the same purpose (personal observation). Some records indicate that tef has also been produced for food in Yemen, Kenya, Malawi and India. Recently, the increasing popularity of Ethiopian restaurants in Europe and North America has encouraged US and South Africa farmers to produce tef commercially (Anonymous, 1996).

As animal fodder, tef is extensively used in South Africa and Australia. Its straw is very palatable to livestock and compared to the residue and hay of other cereals, tef contains higher crude protein and less lignin (Jones, 1988).

1.2.2.2 Ecological Adaptation of tef

Tef can grow in ecologically diversified environmental conditions. The optimum temperatures and rainfall for growth of tef are 25-28°C and 300–500mm, respectively (Ketema, 1993). Although it is susceptible to frost (Kassier, 2001), it grows well at altitudes ranging from sea level to 3000 m in many different soil types (Jones, 1988).

1.2.2.3 Husbandry of tef

Seedbed preparation: Due to its extremely small-sized seeds, planting of tef requires a firm moist seedbed to effect good soil moisture-seed contact (Stallknetch *et al.*, 1991). Ketema (1993) suggested that farmers plough their tef fields 2-5 times depending on soil type, weed infestation and water logging. Where weeds are absent, repeated ploughing might not be necessary, indicating that tef can be produced under reduced tillage. However, yield of tef was observed to increase by 44% with extra tillage (Ketema, 1993).

Planting: Once the field is prepared, seeds are spread on the surface of the soil and left uncovered or, sometimes slightly covered by pulling woody tree branches over the soil surface using oxen (Ketema, 1993). Moderate compaction is recommended in some areas to enhance stand establishment on heavy soils that suffer from soil cracking. A seeding depth of 5-15mm gives best germination and emergence compared to seeding on the soil surface or deeper than 20mm (Ketema, 1993). A planting depth of 12mm is recommended (Stallknetch *et al.*, 1991).

Seeding r ate: The amount of seed required per hectare varies according to the method of planting. For broadcasting by hand, 25-30kg ha^{-1} is recommended, and for a drill or broadcaster, t he a mount of seed per hectare is 15kg (Ketema, 1993), whearas for advanced planters, 2-9kg ha^{-1} is optimum (Stallknetch *et al.*, 1991).

Fertilization: Based on results obtained in Ethiopia, Ketema (1993) recommends 60/23 N/P_2O_5 kg ha^{-1} and 40/26 N/P_2O_5 kg ha^{-1} on heavy (vertisoils) and sandy clay soils, respectively.

Yield: Appropriate cultural practices (i.e., land preparation, sowing and weed control) can double or even triple grain yield of tef (Jones, 1988). In Ethiopia, grain yield varies from 1400-2200kg ha^{-1} (Ketema, 1993).

1.3 SEEDLING AND ROOT DISEASES

Seedling diseases can be a major factor in stand establishment and may be associated with several causal organisms and symptom complexes. Generally, unhealthy (i.e., damaged, weathered, or mouldy) seeds germinate poorly. Even with high seed quality, seed-borne and soil-borne fungi can cause severe reduction in stand and seedling vigour if the prevalent conditions during, or shortly after planting, are conducive to seedling diseases (Forbes *et al.*, 1986). Because no report could be accessed on seedling diseases of tef, only common symptoms of sorghum are presented below.

1.3.1 Symptoms of seedling and root rot diseases of sorghum

Earliest disease expression is the failure of seeds to germinate. Infection of seedlings during, or shortly after germination, or when seedlings have emerged may also cause pre- and post-emergence damping-off (Forbes *et al.*, 1987). Unless a newly planted field is monitored carefully, it is difficult to determine if poor stands occur due to seed rotting before emergence, or if seedlings emerge and then die. In most cases, seedling and root diseases are caused by several fungi, each fungus producing similar symptoms (Odvody, 1986). Positive identification of the pathogen(s) causing stand loss is thus of great importance.

In roots and rootlets, red, brown, or black lesions are formed along the roots causing varying degrees of damage, depending on a number of factors (Odvody, 1986). Elongation of the root is inhalted if infection occurs at the root tip. Other symptoms include water-soaking and basal soft rot (Forbes *et al.*, 1986). Under hot, dry conditions, leaf and plant death may occur due to *Pythium* root rot most frequently near or after plant maturity (Odvody, 1986). Post-emergence damping-off occurs most often if the transitory primary root system is destroyed before the permanent root system has been established from the crown (Forbes *et al.*, 1986). In young

plants, stunting and yellowing of lower leaves is a common symptom. However, in leaves of older plants, rolling and wilting may occur in addition to stunting and yellowing.

1.3.2 Epidemiology

Generally, problems of stand establishment occur when seeds are planted in cold and wet soils (Forbes *et al.*, 1986; Odvody, 1986). Practices that aggravate seedling and root diseases caused by *Pythium* and *Rhizoctonia* include deep sowing, over-sowing in moist soils and poorly prepared seedbeds.

1.3.3 Seedling and root pathogens

Seedling and root diseases are caused by several organisms resulting in complex symptoms. In sorghum, causal organisms for these diseases include *Pythium* spp. (the most important causal agent), *Fusarium* spp., *Aspergillus* spp., *Rhizoctonia* spp. and *Phoma* spp. (Forbes *et al.*, 1986).

1.3.3.1 Pythium *species*

The genus *Pythium* belongs to the family *Pythiaceae* of the Oomycetes (Agrios, 1997). It occurs most abundantly in cultivated soils near the root region in superficial soil layers, less commonly in non-cultivated or acid soils, where *Trichoderma* is responsible for their absence. *Pythium* spp. have also been isolated from soils from arable land, pastures, forests, nurseries, swamps and water (van der Plaats-Niterink, 1981).

Pythium spp. are parasitic on roots and rootlets of many of the graminae family throughout the world (Jones and Clifford, 1983) where they can cause both seedling and root diseases. Species identified as causal agents of root and seedling diseases of sorghum include: *P. graminicola, P. arrhenomanes, P. aphanidermatum, P. myriotylum and P. periplocum* (Pratt and Janke, 1980; Odvody, 1986; Forbes *et al.*, 1987).

Characteristics: Species of *Pythium* can attack the roots of most agricultural crops and weeds and survive in soil for many years. They can cause serious disease in sorghum when cool, wet conditions occur after planting (Forbes *et al.*, 1986). Other stress factors such as low or high pH, herbicides and herbicide antidotes, that delay the establishment of the permanent root system, increase the potential of seedling damping-off. Additionally, early planting of sorghum by producers when soils are typically cooler and wetter, aggravate certain diseases especially root rots caused by *Pythium* spp. (Davis and Bockus, 2001). In cool, wet soil, sorghum may be more vulnerable to *Pythium* attack because of delayed seed germination and seedling emergence, reduced growth of seiminal roots and slower establishment of roots from the crown (Odvody, 1986). A review by Jones and Clifford (1983) shows that unbalanced and poor fertilization can also contribute to infection of plants by weakening the defense mechanism of the host.

Pythium can be spread by water, man and other animals and in a study conducted to see possible transmission of *Pythium* by animals, it was also recovered from bird droppings. Earthworms may also play an important role in transmission of this fungus (van der Plaats-Niterink, 1981).

The fungus survives at a soil depth of 20-30cm (Jones and Clifford, 1983) as oospores (van der Plaats-Niterink, 1981) and germinates when it absorbs energy sources from seed and root exudates (Forbes *et al.*, 1986; Bruehl, 1987). There is no germination if there is no exogenous source of energy (van der Plaats-Niterink, 1981).

Infection takes place when zoospores produce germ tubes or hyphal elements from appressoria and then penetrate the plant by means of infection pegs. Pathogenicity is determined by the availability of enzymes. Inoculum density, soil water content, temperature, light intensity, cation content and pressure of other microorganisms determine infection of seeds and seedlings by *Pythium* species (van der Plaats-Niterink, 1981). Sufficient or excessive water often favours infection and severity of attack. Van der Plaats-Niterink (1981) showed that in susceptible plants, root exudates could cause an accumulation of zoospores and accelerate their encystment and germination, especially in differentiating or injured roots.

Symptoms: Symptoms on large adventitious roots are darkening, blackening and formation of sunken, red-brown or black lesions; sometimes, at root death, lesions or the entire root turns tan in colour (Odvody, 1986). Aboveground symptoms include stunting, reduced tillering, chlorosis and delayed maturity. However, since these symptoms are uniform throughout the field, it may remain unnoticed (Millus and Rothrock, 1997).

Effects of *Pythium*

Effects on stand: Studies have shown that *Pythium* root and/or seed rot could be a serious yield-limiting factor by reducing seedling stands (Pratt and Janke, 1980; Forbes *et al.*, 1987).

Effects on yield: In some areas of the USA, extensive losses and lodging were observed in plants with root rot during 1972 and later years (Pratt and Janke, 1980). In the same regions, Forbes *et al.* (1985) reported severe yield reduction in fields of sorghum due to the presence of *Pythium* spp. However, unlike most other crops, grain yield of sorghum is not necessarily determined by final stand counts. This is because sorghum plant with any crop has the ability to produce tillers and compensate the reduction in stand if the loss is not too high, provided that it has a relatively healthy root system. The most important negative effect of *Pythium* is the chronic root rot that it causes since any impact on the root system has a detrimental effect on water and nutrient uptake and consequently grain yield (Davis and Bockus, 2001).

Control strategies for *Pythium*

Once *Pythium* spp. are established in soil as oospores or zoospores, it becomes difficult and expensive to control disease caused be them (Hendrix and Campbell, 1973). Hence no specific control measure is recommended for its control. However, the following practices have been used as best options.

Cultural: Any practice (e.g., irrigation frequency and timing of final irrigation) that influences the moisture content of the soil, has a direct relation to incidence and severity of *Pythium* on sorghum (Odvody, 1986). The influence of previous crops and other tillage practices on

Pythium root rot is not known but may have some effect on initial inoculum, soil moisture, soil temperature and other disease factors (Odvody, 1986). However, duration of rotation may not have any effect in controlling *Pythium* because it has a wide host range. Seeds producing seedlings of low germination vigour are prone to attack and therefore, seeds of high quality should be used for planting (Jones and Clifford, 1983). Moreover, Jones and Clifford (1983) showed that in various cereals, application of the recommended amount of fertilizer has been used as a means of controlling root rot caused by *Pythium* spp.

Chemical: Many fungicides applied to sorghum seed are ineffective in controlling seedling diseases caused by *Pythium*, probably because either the site of parasitic attack is distal to the seed or the fungicide (captan or thiram) is not effective in controlling the p athogen. S ome systemic fungicides such as metalaxyl, may provide better protection (Odvody, 1986). Sorghum, seed treatment with metalaxyl protected roots from infection by *P. ultimum* for 28 days after planting (Davis and Bockus, 2001) and increased grain yield by 22.7% (Davis and Bockus, 1996). In other crops, previcur was used as a standard fungicide against *Pythium* (Omarjee, 2002).

Resistance: Resistance to a few *Pythium* spp. has been observed in some cultivars of sorghum (Forbes *et al.*, 1986). Genotypes differ in their susceptibility or tolerance toward damage caused by the pathogen. In a study where *Pythium* spp. caused severe seedling disease, Forbes *et al.* (1985) observed that some sorghum hybrids were able to resist attack by this fungus. Where resistance of sorghum to *Pythium* spp. is observed, it is quantitative and may represent a relatively small difference in disease severity or symptoms (Forbes *et al.*, 1987). In some plants, resistance to *Pythium* species may be related to the presence of phenolic compounds and other inhibitory substances in the seed (van der Plaats-Niterink, 1981). In cotton, resistance of seedlings to *Pythium* spp. and *R. solani* has been reported to be associated with release of little or no seed exudates, or slow germination and emergence (Howell, 2002). However, in other plants, such resistance may not be expressed against different isolates of *Pythium* and, the use of resistant cultivars may therefore not be promising.

Biological: To date, there is no record where biological control was applied effectively in controlling seedling and root diseases on sorghum. Despite, excellent control having been achieved in other crops. Biocontrol agents that provided have effective control of *Pythium* damping-off include species of *Bacillus* spp. (Kim *et al.*, 1997a), *Trichoderma* spp. (Howell, 2002), *Pseudomonas fluorescens* (Kim *et al.*, 1997b) and *Gliocladium virens* (Lewis and Papavizas, 1987).

Integrated disease management: As in most other crops, it may be possible to incorporate BCAs with chemical fungicides to supplement other control measures.

1.3.3.2 Rhizoctonia solani

Rhizoctonia solani is a basidiomycete fungus that does not produce any asexual spores and only occasionally will the fungus produce sexual spores (basidiospores). In nature, *R. solani* exists primarily as vegetative mycelium and/or sclerotia (Agrios, 1997). Species of *Rhizoctonia* are often unspecialised, with a very high competitive saprophytic ability so that they can survive, and indeed flourish, in competition with other organisms (Campbell, 1989). The fubgus occurs throughout the world and causes significant losses in maby plant hosts (Lewis and Lumsden, 2001). *Rhizoctonia solani* infects and causes damage to all kinds of plant including vegetables, field crops, ornamentals, shrubs and trees (Agrios, 1997).

Rhizoctonia solani may be introduced into the soil on seed, in contaminated pot mixes, or by residues from greenhouse benches (Lewis and Lumsden, 2001). Infection occurs when prevailing conditions favour germination of the fungus. Saline soils, deep planting and moist soils favour damping-off by *R. solani*. Optimum temperature for growth of this fungus ranges between 25-30°C, with a minimum of 8°C and a maximum of 35°C, depending on the strain (Cook and Baker, 1983). Jurvie (1994) reviewed that for successful infection, propagules of *R. solani* must germinate in the vicinity of, or resume growth towards, the host, form an infection structure, penetrate and become established within the host. To complete this process, the organism requires energy that comes from the soil solution, host exudates and the propagules themselves (internal) (Jarvie, 1994). The sme author pointed out that exudates from the host

effect the pathogenicity of *R. solani* by providing nutritive substances necessary for germination and growth prior to penetration..

Symptoms: Symptoms vary according to prevalent environmental conditions and the crop species (Lewis and Lumsden, 2001). Root rot and damping-off are the most common symptoms of this fungus. Damping-off occurs when seeds are planted in cold wet soils. Young seedlings may die before or during germination or shortly after emergence from the soil. Dark lesions appear in roots just below the soil line, which may enlarge in size and number to include the entire base of the plant and most of the roots. This results in stunting and sometimes mortality (Agrios, 1997).

Control strategies

Although control of *R. solani* is very difficult (Agrios, 1997), certain strategies are commonly used.

Cultural: Preventing the movement of infested soils during cultivation, maintaining adequate soil fertility and good drainage helps to reduce serious losses by *R. solani* (Kiewnick *et al.*, 2001). Wide spacing of plants can also minimize disease severity by providing good aeration (Agrios, 1997). As noted earlier, the pathogenicity of *R. solani* is dependent on the availability of certain soil nutrients. Therefore, modifying the external supply of nutrient can minimize infection by *R. solani* and many other soil-borne pathogens.

Chemical: On turf grasses, the application of contact (ipriodine and chlorothalonil) and systemic (carboxin, triadimefon and methyl) fungicides has provided effective control of *Rhizoctonia* (Agrios, 1997). Benomyl (Benlate) has also been used to provide efficient control of *R. solani* (Ahmed and Baker, 1988). However, many resistant strains of this fungus have been recorded for these chemicals (Sumner *et al.*, 1992).

Resistance: Resistance to *R. solani* in beans, is related to the amount of phenolic compounds that the seed can release and on the ability of the seed coat to maintain its integrity throughout hydration until it is split by the emerging radicle (Jarvie, 1994).

Biological: In the last twenty years, promising results have been achieved in controlling *Rhizoctonia* seedling and root rots by measn of biological control. *Rhizoctonia solani* is more sensitive to attack by several mycoparasites than most soil-borne pathogenic fungi (Cook and Baker, 1983), and has been shown to be parasitized by many fungi, bacteria and nematodes (Agrios, 1997). In different crops, effective control of *R. solani* has been achieved under field and greenhouse conditions with species of *Gliocladium* (Howell, 1982), *Trichoderma* (Elad *et al.*, 1981), *Bacillus* (Kim *et al.*, 1997a and b), *Pseudomonas* (Cook and Baker, 1983) and non-pathogenic binucleate *Rhizoctonia* (Sumner *et al.*, 1992).

1.4 BIOLOGICAL CONTROL

Biological control as a crop protection strategy system emerged as a response to the search for a safe, effective and environmentally friendly approach to replace or supplement the use of chemical pesticides. Biological control of plant diseases involves the use of antagonistic microorganisms to control a pathogen. One form of biological control occurs if the activity of a microorganism, e.g., a plant pathogen, is controlled by another member of the community (Campbell, 1989).

Many rhizosphere competent antagonistic bacteria and fungi are used as BCAs, introduced inconjunction with seed. They colonize seedling roots and survive there for as long as necessary to protect the plant against soil-borne pathogens (Kim *et al.*, 1997a).

Over the past three decades, research has repeatedly demonstrated that several microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). Most commercially available BCAs are intended to control damping-off and improve stand establishment and seedling vigour (Kim *et al.*, 1997a). In addition, an increase in yield of many ornamental plants, vegetables and field crops has been observed as a result of growth stimulation by these

antagonists (Mao, *et al.*, 1998). Some of the most effective BCAs have been processed into products that are commercially available (Whipps and Lumsden, 2001). Successful biological control depends amongst other things, on the production and application of effective BCAs (Mao *et al.*, 1997).

1.4.1 Characteristics of a successful biological control agent

A BCA must firstly be safe to humans, animals and the environment. It should have the ability to grow and colonize the rhizosphere and spermatosphere fast, produce excessive inoculum and survive with the minimum demand for nutrients and favorable environmental conditions (Chao *et al.*, 1986; Lewis and Papavizas, 1987). The BCA should hs also have some means, by which it can survive under unfavourable environmental conditions.

Mao *et al.* (1997) emphasized that active colonization of available substrates by BCAs can greatly reduce pathogen inoculum from a long distance. For instance, infection of seed by *Pythium* and *Rhizoctonia* occurs within six hours after planting. Therefore, such diseases can only be controlled by active and immediate colonization of the seed by the antagonist (Mahaffee and Backman, 1993). In addition, an ideal BCA should be the one that farmers want to use because it can effectively and cheaply control diseases which are of importance to their crops. Moreover, it has to function successfully under different environmental conditions.

1.4.2 Biological control in relation to chemical fungicides

1.4.2.1 Merits of biological control

Chemical treatments are usually easy to apply and relatively inexpensive. However, some potent chemicals such as methyl bromide have been shown to persist in the environment, accumulate in predators at the top of food chains and have long-term effects on non-target organisms (Campbell, 1989). In contrast, biological control is often safe to the environment and humans. In a biological control strategy, a pathogen has limited opportunity to develop

resistance to its the antagonists. Antagonists have also proven their potential to control some soil-borne pathogens by penetrating deep into the soil, which is not possible with chemical control. Moreover, unlike fungicides, some antagonists provide long-term control if they can persist and multiply in the soil (Butt *et al.*, 2001).

1.4.2.2 Drawbacks of biological control

As with any disease management strategy, biological control has its limitations. According to Campbell (1989), biological control has the following drawbacks. Firstly, there is a general belief that introduced "foreign" organisms (antagonists) into a complex environment, such as soil, may operate less successfully compared to an environment without competition (e.g. sterile horticultural composts, fumigated soil or in clean timber). Hence, in more complex environments, colonization, and therefore, efficient control is patchy. Secondly, there is a suspicion that BCAs may deleteriously effect the plant or the soil micoorganisms, move into water supplies or spread to other environments where they could be a problem. Thirdly, there is also a fear of mutation in the BCAs which may result in undesired characteristics that may adversely effect the soil microflora and plant health. Fourthly, BCAs have a limited shelf-life and are sensitive to changes in temperature or osmotic pressure. Finally, in natural soil, many organisms can kill BCAs and therefore, there is a need to deal with several microbes, which would increase the cost of disease management with BCAs.

1.4.2.3 Improvements in control efficiencies of biocontrol agents

Since biological control is holistic in its approach, it is important to combine the manipulation of different aspects that are necessary for the antagonist to achieve maximum plant growth and minimum disease (Campbell, 1989). To increase the efficiency of biological control, combinations of biological, chemical and/or physiological seed treatments have been investigated (Mao *et al.*, 1998).

Manipulation of the soil factors such as pH, water potential and organic matter content (Chet and Baker, 1980; Bruehl, 1987), removing soluble molecules from seed (Mao *et al.*, 1997,

1998), activation of biocontrol formulations with alcohol (Lewis *et al.*, 1998) and growing BCAs in suitable media (Sivan *et al.*, 1984) have been used to improve biocontrol efficiency. Campbell (1989) has also cited that applying the correct dosage of BCA inoculum, controlling growth conditions, using appropriate formulations and storage and placing inoculum in favourable positions can facilitate active colonization of emerging roots by the antagonist. Induction of mutation in less rhizosphere competent strains of *Trichoderma* resulted in strains that are fungicide tolerant and better root colonizers compared to their wild parents (Ahmed and Baker, 1988). Such improvements can reduce the risk of rhizosphere colonization and enhance the biocontrol activity of antagonists.

1.4.2.4 Combination of BCAs and chemicals in an integrated disease management programme

Antagonistic microbes have been used in combination with chemical fungicides in a system of integrated plant disease management resulting in enhanced disease control. For example, Strashnow *et al.* (1985) and Whipps *et al.* (1988) showed that the application of *T. harzianum* with methyl bromide at 20-40% of the recommended dosage provided complete control of *R. solani*, which was only achieved by the the fumigant alone where the dosage was raised 100%. Similarly, Berger *et al.* (1996) using iprodione (Rovral), a fungicide effective against many soil-borne fungi (but not to *Phytophthora* and *Pythium)*, together with *B. subtilis,* control of a wide range of soil-borne diseases (including those caused by *Phytophthora* and *Pythium).* In sugar beet, *Bacillus* sp. applied with a low rate of azoxystrobin increased control of *R. solani* compared to applying these two treatments independently (Kiewnick *et al.*, 2001). These synergistic effects are most likely due to a combination of mechanisms that inhibited the pathogen (Kiewnick *et al.*, 2001).

Tronsmo and Hjeljord (1998) highlited three main advantages when combining fungicides and BCAs. Firstly, in integration of biological and chemical treatments can control the pathogen in climatic conditions beyond the effective range of the bio-protectant. Secondly, by replacing some chemicals with BCAs, environmental pollution is reduced and the likelyhood of the

pathogen developing resistance is lessened. Finally, the combination of a BCA and chemical provides localized and persistent control.

1.4.3 Application of biocontrol agents

Appropriate application of effective antagonist(s) to the proper ecological niche at the correct time is one of the major considerations for successful biocontrol of soil-borne pathogens (Mao *et al.*, 1997 and 1998). There are various means by which microbial antagonists are incorporated into the soil in order to provide effective control of seed and seedling blights caused by species of *Pythium* and *Rhizoctonia*.

Commonly, antagonists are applied either to infection courts on the plants where protection is needed most, or they are incorporated into the rooting medium at an inoculum density sufficient to ihibit the pathogen (Lewis and Papavizas, 1987; Kim *et al.*, 1997b).

Biocontrol agents can be directly introduced into the soil by distributing inoculum evenly throughout the soil, or by application of ioculum to the seed furrow at the time of sowing. Direct application of BCAs is used to destroy pathogen inoculum, prevent recolonization of treated soil by a pathogen and protect germinating seeds and seedlings from infection. Application of BCAs into field plots using this technique may not be economically feasible until the more distant future. But, in greenhouses it is currently practical (Cook and Baker, 1983).

Biocontrol agents can also be introduced into soil by means of seed treatment (Harman *et al.*, 1980). This is a more economical and often very effective method of application (Cook and Baker, 1983). Seed treatment with BCAs involves application of a biocontrol formulation to seed in a liquid or powder form. Using this technique, significant success has been recorded in field crops such as barley, beans, corn, radish, rice, soybean and wheat (Mao *et al.*, 1998). This is particularly true if the applied antagonists are able to proliferate and colonize germinating roots sufficiently (Mahaffee and Backman, 1993; Mao *et al.*, 1998). One method of seed treatment, i.e., seed coating, involves sticking a BCA to seed by soaking clean seed in

a mixture of microbial suspension and a sticker for a few minutes before drying the seed. This allows the antagonist to colonize the seed prior to the pathogenic fungi. For maximum efficiency, treated seeds are sown within five days after treatment.

Compared to introducing BCAs directly into the soil, seed treatment takes a smaller volume of inoculum to treat a large amount of seed and allows prior colonization of the emerging roots by the antagonist (Hadar *et al.*, 1984; Harman, 1991). Moreover, a farmer can utilize this system without changing his planting equipment and procedures (Tronsmo and Hjeljord, 1998).

1.4.4 Mechanisms of action of biocontrol agents

During the development of beneficial microbes for the control of soil-borne diseases, scientists recognized that an effective delivery system requires a thorough understanding of the biological relationship with its target (Bateman and Chapple, 2001).

There are at least four main mechanisms by which a BCA can operate against a target soil-borne pathogen: competition for resources, antibiosis, mycoparasitism and induction of host resistance (Campbell, 1989).

One mechanism used by BCAs to protect seeds and roots from pathogens is through competition for soluble substrates that contain carbon, nitrogen, oxygen, iron and other micronutrients (Mao *et al.*, 1997). Competition between soil microorganisms occurs when two (or more) organisms require the same resource (Tronsmo and Hjeljord, 1998) and the use by one reduces the amount available to the other (Campbell, 1989). In such cases, one organism uses most of the nutrients and grows, while the other has insufficient nutrients for its growth and dies. This is typical for a fungus or bacterium that grows very fast and overwhelms the target organism. Some bacteria can also produce low-molecular-weight compounds, called siderophores which are efficient at binding Fe^{3+} thereby making the iron inaccessible to other microorganisms, including pathogens (Fravel, 1988). The role of siderophores in biological control of diseases has been reviewed by Leong (1986).

Antibiosis occurs when the production of toxic metabolites or antibiotics of one organism has a direct negative effect on another organism. In pure culture, antibiotic production is common in many potential BCAs (Tronsmo and Hjeljord, 1998). It appears to be important to the survival of microorganisms through elimination of microbial competition for food sources, which are usually very limited in soil. Berger *et al.* (1996) showed that antibiotic production was a primary mode of action involved by *B. subtilis* in controlling species of *Pythium* and *Phytophthora*. Antibiotics produced by *Bacillus* spp. has been shown to inhibit root pathogens and their toxins, enabling plants to grow better (Cook and Baker, 1983). Many *Trichoderma* spp. are also known for their antibiotic production which is effective against a wide range of soil pathogens (Chet *et al.*, 1981). However, there is little evidence that production of such compounds is of major importance in disease control under field conditions (Tronsmo and Hjeljord, 1998).

Another mechanism utilized by BCAs is mycoparasitism. This is a parasitism of one fungus by another fungus. It involves direct contact between the fungi resulting in death of the host (plant pathogen), and nutrient absorption by the parasite (antagonist) (Whipps *et al.*, 1988). To break down the walls of their host, mycoparasites possess various enzymes such as: cellulases, chitinases, β-1,3-glucanases and proteases (Chet and Baker, 1980; Elad *et al.*, 1980; Campbell, 1989; Benhamou and Chet, 1993; Migheli *et al.*, 1998). The interaction between mycoparasites and their target fungi occurs in four sequential, but overlapping phases: target location, recognition, contact and penetration (Whipps *et al.*, 1988). In the first stage, a chemical stimulus from the pathogenic fungus attracts the parasite (the antagonist). The second step involves attack of the target pathogen by the mycoparasite with the help of enzymes. In the third step, the mycoparasitic antagonist is attached to the host (pathogenic fungi) either by coiling around or growing alongside it. In the final step, the mycoparasitic fungus degrades the pathogenic cell wall by producing various enzymes (Tronsmo and Hjeljord, 1998). Other BCAs may simply act by making the plant grow faster and escape infection, or they trigger the host defense mechanisms (Campbell, 1989).

Identifying the mechanism(s) of action by which a BCA acts in suppressing pathogens is a prerequisite for the rational utilization of any potential antagonist (Fravel, 1988). Antagonists

that inhibit the growth of a pathogen under laboratory conditions are selected as potential BCAs. A contradictory review by Deacon (1991) indicates that *in vitro* screening based on mode of action is a poor predictor of practical success because there is often little correlation between the ability of a microorganism to inhibit the growth of a pathogen in a Petri dish and its effectiveness in disease suppression in the field. However, most of the effective BCAs that are presently in the market have passed through *in vitro* screening.

1.4.5 Commonly used biocontrol agents

The following microbes are some of the most commonly used antagonists against many soil-borne fungi.

1.4.5.1 Trichoderma *species*

The genus *Trichoderma* is a fungus belonging to the class deuteromycetes (Benitez *et al.*, 1998). It occurs in all types of soil worldwide. Species of *Trichoderma* are known for their ability to stimulate growth of plants and biocontrol activity on a wide range of plant pathogens, including species of *Pythium*, *Phytophthora*, *Rhizoctonia* and *Sclerotium* (Chet and Baker, 1981; Elad and Hadar, 1981; Elad *et al.*, 1982; Gams and Meyer, 1998). This wide range of application is due to the various antagonistic mechanisms found in different *Trichoderma* isolates enabling them to function as potent BCAs on many different crops, against a wide range of pathogens and in several ecological situations (Tronsmo and Hjeljord, 1998).

Trichoderma spp. grow fast *in vitro* in a wide range of carbon sources. When introduced into a field, they have the ability to survive for more than 130 days even without food bases (Cook and Baker, 1983). Maximum growth of *Trichoderma* spp. is achieved in temperature ranges of 25-35°C (Whipps *et al.*, 1988) and pH ranges of 4-7 (Bruehl, 1987). The optimum pH level for germination of conidia in *T. harzianum* is between 4-5, and under acidic conditions, production of of β-1,3 glucanase and chitinase reaches a maximum. In *T. viride*, production of β-1,3-glucanase and chitinase is optimum at pH levels of 4.5 and 5.3, respectively (Bruehl,

1987). Some strains of *Trichoderma* have the ability to resist certain fungicides, which enables the use of integrated disease management to be successful.

Trichoderma harzianum, *T. virens*, *T. viride* and *T. hamatum* are the most common species of *Trichoderma* used against many soil-borne diseases (Campbell, 1989; Tronsmo and Hjeljord, 1998).

1.4.5.2 Bacillus *species*

The bacterial genus *Bacillus* belongs to the family *Bacillaceae* (Priest, 1993). It occurs in all types of soils. As a group, *Bacillus* species are easy to isolate and prepare and have a long shelf-life (Campbell, 1989). The ability of many *Bacillus* strains to produce endospores has solved the question of shelf-life and persistence in the soil. In addition, the ability of this genus to produce several antibiotics with a broad-spectrum activity has drawn the attention of many researchers. Many *Bacillus* strains are known to suppress a number of soil-borne pathogens by producing peptide antibiotics, which are assumed to be responsible for *in vivo* biocontrol of diseases (Leifert *et al.*, 1995). Furthermore, Yobo (2000) noted that *Bacillus* spp. produce a range of other metabolites such as biosurfactants, chitinase and other fungal cell wall-degrading enzymes, volatiles and compounds that trigger plant resistance mechanisms.

Bacillus has also been observed to increase grain yield of several crops grown under field conditions (Cook, 1985; Handelsman *et al.*, 1990; Zaki *et al.*, 1998). Compared to *Pseudomonas,* many *Bacillus* spp. are less efficient as root colonizers and are nutritionally less versatile (Campbell, 1989). Perhaps for this reason, more focus has been given to fluorescent *Pseudomonas* as a BCA for introduction into the rhizosphere (Kim *et al.*, 1997b). However, there are numerous reports showing rhizosphere colonization and root disease control with *Bacillus* introduced as seed inoculants for control of damping-off in several crops (Kim *et al.*, 1997a). For instance, seed treatment of wheat with *Bacillus* resulted in rhizosphere population of 10^5 c.f.u. g^{-1} of root tissue of inoculated bacteria after one month of inoculation (Juhnke *et al.*, 1987). These recent findings are drawing more attention toward *Bacillus* than

Pseudomonas. *Bacillus subtilis* and *B. pumulus* and *B. cereus* are the most studied species of *Bacillus*.

1.4.5.3 Other potential biocontrol agents for Pythium *and* Rhizoctonia

In addition to *Bacillus* and *Trichoderma*, seedling damping-off caused by *Pythium* and *Rhizoctonia* has been controlled by several beneficial bacteria and fungi, including, binucleate *Rhizoctonia* (Sumner *et al.*, 1992), *Gliocladium* spp., *Pseudomonas* spp., *Penicillium* spp., non-pathogenic *Pythium*, *Sclerotinia* spp. and *Streptomyces* spp. (Cook and Baker, 1983).

Among these microbes, *Gliocladium* and *Pseudomonas* are considered by most workers as good BCAs for most soil-borne pathogens. They are easy to isolate and grow in the laboratory and are nutritionally versatile (Cook and Baker, 1983; Campbell, 1989,).

1.4.6 Strategies for use of antagonists for biological control of plant diseases

The key for successful development and use of biocontrol strategy, as discussed earlier, is an understanding of the behaviour of the crop plant, the plant pathogen and the antagonist in a natural environment. There have been many failures in field experiments as a result of insufficient knowledge regarding these issues.

Knowledge of the crop: According to Cook and Baker (1983), the host plant plays a decisive role in the biological control of disease. It can cause fluctuation of temperature, water potential, pH, organic and inorganic nutrients and partial pressures of biologically important gases of the soil. This in turn affects the composition and density of microorganisms in the soil. For example, release of exudates from plant roots often results in rapid growth of several microbes, including pathogens, around the rhizosphere for limited periods of time followed by dominance with a few, but more rhizosphere competent microbes, usually antagonists. Resistance of host plants to attack by pathogens can also determine severity of disease and survival of pathogenic organisms and thereby efficiency of biological control. Previous studies indicate that performance of some BCAs is specific to certain crops or genotypes. Therefore

knowledge on the genotype, ecology and agronomy of the crop is very important for effective control plant diseases with BCAs.

Knowledge of the plant pathogen: A good understanding of the biology and behavior of the pathogen is necessary for effective biological control of the disease. Identifying the structure of the propagule through which it survives and infects the host plant, knowledge of the inoculum density responsible for disease initiation, location and distribution of the propagule in the soil and the time at which the pathogen infects the host are important points that dictate the dose, time and method of application of BCA into the soil (Ayers and Adams, 1981).

Knowledge of the antagonist: The biology and behavior of an antagonist in the soil or natural environment should be known. *In vitro* modes of action employed by antagonists against the target pathogen must be considered. The concentration of the antagonist that must be applied to bring a significant change in the population of the pathogen and disease severity should be precisely known. The time required for the BCA to destroy propagules of the pathogen is an important consideration that may determine when and how to apply it as a BCA (Ayers and Adams, 1981).

Effects of soil factors such as temperature, pH, moisture, texture and soil microflora on the survival, multiplication and aggressiveness of the pathogen should be determined. Similarly, effects of these factors should be investigated on the survival and activity of the antagonist if the efficiency of the beneficial microbe is to be assessed under various field conditions (Ayers and Adams, 1981).

Designing field tests: Potential antagonists must be evaluated for their activity under natural conditions before they are released to the market as effective BCAs. For successful field evaluation, the following points should be considered:

Ideally, a field should be naturally infested with sufficient inoculum of the pathogen to ensure a uniformly severe disease on the crop. Artificial inoculation of the field with a pathogen may not reflect the realistic infection process. However, natural infestation is more realistic for

biological control. Other important issues in designing field trial are size and orientation of plots. These depends on the nature of the crop plant, the cultivation practices that may be used, the duration of the field test and the number of plants that will be collected for sampling (Ayers and Adams, 1981).

1.5 REFERENCES

Agrios, G.N. 1997. Plant pathology. 4th ed. Academic Press, London, UK.

Ahmed, J.S. and Baker, R. 1988. Rhizosphere competence of benomyl-tolerant mutants of *Trichoderma* spp. Canadian Journal of Microbiology **34**, 694-696.

Anonymous. 1996. Lost crops of Africa: grains. Vol. 1. National Academy Press, Washington D.C., USA.

Ayers, A. and Adams, B. 1981. Mycoparasitism and its application to biological control of plant diseases. In: G.C. Papavizas (Ed.). Biological control in crop production. Allanheld, Osmum, USA. Pp. 91-105.

Bateman, A. and Chapple, A. 2001. The spray application of mycopesticide formulations. In: T.M. Butt, C.W. Jackson and N. Megan (Eds.). Fungi as biocontrol agents: progress, problems and potential. CABI, Wallingford, UK. Pp. 289-310.

Benhamou, N. and Chet, I. 1993. Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology **83**, 1062-1071.

Benitez, T., Limon, C., Delgando-Jarana, J. and Rey, M. 1998. Glucanolytic and other enzymes and their genes. In: G.E. Harman and C.P. Kubicek (Eds.). *Trichoderma* and *Gliocladium*: enzymes, biological control and commercial applications. Vol. 2. Taylor and Francis, London, UK. Pp. 101-127.

Berger, F., Li, H., White, D., Frazer, R. and Leifert, C. 1996. Effect of pathogen inoculum, antagonist density and plant species on biological control of *Phytophthora* and *Pythium* damping-off by *Bacillus subtilis* Cot1 in high-humidity fogging glasshouses. Phytopathology **86,** 428-433.

Bruehl, G. W. 1987. Soil-borne plant pathogens. MacMillan, New York, USA.

Butt, T.M., Jackson, C. and Magan, N. 2001. Fungal biological control agents: progress, problems, and potential. In: T.M. Butt, C.W. Jackson and N. Megan (Eds.). Fungi as biocontrol agents: progress, problems and potential. CABI, Wallingford, UK. Pp. 377-384.

Campbell, R. 1989. Biological control of microbial plant pathogens. Cambridge University Press, Cambridge, UK.

Chantereau, J. and Nicou, R. 1994. Sorghum: the tropical agriculturalist series. MacMillan, New York, USA.

Chao, W.L., Nelson, E.B., Harman, G.E. and Hoch, H.C. 1986. Colonization of the rhizosphere by biological control agents applied to seeds. Phytopathology **76**, 60-65.

Chet, I. and Baker, R. 1980. Induction of suppressiveness to *Rhizoctonia solani* in soil. Phytopathology **70**, 994-998.

Chet, I. and Baker, R. 1981. Isolation and biocontrol potential *Trichoderma hamatum* from soil naturally suppressive to *Rhizoctonia solani*. Phytopathology **71,** 286-29.

Chet, I., Harman, G.E. and Baker, R. 1981. *Trichoderma hamatum*: its hyphal interaction with *Rhizoctonia solani* and *Pythium* spp. Microbial Ecology **7**, 29-38.

Cook, R.J. 1985. Biological control of plant pathogens: theory to application. Phytopathology **75**, 25-29.

Cook, R.J. 2000. Advances in plant health management in the 20th century. Annual Review of Phytopathology **38,** 95-116.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens. APS Press, St Paul, Minnesota, USA.

Davis, M.A. and Bockus, W.W. 1996. Evidence for *Pythium* as a yield reducer under a continuous grain sorghum in Kansas. Plant Disease **86**, s55.

Davis, M.A. and Bockus, W.W. 2001. Evidence for *Pythium* as a yield reducer under a continuous grain sorghum. Plant Disease **85**, 780-784.

Deacon, J.W. 1991. Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Science and Technology **1,** 5-20.

Doggett, H. 1988. Sorghum. 2nd ed. Longman Scientific and Technical, New York, USA.

Elad, Y. and Hadar, Y. 1981. Biological control of *Rhizoctonia solani* by *Trichoderma harzianum* in carnation. Plant Disease **65,** 675-677.

Elad, Y., Chet, I. and Henis, Y. 1981. Biological control of *Rhizoctonia solani* in strawberry fields by *Trichoderma harzianum*. Plant and Soil **60,** 245-254.

Elad, Y., Chet, I. and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian Journal of Microbiology **28,** 719-725.

Elad, Y., Chet, I. and Katan, J. 1980. *Trichoderma harzianum*: a biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology **70,** 119-121.

Fageria, N.K., Baligar, V.C. and Jones, C.A. 1991. Growth and mineral nutrition of field crops. Marcel Dekker, New York, USA.

Finch, S. 1992. Appraisal of integrated pest management systems for controlling insects of vegetable crops. In: J.C. van Lateren, A.K. Minks and O.M.B. de Ponti (Eds.). Biological control and integrated crop protection: towards environmentally safer agriculture. Podac Scientific, Wageningen, The Netherlands. Pp. 49-60.

Forbes, G.A., Collins, D.C., Odvody, G.N. and Frederiksen, R.A. 1985. A seedling epiphytotic of sorghum in Texas caused by *Pythium arrhenomanes*. Plant Disease **69,** 726.

Forbes, G.A., Odvody, G.N. and Teri, J.M. 1986. Seedling diseases: In: R.A. Frederiksen (Ed.). Compendium of sorghum diseases. APS, New York, USA. Pp. 7-8.

Forbes, G.A., Ziv, O. and Frederiksen, R.A. 1987. Resistance in sorghum to seedling disease caused by *Pythium arrhenomanes*. Plant Disease **71,** 145-148.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annual Review of Phytopathology **26,** 75-91.

Gams, W. and Meyer, W. 1998. What exactly is *Trichoderma harzianum*? Mycologia **90,** 904-915.

Hadar, Y., Harman, G.E. and Taylor, A.G. 1984. Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soils for biological control of seed rot caused by *Pythium* spp. Phytopathology **74,** 106-110.

Handelsman, J., Raffel, S., Mester, E.H., Wunderlich, L. and Grau, C.R. 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Applied and Environmental Microbiology **56,** 713-718.

Harman, G.E. 1991. Seed treatment for biological control of root diseases. Crop Protection **10,** 795-798.

Harman, G.E., Chet, I. and Baker, R. 1980. *Trichoderma hamatum* effects on seed and seedling diseases induced in radish and peas by *Pythium spp.* and *Rhizoctonia solani*. Phytopathology **70,** 1167-1172.

Hendrix, F.F. and Campbell, W.A. 1973. *Pythium* as a plant pathogen. Annual Review of Phytopathology **11,** 77-98.

House, L.R., Gomez, M., Sun, Y., Murty, D.S. and Verma, B.N. 2000. Development of some agricultural industries in several African and Asian countries. In: C.W. Smith and R.A. Frederiksen (Eds.). Sorghum: origin, history and production. John Wiley and Sons, New York, USA. Pp. 131-190.

Howell, C.R. 1982. Effects of *Gliocladium* virens on *Pythium ultimum*, *Rhizoctonia solani* and damping-off of cotton seedlings. Phytopathology **72,** 496-498.

Howell, C.R. 2002. Cotton seedling pre-emergence damping-off incited by *Rhizopus oryzae* and *Pythium spp.* and its biological control with *Trichoderma spp.* Phytopathology **92,** 177-180.

Jarvie, J.A. 1994. The occurrence of resistance to *Rhizoctonia solani* and its inheritance in dry beans. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Jones, D.G. and Clifford, B.C. 1983. Cereal diseases their pathology and control. 2nd ed. John Wiley and Sons, Chichester, UK.

Jones, G. 1988. Endemic crop plants of Ethiopia: tef (*Eragrostis tef*). Wallya, Addis Ababa, Ethiopia.

Juhnke, M.E., Mathre, D.E. and Sands, D.C. 1987. Identification and characterization of rhizosphere-competent bacteria of wheat. Applied and Environmental Microbiology **53,** 2793-2799.

Jutsum, A.R. 1988. Commercial application of biological control: status and prospects. In: R.K.S. Wood and M.J. Way (Eds.). Biological control of pests, pathogens and weeds: developments and prospects. Vol. 318. Royal Society of London, UK. Pp. 357-370.

Kassier, S.B. 2001. Comparative response of fodder and grain tef (*Eragrostis tef* (Zucc.) Trotter) cultivars to spatial, temporal and nutritional management. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Kerry, B.R. 1992. The biological control of soil-borne pests and diseases. In: J.C. van Lateren, A.K. Minks and O.M.B. de Ponti (Eds.). Biological control and integrated crop protection: towards environmentally safer agriculture. Podac Scientific, Wageningen, The Netherlands. Pp. 117-124.

Ketema, S. 1993. Tef (*Eragrostis tef*): breeding, agronomy, genetic resources, utilization and role in Ethiopian agriculture. Institute of Agriculture Research. Addis Ababa, Ethiopia.

Kiewnick, S., Jacobsen, B.J., Braun-Kiewnick, A., Eckhoff, J.L.A. and Bergman, J.W. 2001. Integrated control of *Rhizoctonia* crown and root rot of sugar beet with fungicide and antagonistic bacteria. Plant Disease **85,** 718-722.

Kim, D.S., Cook, R.J. and Weller, D.M. 1997a. *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology **87,** 551-558.

Kim, D.S., Weller, D.M. and Cook, R.J 1997b. Population dynamics of *Bacillus* sp. L324-92R_{12} and *Pseudomonas fluorescens* 2-79RN_{10} in the rhizosphere of wheat. Phytopathology **87,** 599-564.

Kimber, C.T. 2000. Origin of domesticated sorghum and its early diffusion to India and China. In: C.W. Smith and R.A. Frederiksen (Eds.). Sorghum: origin, history and production. John Wiley and Sons, New York, USA. Pp. 3-98.

Landa, B.B., Navas-Cortes, J. A., Hervas, A. and Jimenez-Diazet, R.M. 2001. Influence of temperature and inoculum density of *Fussarium oxysporum* f. sp. *ciceris* on suppression of *Fusarium* wilt of chickpea by rhizosphere bacteria. Phytopathology **91,** 807-816.

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. and Harbour, A. 1995. Antiobiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Bacteriology **78,** 270-279.

Leong, J. 1986. Siderophores: their biochemistry and possible role in the biocontrol of plant pathogens. Annual Review of Phytopathology **24,** 187-209.

Lewis, J.A. and Lumsden, R.D. 2001. Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with formulation of *Trichoderma* spp. Crop Protection **20,** 49-56.

Lewis, J.A. and Papavizas, G.C. 1987. Application of *Trichoderma* and *Gliocladium* in alginate pellets for control of *Rhizoctonia* damping-off. Plant Pathology **36,** 438-446.

Lewis, J.A., Larkin, R.P. and Rogers, D.L. 1998. A formulation of *Trichoderma* and *Gliocladium* to reduce damping-off caused by *Rhizoctonia solani* and saprophytic growth of the pathogen in soilless mix. Plant Disease **82,** 501-506.

Mahaffee, W.F. and Backman, P.A. 1993. Effects of seed factors on spermatosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. Phytopathology **83,** 1120-1125.

Mao, W., Lewis, J.A., Hebbar, P.K. and Lumsden, R.D. 1997. Seed treatment with a fungal or bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium*. Plant Disease **81,** 450-454.

Mao, W., Lumsden, R.D., Lewis, J.A. and Hebbar, P.K. 1998. Seed treatment using pre-infiltration and biocontrol agents to reduce damping-off of corn caused by species of *Pythium* and *Fusarium*. Plant Disease **82,** 294-299.

Martin, H., Leonard, W.H. and Stamp, D. 1976. Principles of field crop production. 3rd ed. MacMillan, New York, USA.

Maunder, A.B. 2002. Sorghum worldwide. In: J.F. Leslie (Ed.). Sorghum and Millets diseases. Iowa State Press, Iowa, USA. Pp. 11-18.

McLaren, N.W. 1987. Studies on pre- and post-emergence damping-off and seedling blight of sorghum. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Metcalfe, D.S. and Elkins, D.M. 1980. Crop production: principles and practices. 4th ed. MacMillan, New York, USA.

Migheli, Q., Gonzalez-Kandelas, L., Dealessi, L., Camponogara, A. and Ramon-Vidal, D. 1998. Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4-endoglucanase gene *egl1* show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology **88,** 673-677.

Milus, E.A. and Rothrock, C.S. 1997. Efficacy of bacterial seed treatments for controlling *Pythium* root rot of winter wheat. Plant Disease **81,** 180-184.

Odvody, G.N. 1986. *Pythium* root rot. In: R.A. Frederiksen (Ed.). Compendium of sorghum diseases. APS, New York, USA. Pp. 32-33.

Omarjee, J. 2002. Studies on the application of biocontrol agents for the control of seedling diseases. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Pratt, R.G. and Janke, G.D. 1980. Pathogenicity of three species *Pythium* to seedlings and mature plants of grain sorghum. Phytopathology **70,** 766-771.

Priest, F.G. 1993. Systematics and ecology of *Bacillus*. In: A.L. Senenshein, J. Hoch and R. Losick (Eds.). *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology and molecular genetics. American Society for Microbiology, Washington, D.C., USA. Pp. 3-16.

Schippers, B. 1988. Biological control of pathogens with rhizobacteria. In: R.K.S. Wood and M.J. Way (Eds.). Biological control of pests, pathogens and weeds: developments and prospects. Vol. 318. Royal Society of London, UK. Pp. 283-290.

Sivan, A., Elad, Y. and Chet, I. 1984. Biological control effects of new isolate of *Trichoderma harzianum* on *Pythium aphanidermatum*. Phytopathology **74,** 498-501.

Skerman, P.J. and Riveros, F. 1990. Tropical grasses. FAO, Rome, Italy.

Stallknetch, G.F., Gilbertson K.M. and Eckhoff, J.L. 1991. Teff: food for humans and animals. In: J. Janick and J.E. Simon (Eds.). New crops. John Wiley and Sons, New York, USA. Pp. 231-234.

Strashnow, Y., Elad, Y., Sivan, A. and Chet, I. 1985. Integrated control of *Rhizoctonia solani* by methyl bromide and *Trichoderma harzianum*. Plant Pathology **34,** 146-151.

Sumner, D.R., Lewis, J.A. and Gitaitis, R.D. 1992. Chemical and biological control of *Rhizoctonia solani* AG-4 in snap bean double-cropped with corn. Crop Protection **11,** 121-126.

Taylor, J.R.N. and Belton, P.S. 2002. Sorghum. In: J.R.N. Taylor, and Belton P.S. (Eds.). Pseudocereals and less common cereals: grain properties and utilization potential. Springer-Verlag, Heidelberg, Germany. Pp. 25-91.

Tronsmo, A. and Hjeljord, L.G. 1998. Biological control with *Trichoderma* species. In: G.S. Boland and L.D. Kuykendall (Eds.). Plant-microbe interactions and biological control. Marcel Dekker, New York, USA. Pp. 111-126.

van der Plaats-Niterink, A.J. 1981. Monograph of the genus *Pythium*: studies in mycology. Vol. 21. Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Whipps, J.M. and Lumsden, R. 2001. Commercial use of fungi as plant disease biological control agent: status and prospects. In: T.M. Butt, C.W. Jackson and N. Megan (Eds.). Fungi as biocontrol agents: progress, problems and potential. CABI, Wallingford, UK. Pp. 9-22.

Whipps, J.M., Lewis, K. and Cook, R.C. 1988. Mycoparasitism and plant disease control. In: M.N. Burge (Ed.). Fungi in biological control systems. Manchester University Press, Manchester, UK. Pp. 161-187.

Yobo, K.S. 2000. Utilization of *Bacillus* spp. as plant probiotics. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Zaki, K., Misaghi, A. and Shatla, M.N. 1998. Control of cotton seedling damping-off in the field by *Burkholderia (Pseudomonas) cepacia*. Plant Disease **82,** 291-293.

CHAPTER 2

ISOLATION AND *IN VITRO* SCREENING OF *BACILLUS* SPP. AGAINST *RHIZOCTONIA SOLANI* AND *PYTHIUM* SP.

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Abstract

Bacillus colonies were investigated *in vitro* for their potential biocontrol activity against *Rhizoctonia solani* and *Pythium* sp. Bacterial colonies were isolated from soil samples using Tryptone Soy Agar. A simple, rapid assay was developed to screen bacteria for their ability to inhibit *in vitro* growth of *R. solani* and *Pythium* sp. Most isolates were more effective against *R. solani* than *Pythium* sp. Of all isolates tested, endospore forming *Bacillus* strains H44 and H51 gave higher antifungal activity against the two test-pathogens in three consecutive tests. Results suggest that both H44 and H51 have potential as biocontrol agents against diseases caused by these two pathogenic fungi.

2.1 INTRODUCTION

Control of soil-borne plant diseases by incorporating antagonistic bacteria and fungi into soil has been widely investigated over the last three decades. The most commonly used antagonists are the genus of *Bacillus* spp. (Weller, 1988; Turner and Backman, 1991; Kim *et al.*, 1997a and b). *Bacillus* spp. have been isolated from soil and tested *in vitro* and *in vivo* for their ability to control plant pathogens and enhance plant growth (Bron *et al.*, 1999). The ability of many *Bacillus* spp. to produce antibiotics with broad-spectrum activity has drawn the attention of numerous researchers (Zuber *et al.*, 1993). Recently, some of the more promising isolates of *Bacillus* spp. have been further developed and marketed to farmers as an alternative to traditional chemical-based fungicides (Mathre *et al.*, 1999; Paulitz and Belanger, 2001).

Under laboratory conditions, *Bacillus* spp. produce several peptide antibiotics (Ochi and Ohsawa, 1984) that kill or have a detrimental effect on several soil-borne plant pathogens (Handelsman *et al.*, 1990). Some of these antibiotic-producing strains have also been shown to suppress fungal plant diseases *in vivo* (Leifert *et al.*, 1995).

In addition to antibiotics, *Bacillus* spp. are also known for their production of chitinase and other cell wall degrading substances (Pelletier and Sygusch, 1990), volatiles (Fiddaman and Rossal, 1993 and 1994) and compounds that elicit plant resistance mechanisms (Leifert *et al.*, 1995). However, there has apparently been little correlation between *in vitro* production of such compounds and disease control under field conditions (Deacon, 1991). Nevertheless, results of recent investigations by Handelsman *et al.* (1990) and Georgakopoulos *et al.* (2002) confirm that antagonists capable of inhibiting *in vitro* growth of a pathogen are effective biocontrol agents in the soil. In addition, there is sufficient evidence, mostly obtained from genetic investigations, indicating that antibiotics are involved in disease control (Fravel, 1988).

According to Campbell (1989) and Georgakopoulos *et al.* (2002), any biocontrol agent intended to control soil-borne pathogens has to demonstrate adequate shelf-life, wide-spectrum activity and repeatable results. The ability of *Bacillus* spp. to resist adverse environmental conditions and produce antibiotics has been attributed to the ability of the organism to sporulate (Sneath, 1986; Priest, 1993; Bron *et al.*, 1999).

Less than 10% of the total population of bacteria in the rhizosphere have been found to have the ability to provide biological control against soil-borne diseases (Weller, 1988). Based on this assumption, Weller (1988) suggested that the chance of selecting effective strains may be improved by first selecting bacteria from the environment in which they will be used, e.g., selecting from a maize (*Zea mays*) rhizosphere if the target pathogen causes a root disease of maize. The objective of this investigation was to isolate *Bacillus* from soil where sorghum (*Sorghum bicolor*) and tef (*Eragrostis tef*) were grown for more than two years and screen spore-forming *Bacillus* isolates antagonistic to the pathogens, *R. solani* and *Pythium* sp., responsible for damping-off and seedling diseases of many plants, including sorghum and tef.

2.2 MATERIALS AND METHODS

2.2.1 Isolation of *Bacillus* spp.

Soil samples were collected from Cedara, KwaZulu-Natal, South Africa, on lands where sorghum and tef had been grown for more than two years. Soil samples were heat-treated at 80°C for 10min on a water-bath so that endospores could be separated from vegetative cells (Foldes *et al.*, 2000). The heat-treated soil suspension was then diluted serially, plated onto Tryptone Soy Agar (TSA) and incubated overnight at 30°C. Colonies that resembled *Bacillus* spp. were identified using a Gram-reaction, streaked onto Potato Dextrose Agar (PDA) and incubated for two days at 30°C.

2.2.2 Pathogenic fungi

Pure cultures of *R. solani* and *Pythium* sp. were obtained from C. Clark[1] and maintained in McCarthy bottles containing V8 agar slants.

2.2.3 Dual culture test

To test the efficacy of the *Bacillus* isolates in inhibiting hyphal growth of *R. solani* and *Pythium* sp., dual culture tests used by Landa *et al.* (2001), were prepared on PDA. Briefly, four isolates of *Bacillus* were spotted with a sterile loop 10mm from the edge of a Petri plate of PDA and incubated at 30°C. After 24h of incubation, an 8mm-diameter agar plug, taken from the leading edge of a five day old pathogen culture, was placed in the centre of the plate. Control treatments were prepared by plating the pathogen only. After four days of incubation at 30°C, the width of the inhibition zone between each bacterial and fungal colony and the length of the hyphal growth toward the bacteria and that of the control were measured.

Antifungal activity of each *Bacillus* isolate on the two fungi were scored numerically with a scale ranging from 0 to 4, where 0 = no inhibition (the fungal mycelium overgrew the bacterial

[1] C. Clark, Discipline of Plant Pathology, School of Applied Environmental Sciences, University of Natal, Private Bag X01, Scottsville, 3209, South Africa

colony); 1 = poor inhibition (fungal mycelium does not touch the bacterial colony); 2 = moderate inhibition (formation of inhibition zone of 1-2mm); 3 = strong inhibition (inhibition zone of 2-5mm); and 4 = very strong inhibition (inhibition zone of >5mm). Each combination of the antagonists and pathogen and that of the controls were replicated on four plates. The trial was repeated once. Isolates that showed biocontrol activity in the two trials against both pathogens were spore stained to check for the presence of endospores before they were tested a third time. Isolates that consistently yielded the best results were selected for further studies.

2.3 RESULTS

Numerous bacterial colonies were obtained from soil samples collected from sorghum and tef rhizospheres. A total of 80 Gram-positive colonies were selected for *in vitro* antibiosis. Isolates were screened in in vitro bioassay (Fig. 2.1, Plates A and B) for their ability to inhibit growth of *Pythium* sp and *R. solani*. When the two fungi were grown on PDA separately, they covered the entire plate within two days of incubation. In contrast, growth of all isolates of *Bacillus* tested was small in diameter when grown on the same medium (<15mm).

Interaction between the hyphae of the pathogenic fungi and *Bacillus* isolates was evident by the second day of incubation, when the two organisms grew toward each other. After the second day of incubation, an ambiguous zone of inhibition was formed around the *Bacillus* colony. As incubation continued, the inhibition zone became more and more pronounced and as a result no visible hyphal growth toward the bacterial colony was observed (Fig. 2.1, Plates A and B). On the fourth day of incubation, the average inhibition zone ranged from 0-15mm in diameter and colony growth was 1-23mm (Table 2.1).

Of the 80 *Bacillus* isolates tested for nhibition of *R. solani* growth, 24 isolates showed antibiotic activity. Only three of them could stop growth of the pathogen without any inhibition zone, nine isolates produced small inhibition zones of 1-2mm, and 12 isolates inhibited the fungus, creating inhibition zones of 3mm to > 5mm. However, only 13 *Bacillus* isolates showed antagonistic activity against *Pythium* sp. Of these, six inhibited growth of the pathogenic fungus beyond the contact area; two isolates provided slight activity with

inhibition zones of 1-2mm; and four isolates showed strong antifungal activity. Overall, nine isolates showed antibiotic activity against both pathogens ranging from partial to strong antifungal activities (Table 2.1). In three consecutive tests, H44 and H51 provided consistently greater control against *Pythium* sp. and *R. solani* with inhibition zones of 5-12mm. Antifungal activities of these two isolates on both fungi, is illustrated in Fig. 2.1, Plates C and D.

Table 2.1 *In vitro* bioassay rating of selected isolates of *Bacillus* on *Rhizoctonia solani* and *Pythium* sp. rated four days after incubation at 30°C

***Bacillus* strain**	**Antifungal rating**	
	R. solani	***Pythium* sp.**
H22	3^{C}	2^{C}
H28	2^{NC}	1^{NC}
H33	1^{NC}	2^{NC}
H44	4^{C}	4^{C}
H51	4^{C}	4^{C}
H62	3^{NC}	2^{NC}
H77	2^{NC}	1^{NC}
H125	4^{NC}	2^{NC}
H127	4^{NC}	3^{NC}

Key: 1 = Poor inhibition (fungal mycelium does not overgrow the bacterial colony)

2 = Moderate inhibition (zone of inhibition between 1 and 2mm)

3 = strong inhibition (zone of inhibition between 2 and 5mm)

4 = Very strong inhibition (zone of inhibition >5mm)

C = Consistency of results in three consecutive tests

NC = Non-consistency of results in three consecutive tests.

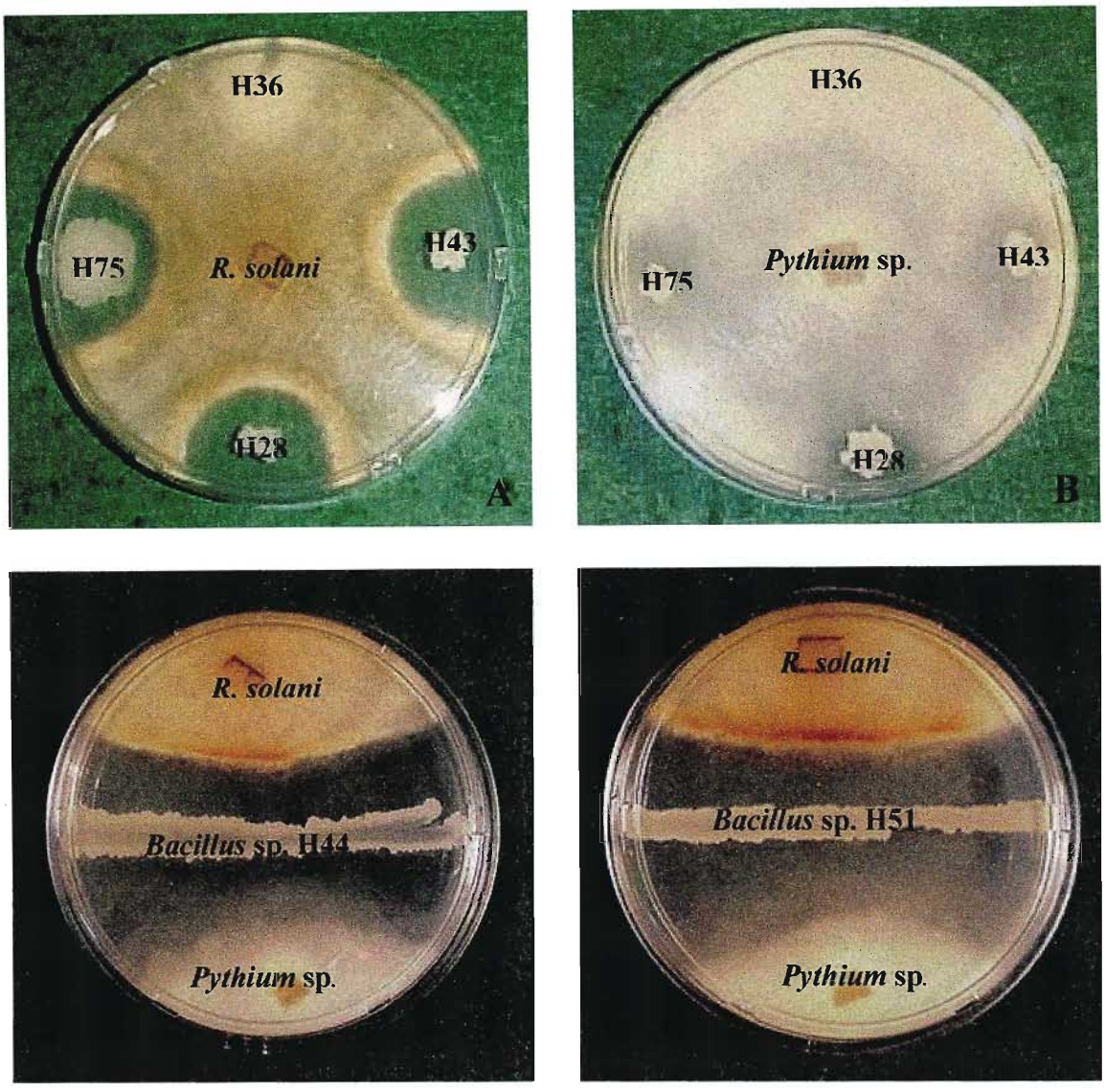

Fig. 2.1 Plates showing antibiotic activities of some isolates of *Bacillus* (H28, H36, H43 and H75) against *Rhizoctonia solani* and a *Pythium* sp. after incubation at 30°C for four days. Plates A and B are examples of the *in vitro* bioassay tests used in screening *Bacillus* isolates for their antifungal activities against *R. solani* and *Pythium* sp., respectively. Note that *R. solani* was more sensitive than *Pythium* sp. toward the same *Bacillus* isolates. Plates C and D demonstrate potency of H44 and H51 in controlling *in vitro* growth of *R. solani* and *Pythium* sp., respectively.

2.4 DISCUSSION

The development of a proper isolation and *in vitro* screening protocol that provides rapid, repeatable and reliable results is an important initial step in screening efficient bacterial antagonists for biocontrol of plant diseases. The success of all subsequent stages depends on the ability of the initial screening procedure to identify an appropriate candidate (Anith *et al.*, 2003).

In the present study, *in vitro* growth of a *Pythium* sp. and *R. solani* was inhibited by some isolates of *Bacillus*. Formation of a clear inhibition zone between the pathogenic fungi and bacterial colonies by Day 4, indicated strong antibiotic activity of the bacteria. This probably resulted when bacterial colony released inhibitory metabolites, possibly peptide antibiotics (Ochi and Ohsawa, 1984), which impair hyphal growth.

The degree of inhibition formed between the pathogens and their antagonists varied depending on the isolate and the target fungus. Some isolates showed greater inhibition against *R. solani* than the *Pythium* sp. and *viceversa*. In general, *R. solani* (Fig 2.1 Plate A) was more susceptible to *Bacillus* isolates than *Pythium* sp. (Fig. 2.1, Plate B). This agrees with findings by Cook and Baker (1983) that *R. solani* is more sensitive to attack by several antagonists than most soil-borne pathogenic fungi. Similar discoveries were recorded by Kim *et al.* (1997a) when they tested *Bacillus* sp. L324-92 against *R. solani* and *Pythium* sp.

Differences in susceptibility among the two pathogenic fungi toward antagonistic *Bacillus* isolates may be due to the differences in their cell wall composition. The cell wall components of *R. solani* are made up of 1,3-β-glucan and chitin (Bartnicki-Garcia, 1968; Papavizas, 1985) and that of *Pythium* are 1,3-β-glucan and/or 1,6-β-glucan covered by fibrillar cellulose (Thrane *et al.*, 1997). Each cell wall component can only be degraded by a specific extracellular enzyme. Priest (1989) pointed out that several enzymes such as glucanase, chitinase and cellulase are produced by certain strains of *Bacillus*. These enzymes have long been known to dissociate glucan, chitin and cellulose which are the main components of cell

walls of many soil-borne fungi (Bartnicki-Garcia, 1968). One or more of this group of these enzymes maight have been released by the *Bacillus* isolates tested in this trial.

Investigations confirm that synthesis of several antibiotics is initiated after the antagonist has passed the rapid growth phase (Priest, 1993) or when it is under conditions of nutritional stress (Bron *et al.*, 1999). In this test, antibiotic production by *Bacillus* isolates was triggered when they were incubated at 30°C for 24h before the test fungi (Foldes *et al.*, 2000). It is believed that the possible function of antibiotics is to kill or inhibit growth of other microorganisms in nature thereby providing competitive advantages to the producing microbe (Priest, 1993; Zuber *et al.*, 1993).

After keeping the plates in the same environment for more than one week, formation of dark rings on the edges of the *R. solani* cultures were noticed together with further shrinkage of the mycelial mat of *Pythium* sp. This indicates the potency of some isolates in controlling growth of these two fungi even after an extended time. This was true for most isolates that showed strong antibiotic activity. However, isolates with less activity were overgrown, especially by *Pythium* sp. This could be attributed to the nature of the specific antibiotic. Certain *Bacillus* strains can produce volatile antibiotics such as ammonia that can initially inhibit growth of *Pythium* sp. and *R. solani* but wares over time (Fiddaman and Rossal, 1993). This might be the possible reason why in the present study some of the isolates used were overgrown by the fungi after showing some degree of antibiosis at the beginning of the bioassay tests. In contrast to these findings, Georgakopoulos *et al.* (2002) reported volatile antibiotics are seldom detected and hence, screening based on the dual culture method was not recommended.

The potency of *Bacillus* strains to produce peptide antibiotics and resist adverse environmental conditions is directly related to their ability to sporulate (Ochi and Ohsawa, 1984; Sneath, 1986). Screening of *Bacillus* has focused on endospore-formers. Kim *et al.* (1997a) recovered the endospore-forming *Bacillus* sp. L324-92 by treating macerated roots at 80°C for 30 min. This isolate was considered to be one of the most promising strains of *Bacillus* developed for disease control and growth stimulation (Kim *et al.*, 1997b; Mathre *et al.*, 1999).

Spores in a natural environment may possess different heat sensitivities from those which have been cultured following such treatment (Sneath, 1986). It is clear that an isolate treated for longer periods of time is more likely to loose its character than those treated for shorter periods. Hence, checking the presence of spores before or after passing the dual culture test using spore-staining techniques appears to be safer in preserving the biochemical characteristics of the antagonistic. In this study, besides treating soil at 80°C for 10 min, no attempt was made to screen spore-formers before the dual culture test. However, all strains were spore-formers, indicating that exposure to heat for 10min is enough to eliminate vegetative bacteria (Foldes *et al.*, 2000).

Bacillus strains H44 and H51, that inhibited *in vitro* growth of *R. solani* and *Pythium* sp. in three subsequent tests were maintained as the best isolates. In all antagonistic interactions between these two organisms, antibiosis seems to be the mechanism by which isolates of *Bacillus* inhibited *in vitro* growth of the two pathogenic fungi. Results of this trial suggest that *Bacillus* strains H44 and H51 produced either broad-spectrum antifungal compounds, or several compounds with different activities.

The screening protocol followed in the present study fulfills several important requirements. Firstly, the biocontrol agents screened could inhibit *in vitro* growth of the two target pathogenic fungi. Secondly, these potential agents are endospore-formers, which enable them to survive adverse environmental conditions and storage for extended period of time. Thirdly, isolates that were maintained as potential antagonists were the ones that showed reproducible results. These three points partially answer the questions of spectrum activity, shelf-life and reproducibility of results. Finally, the isolation and screening techniques followed, permitted the screening of many potential biocontrol agents within a short time.

In this trial, the potential of certain isolates in inhibiting *in vitro* growth of two groups of fungi, i.e., oomycetes (*Pythium* sp.) and basidiomycetes (*R. solani*) was demonstrated. Further work is needed to extend the range of fungi tested and investigate the nature of antibiotics produced by antagonistic isolates. In addition, since no relationship is said to exists between the ability of an antagonist to inhibit a pathogen *in vitro* and suppress disease *in vivo* (Deacon,

1991; Leifert *et al.*, 1995), strains producing largest zones of inhibition on plates may not necessarily be the best biocontrol agents. Hence, greenhouse and field trials must be conducted to determine the efficiency of these isolates in controlling diseases caused by the fungi tested in the present study.

2.5 REFERENCES

Anith, K.N., Radhakrishnan, N.V. and Manomohandas, T.P. 2003. Screening of antagonistic bacteria for biological control of nursery wilt of black pepper (Piper nigrum). Microbiological Research 158, 91-97.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Annual Review of Microbiology 22, 87-108.

Bron, S., Meima, R., van Dijl, J.M., Wilpat, A. and Harwood, C.R. 1999. Molecular biology and genetics of Bacillus spp. In: A.L. Demain, J.E. Davis and R.M. Atlas (Eds.). Manual of industrial microbiology and biotechnology. 2nd ed. American Society of Microbiology, Washington D.C., USA. Pp. 392-416.

Campbell, R. 1989. Biological control of microbial plant pathogens. Cambridge University Press, Cambridge, UK.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens. APS Press, St Paul, Minnesota, USA.

Deacon, J.W. 1991. Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Science and Technology 1, 5-20.

Fiddaman, P.J. and Rossal, S. 1993. The production of antifungal volatiles by Bacillus subtilis. Journal of Applied Bacteriology 74, 119-126.

Fiddaman, P.J. and Rossal, S. 1994. Effect of substrate on the production of antifungal volatiles by Bacillus subtilis. Journal of Applied Bacteriology 76, 395-405.

Foldes, T., Banhegyi, I., Herpai, Z., Varga, L. and Szigeti, J. 2000. Isolation of Bacillus strains from rhizosphere of cereals and in vitro screening for antagonism against phytopathogenic, food-borne pathogenic and spoilage microorganisms. Journal of Applied Microbiology 88, 840-846.

Fravel, D.R. 1988. Role of antibiosis in biocontrol of plant diseases. Annual Review of Phytopathology **26,** 75-91.

Georgakopoulos, D.G., Fiddaman, P., Leifert, C. and Malathrakis, N.E. 2002. Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists. Journal of Applied Microbiology **92,** 1078-1086.

Handelsman, J., Raffel, S., Mester, E.H., Wunderlich, L. and Grau, C.R. 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Applied and Environmental Microbiology **56,** 713-718.

Kim, D.S., Cook, R.J. and Weller, D.M. 1997a. *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology **87,** 551-558.

Kim, D.S., Weller, D.M. and Cook, R.J. 1997b. Population dynamics of *Bacillus* sp. L324-92R_{12} and *Pseudomonas fluorescens* 2-79RN_{10} in the rhizosphere of wheat. Phytopathology **87,** 599-564.

Landa, B.B., Navas-Cortes, J.A., Hervas, A. and Jimenez-Diazet, R.M. 2001. Influence of temperature and inoculum density of *Fusarium oxysporum* f. sp. *ciceris* on suppression of *Fusarium* wilt of chickpea by rhizosphere bacteria. Phytopathology **91,** 807-816.

Leifert, C., Li, H., Chidburee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. and Harbour, A. 1995. Antiobiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology **78,** 97-108.

Mathre, D.E., Cook, R.J. and Callan, N.W. 1999. From discovery to use: traversing the world of commercializing biocontrol agents for plant disease control. Plant Disease **83,** 979-983.

Ochi, K. and Ohsawa, S. 1984. Isolation of antibiotic production by the stringent response of *Bacillus subtilis* Marburg. Journal of General Microbiology **130,** 2473-2482.

Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. Annual Review of Phytopathology **23,** 23-54.

Paulitz, T. and Belanger, R.R. 2001. Biological control in greenhouse systems. Annual Review of Phytopathology **39,** 103-133.

Pelletier, A. and Sygusch, J. 1990. Purification and characterization of three chitinase activities from *Bacillus megaterium* P1. Applied and Environmental Microbiology **56,** 844-848.

Priest, F.G. 1989. Products and applications. In: Harwood, C.R. (Ed.). *Bacillus*. Plenum Press, New York, USA. Pp. 293-315.

Priest, F.G. 1993. Systematics and ecology of *Bacillus*. In: A.L. Senenshein, J. Hoch and R. Losick (Eds.). *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology and molecular genetics. American Society for Microbiology, Washington, D.C., USA. Pp. 3-16.

Sneath, P.H.A. 1986. Endospore forming Gram-positive rod and cocci. In: P.H.A. Sneath, N.S. Mair, M.E Sharpe and J.G. Holt (Eds.). Bergey's manual of systematic bacteriology. Vol. 2. Williams and Welkins, Baltimore, USA. Pp. 1104-1115.

Thrane, C., Tronsmo, A. and Jensen, D.F. 1997. Endo-1,3-β-glucanases and cellulases from *Trichoderma harzianum*: purification and partial characterization, induction of biological activity against plant pathogenic *Pythium* spp. European Journal of Plant Pathology **103,** 331-344.

Turner, J. and Backman, P.A. 1991. Factors relating to peanut increase after seed treatment with *Bacillus subtilis*. Plant Disease **75,** 437-353.

Weller, D.M. 1988. Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology **26,** 379-407.

Zuber, P., Nakano, M.M. and Marahiel, M.A. 1993. Peptide antibiotics. In: A.L. Senenshein, J.A. Hoch and R. Losick (Eds.). *Bacillus subtilis* and other Gram-positive bacteria: biochemistry, physiology and molecular genetics. American Society for Microbiology, Washington, D.C., USA. Pp. 897-916.

CHAPTER 3

IN VITRO AND ULTRASTRUCTURE OF HYPHAL INTERACTIONS BETWEEN *TRICHODERMA* SPP. AND *PYTHIUM* SP.

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Abstract

The modes of hyphal interaction between three isolates of *Trichoderma* (*T. harzianum* Eco-T, *Trichoderma* sp. SY3 and *Trichoderma* sp. SY4) and *Pythium* sp. were investigated using *in vitro* bioassays and environmental scanning electron microscopy (ESEM). Visual observation on the dual culture test revealed that growth inhibition of the pathogen by these antagonists started soon after hyphal contact within 3-4 days of incubation. By means of ESEM all three isolates of *Trichoderma* were shown to grow toward the pathogen, attach to the host's cell wall with hook-like structures, coil around the hyphae of the pathogen, perforate the surface of the pathogen and penetrate the hyphae of the host, leading to collapse and disintegration of the host's cell wall. Degradation of the host's cell wall was attributed to the production of lytic enzymes. Based on these observations, antibiosis (only by Eco-T) and mycoparasitism (by all three isolates) were the mechanisms of action by which *in vitro* growth of *Pythium* sp. was suppressed by the three *Trichoderma* isolates.

3.1 INTRODUCTION

Trichoderma has been documented as a potential biological control agent against several soil-borne fungi and has drawn much attention in the last two decades (Cook and Baker, 1983; Gams and Meyer, 1998). Understanding the mechanism(s) of action underlying the antifungal activity is a prerequisite for rational utilization of any potential antagonist (Fravel, 1988). Several *in vitro* investigations have revealed that growth of *Pythium* (Benhamou and Chet,

1997; Thrane *et al.*, 1997) and *Rhizoctonia* (Chet *et al.*, 1981; Elad *et al.*, 1982) was inhibited by certain species of *Trichoderma*. However, the mechanism of action involved by antagonistic *Trichoderma* spp. in controlling these two fungal pathogens was not fully known.

Biocontrol activity of *Trichoderma* may involve competition (Sivan and Chet, 1989; Howell, 2002), antibiosis (Ghisalberti and Sivasinthamparan, 1991), mycoparasitism (Ayers and Adams, 1981; Whipps *et al.*, 1988) and the induction of plant resistance (Inbar *et al.*, 1994). These modes of action can operate separately or in combination with each other. Competition between soil microorganisms occurs when two (or more) organisms utilize the same resource(s) (Tronsmo and Hjeljord, 1998). The use of the resources by one microorganism reduces its availability to the other (Campbell, 1989). In pure culture, many *Trichoderma* spp. produce diffusible inhibitors such as antibiotics and mycotoxins (Benhamou and Chet, 1993) and volatile inhibitors (Fravel, 1988) that are effective against a wide range of soil-borne pathogens (Chet *et al.*, 1981). Mycoparasitism, a process that involves several successive and overlapping steps, may also play an important role in the antagonistic nature of *Trichoderma*. During the process of parasitism, *Trichoderma* has been shown to grow toward the pathogen, attach itself to the host cell wall, coil around the hyphae of the pathogen and then excrete several extracellular enzymes (e.g., cellulases, chitinases, β-1,3-glucanases and proteases) that degrade the host cell wall (Chet and Baker, 1980; Whipps *et al.*, 1988; Campbell, 1989; Migheli *et al.*, 1998). Finally, the antagonist may penetrate the pathogen (Elad *et al.*, 1982).

Recently, dual culture tests and the ultrastructure of hyphal interactions between *T. harzianum* Eco-T (previously named as *T. harzianum* KMD) (Omarjee, 2002), *Trichoderma* spp. SY3 and SY4 (K.S. Yobo[1], personal communication) and *R. solani* were studied. *In vitro* and scanning electron microscopy (SEM) observations of these three *Trichoderma* isolates revealed that mycoparasitism was the main mode of action involved in controlling *R. solani*. However, none of these investigators dealt with the biocontrol mechanisms that may be involved in controlling *Pythium* sp. The objective of this study was therefore, to determine the mechanism(s) of action involved by these *Trichoderma* isolates on *in vitro* suppression of

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Pythium sp. growth using *in vitro* bioassays together with environmental scanning electron microscopy (ESEM).

3.2 MATERIALS AND METHODS

3.2.1 Source of fungal isolates

Pre-selected isolates of *T. harzianum* Eco-T and *Trichoderma* spp. SY3 and SY4 were supplied by Plant Health Products[2] in formulation. A culture of *Pythium* sp. was obtained from C. Clark[3]. Originally Eco-T was isolated from soil obtained from Tala Valley, KwaZulu-Natal, South Africa (Omarjee, 2002) and SY3 and SY4 were isolated from compost soil (K.S. Yobo, personal communication). All *Trichoderma* isolates were cultured by spreading them directly onto V8 agar. Cultures were stored in agar slants and subcultured on V8 plates when needed.

3.2.2 Dual culture tests

To study hyphal interactions between *Trichoderma* spp. and *Pythium* sp., dual culture tests used by Bell *et al.* (1982) were followed *in vitro*. Mycelial agar plugs (8mm in diameter, cut from the leading edge of a five day old mycelial mat on V8 agar) of three *Trichoderma* spp. and a *Pythium* sp. were placed on opposite sides of Petri dishes of V8 medium. Control treatments were similarly prepared by placing the same size of the antagonists and the pathogen in separate Petri dishes. Four replications were prepared for each isolate and incubated for 10 days on a laboratory bench at a temperature of 25-26°C under a constant "daylight" fluorescent light. Visual observations on the hyphal interactions were conducted daily. After one week of incubation, mycelial plugs (3mm in diameter) were taken from zones of interaction on the agar plates and processed for ESEM investigations.

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3.2.3 Scanning electron microscopy studies

Mycelial plugs (3mm in diameter) were cut from the interaction zone of *Trichoderma* and *Pythium* and fixed overnight in 3% glutaraldehyde in cacodalate buffer (0.1M; pH7). Samples were then dehydrated in a graded alcohol series. Specimens were critical point dried with carbon dioxide as a transfusion fluid in a Hitachi HCP-2. Dried specimens were mounted on copper stubs using double-sided carbon tape. All stubs were then coated with gold-palladium in a Polaron E500 Sputter Coater and viewed under Philips XL30 environmental scanning electron microscope (ESEM) operating at 10 kV.

3.3 RESULTS

3.3.1 Fungal growth and interaction in dual cultures

Growth of fungi began on the second day of incubation. When the *Pythium* sp. and *Trichoderma* spp. were cultured on V-8 agar separately, *Pythium* grew as a fluffy white mycelium, while the three isolates of *Trichoderma* produced green mycelia with copious production of spores. The *Pythium* sp. and SY4 grew fast, and covered the entire plate within three days of incubation. In contrast, Eco-T and SY3 grew slowly, requiring 5-6 days of incubation to cover the same area on the plate. In dual culture tests, contact between hyphae of the antagonists and the pathogenic fungus started on the third day of incubation. On their first day of contact, the percentage of growing medium colonized by the *Pythium* sp. was based on the growth rate of the antagonist involved. In plates containing Eco-T, the pathogen covered about 70% of the plate (Fig. 3.1, Plate A). On plates overgrown by SY3 and SY4, *Pythium* sp. colonized only 65% (Fig. 3.1, Plate C) and 50 % (Fig. 3.1, Plate E) of the plate, respectively. In the interaction between SY3 and SY4 and *Pythium* sp., hyphae of the pathogen started to thicken at the point of contact and its further growth was inhibited (Fig. 3.1, Plates C and E). In the subsequent few days of contact, the mycelia of the pathogen was overgrown by these two *Trichoderma* isolates which produced spores on the mycelium of the pathogen. At 9-10 days after inoculation, the fluffy growth of *Pythium* sp. started to shrink and remain as a thin layer, entirely overgrown by SY3 and SY4, confined to the surface of the agar showing that the pathogen had lost its turgor and was collapsing (Fig. 3.1, Plates D and F). In the course of

this interaction, there was no formation of an inhibition zone between these two antagonists and the host. However, when Eco-T was plated against *Pythium* sp., a small inhibition zone was formed between the antagonist and the host as they grew toward each other on the third day of inoculation (Fig. 3.1, Plate A). Thereafter, Eco-T continued proliferating forward, lysing the hyphae of *Pythium* sp. and widening the inhibition zone. At 9-10 days of incubation, the area covered by *Pythium* sp. was reduced to less than 25% (Fig. 3.1, Plate B). Although the mechanism was mainly antibiosis, a few spores of Eco-T were visible on the dying hyphae of *Pythium* sp. (Fig. 3.1, Plate B).

3.3.2 ESEM observations on hyphal interactions

Samples from the interaction region of dual culture tests of *Pythium* sp. and three isolates of *Trichoderma* were investigated using ESEM (Fig. 3.2, Plates 1-8). The hyphal diameters of pure cultures of the two groups were used as distinguishing features. Generally, *Pythium* sp. was wider in diameter than the three isolates of *Trichoderma* used in this study. The three *Trichoderma* isolates produced lateral loop-like branches that were used for attachment, coiling around and penetration of the host cell wall. Among the three isolates, Eco-T and SY3 had slow growing mycelia accompanied by excessive production of spores. In contrast, SY4 had actively growing mycelia with fewer spores.

Microscopic observations revealed that different but overlapping events were characterstic in the three antagonists in controlling *Pythium*. Recognition of the host (*Pythium* sp.) as a result of a chemical stimulus released by the host (Fig. 3.2, Plate 4), attachment (Fig. 3.2, Plates 1, 4, 5 and 7), coiling around the hyphae of the pathogen (Fig. 3.2, Plates 4, 5 and 7), penetration (Fig. 3.2, Plates 1, 2 and 5) and degradation of the host cell wall due to release of lytic enzymes by the antagonists (Fig. 3.2, Plates 6 and 8) were observed for all three isolates. In addition, loss of turgor pressure of the hyphae of *Pythium* sp. without contact with *Trichoderma* was observed for Eco-T (Fig. 3.2, Plate 3).

Fig. 3.1 Dual culture tests on hyphal interactions between *Trichoderma* spp. and *Pythium* sp. at four days (left column) and nine days (right column) of incubation.

Plate A: Early stages of hyphal interaction showing formation of inhibition zone (I) between *T. harzianum* (Eco-T) and *Pythium* sp.

Plate B: Advanced stage, *T. harzianum* (Eco-T) advances its coverage and parasitizes the mycelia of *Pythium* sp. (M), with an increased inhibition zone (I).

Plate C: Early stage of hyphal interaction between *Trichoderma* sp. SY3 and *Pythium* sp. Hyphal growth of *Pythium* sp. discontinues, thickens at the intersection zone, SY3 starts to overgrow the pathogen showing early signs of mycoparasitism (M).

Plate D: Advanced mycoparasitism (M), *Trichoderma* sp. SY3 overgrowing *Pythium* sp. and dominating the entire plate.

Plate E: Early stage of interaction between *Trichoderma* sp. SY4 and *Pythium*. Hyphae of *Pythium* receed at the intersection zone and discontinue growth while SY4 starts to overgrow the pathogen (M).

Plate F: Advanced stage of mycoparasitism (M), *Trichoderma* sp. SY4 overgrows *Pythium* sp. and dominates the entire plate.

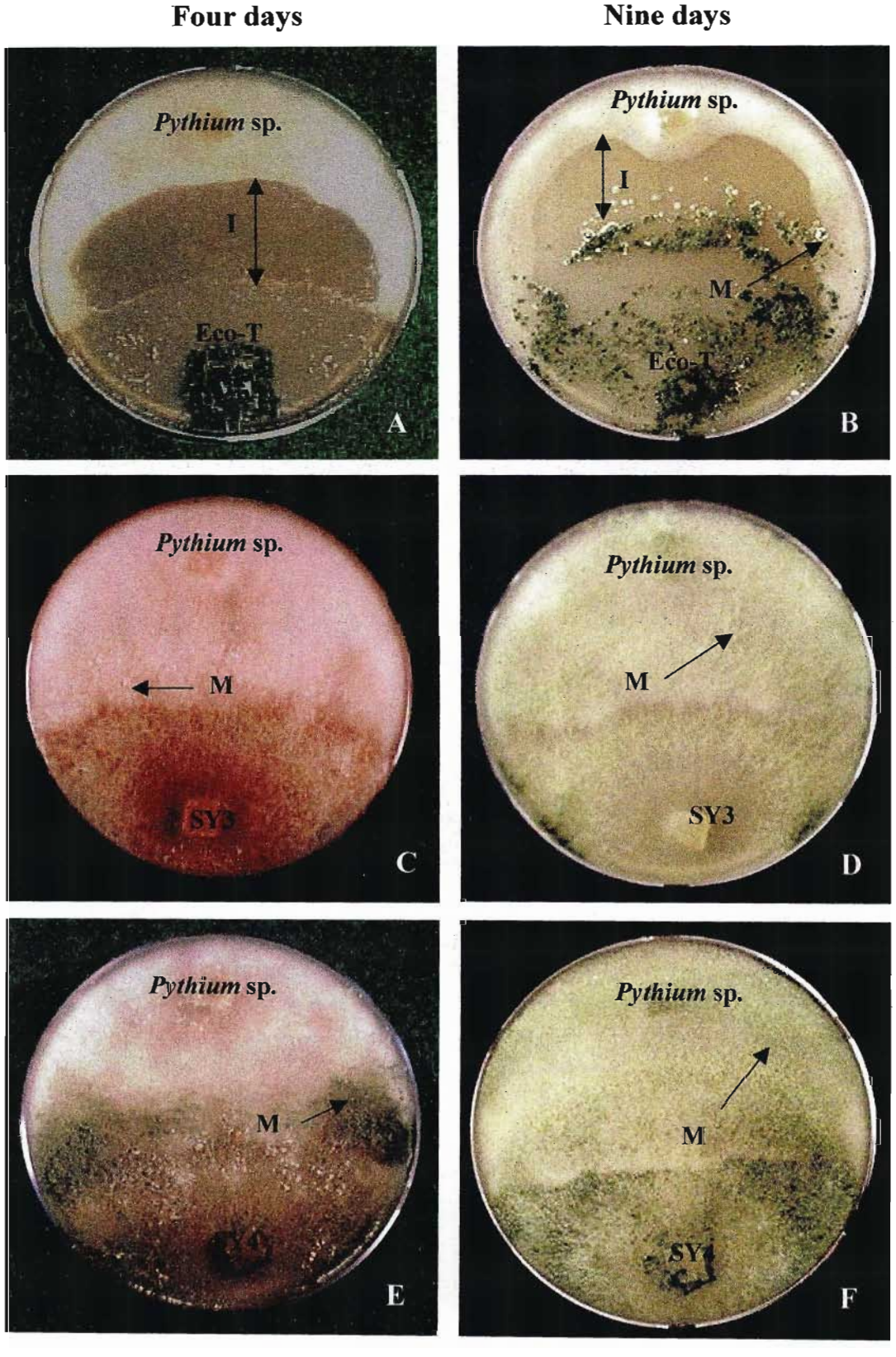

Four days
Nine days
Pythium sp.
I
Eco-T
A
Pythium sp.
I
M
Eco-T
B
Pythium sp.
M
SY3
C
Pythium sp.
M
SY3
D
Pythium sp.
M
E
Pythium sp.
M
F

Fig. 3.2 ESEM observation on hyphal interaction between *Trichoderma* spp. and *Pythium* at various stages of mycoparasitism.

Plate 1: *Trichoderma harzianum* Ecto-T attached to the hyphae of *Pythium* with its appressoria-like structure and penetrating through the penetrating hole with its hook-like structures.

Plate 2: Eco-T penetrating the hyphae of *Pythium*.

Plate 3: Advanced stage of antibiosis, *Pythium* losing its turgor after incubation with Eco-T.

Plate 4: Recognition and attachment by SY3.

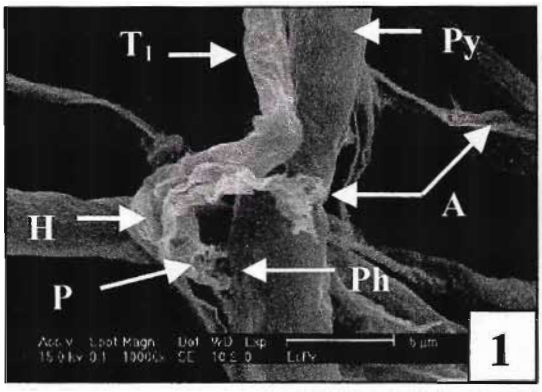
Plate 5: Attachment and coiling of hyphae of SY3 around the hyphae of *Pythium*, resulting in lysis of the host cell wall.

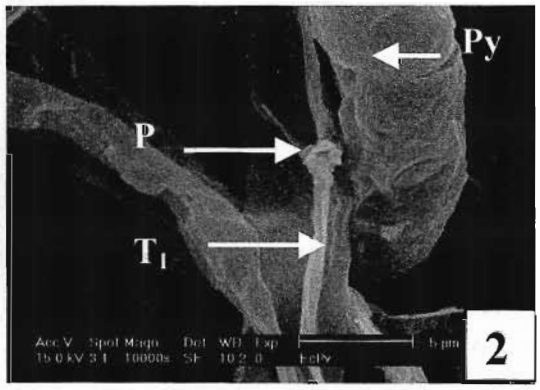
Plate 6: Degradation of *Pythium* by SY3.

Plate 7: SY4 attaching, coiling and penetrating *Pythium* with its hook-like structures after making penetration holes on *Pythium*.

Plate 8: Degradation of *Pythium* by SY4.

Key: A = Appressorium-like structure
At = Attachement
C = Coiling
D = Degradation of *Pythium* sp. by *Trichoderma* spp.
H = Hook-like structure
L = Lysis
P = Penetration
Ph = Penetration hole
Py = *Pythium* sp.
T_1 = *T. harzianum* Eco-T
T_2 = *Trichoderma* sp. SY3
T_3 = *Trichoderma* sp. SY4


T1
Py
A
H
P
Ph
1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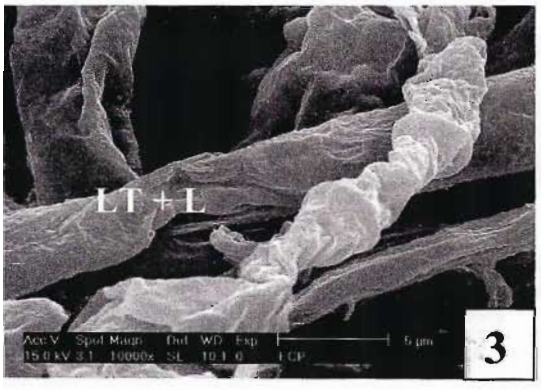
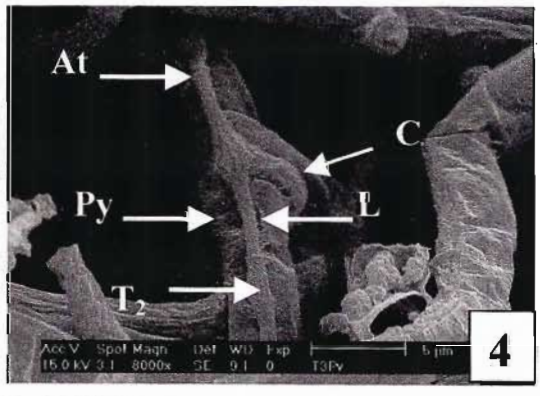
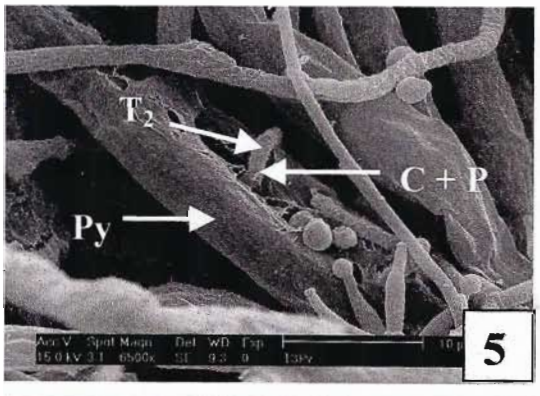
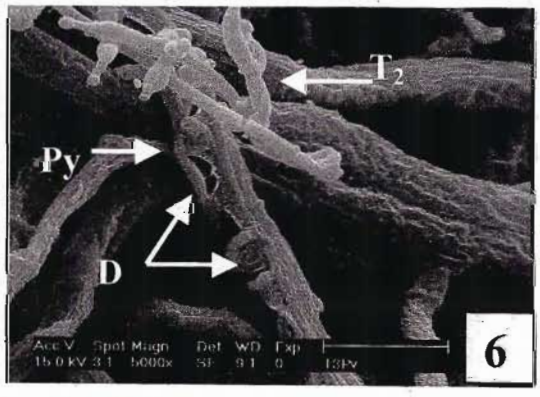
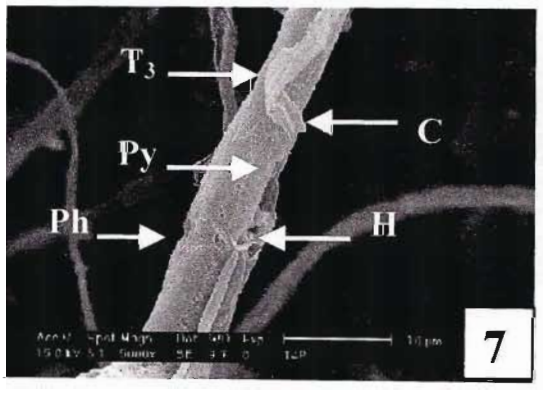
P
T1
2


LT + L
3


At
C
Py
L
T2
4


T2
C + P
Py
5


T2
Py
D
6


T3
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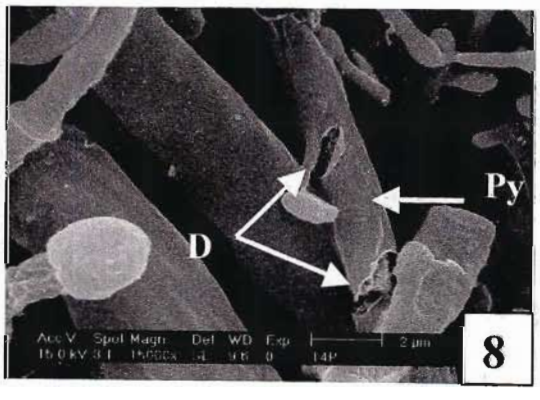
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3.4 DISCUSSION

In vitro results of the dual culture test revealed that the three isolates of *Trichoderma* were effective in inhibiting growth of *Pythium* and completely overgrew the pathogen within 5-6 days of incubation. Visual observations on hyphal interaction between the two organisms showed that growth inhibition of *Pythium* by SY3 and SY4 occurred soon after contact. This illustrates that the antagonistic action of SY3 and SY4 on *Pythium* did not result from the action of diffusible substances produced in advance of contact. Therefore, sporulation of SY3 and SY4 over the mycelium of *Pythium* was a result of mycoparasitism (Elad *et al.*, 1983a). In contrast, Eco-T inhibited growth of *Pythium* before contact, showing that diffusible inhibitory substances were released by this antagonist to attack its host. Although rare, Eco-T also sporulated over the mycelia of *Pythium*. This shows that the pathogenic fungus was being paratisized by Eco-T. Involvement of these two different actions indicates that combinations of antibiosis and mycoparasitism are involved by Eco-T in controlling *in vitro* growth of *Pythium*.

ESEM investigations on hyphal interactions showed that damage to *Pythium* began soon after contact with the antagonists. This triggered a series of events resulting in degradation of the host mycelium. These different and overlapping steps of hyphal interactions are largely the result of mycoparasitic interactions.

Interaction began when chemotrophic substances, released from the pathogen, stimulated growth of the antagonist toward the host (Thrane *et al.*, 1997). This step was demonstrated in Fig. 3.2, Plates D and G. Frequently, the three species of *Trichoderma* were shown to attach themselves to the target host with their hook-like branches and appressorium-like structures (Fig. 3.2, Plates A, D, E and G). Elad *et al.* (1983a) and Benhamou and Chet (1997) made similar observations. According to Chet (1987) this occurrence is facilitated by certain chemicals that adhere onto the host's cell wall. This appears to be the second step following recognition. Coiling of *Trichoderma* hyphae around that of the pathogen was assumed to be the successive step for recognition. Dennis and Webster (1971) studied the interaction of

Trichoderma spp. with plastic threads of similar diameter. They reported that *Trichoderma* never coiled around the threads, suggesting that coiling was not merely a contact stimulus.

In a numerous studies, release of several extracellular lytic enzymes such as β-1,3-glucanases, chitinases, lipase, proteases and cellulase by *Trichoderma* have been noticed when the hyphae of *Trichoderma* coiled around a target soil-borne pathogenic fungi (Lorito *et al.*, 1993; Migheli *et al.*, 1998; Tronsomo and Hjeljord, 1998). These lytic extracellular enzymes are capable of degrading the host cell wall. The significance of each enzyme, however, varies depending on the target host. When *Trichoderma* is grown on the cell wall of *Rhizoctonia* or *Sclerotium rolfsii*, it releases β-1,3-glucanase and chitinase to degrade β-1,3-glucan and chitin which are the main components of the cell wall of these two pathogenic fungi (Elad *et al.*, 1983b). In *Pythium*, since its cell wall is largely made up of cellulose (Bartnicki-Garcia, 1968), significant levels of cellulase activity have also been demonstrated with *Trichoderma* spp. (Elad *et al.*, 1983a; Ahmed and Baker, 1987). In this study, production of some of these lytic enzymes by antagonists, in response to their contact, was noted by perforation and disintegration of the mycelia of *Pythium* (Fig. 3.2, Plates 1, 4, 5, 7 and 8). Once perforations were formed on the surface of *Pythium, Trichoderma* penetrated through these openings with its hook-like structures (Fig. 3.2, Plates 1, 2 and 7). A similar occurrence was reported by Benhamou and Chet (1997) when they studied hyphal interactions between *Trichoderma* and *Pythium* sp. Piercing of the host cell wall might have caused leakage of cytoplasmic constituents that could probably be utilized by the parasite. *Trichoderma* parasitizes a target host if it obtains its nutrients from the host (Chet *et al.*, 1981).

Formation of appressoria-like structures around the perforated zone is associated with release of lytic enzymes (Bertagnolli *et al.*, 1996; Gupta *et al.*, 1999). Therefore, formation of perforations on the host cell wall and appressoria like structures by antagonists is strong evidence that lytic enzymes are released.

Mycoparasitism, as noted in this and other studies undertaken by K.S. Yobo (personal communication), seemed to be the mode of action by which isolates SY3 and SY4 attack *Pythium* and *Rhizoctonia*. Eco-T demonstrated antibiosis and mycoparasitism involved in

antagonizing *Pythium,* compared to only mycoparasitism against *Rhizoctonia* (Omarjee, 2002).

In several field studies of soil-borne diseases, promising control has been achieved by introducing *Trichoderma* into the soil. However, in most reports, the actual mechanism is not known and it appears to be the result of interaction between competition, antibiosis and mycoparasitism results in disease suppression. Each of these mechanisms could play a vital role alone, or in combination with other mechanisms.

Most of the understanding on mycoparasitism and antibiosis comes from research conducted under laboratory conditions. Therefore, interaction between the pathogen (*Pythium* sp.) and its antagonist (*Trichoderma* sp.) should be investigated in natural environments to uncover the contribution of the different mechanisms in disease suppression. Moreover, since competition for limited resources may be important in disease control, either alone or in combination with other mechanisms, there is a need to emphasise its significance both *in vitro* and *in vivo.*

3.5 REFERENCES

Ahmed, J.S. and Baker, R. 1987. Competitive saprophytic ability and cellulolytic activity of rhizosphere-competent mutant of *Trichoderma harzianum*. Phytopathology **77,** 358-362.

Ayers, W.A. and Adams, P.B. 1981. Mycoparasitism and its applications to biological control of plant diseases. In: G.C. Papavizas (Ed.). Biological control in crop production. Allanheld, Osmum, London, UK. Pp. 91-103.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis and taxonomy of fungi. Annual Review of Microbiology **22,** 87-108.

Bell, D.K., Wells, H.D. and Markham, C.R. 1982. *In vitro* antagonism of *Trichoderma* species against six fungal plant pathogens. Phytopathology **72,** 379-382.

Benhamou, N. and Chet, I. 1993. Hyphal interaction between *Trichoderma harzianum* and *Rhizoctonia solani*: ultrastructure and gold cytochemistry of the mycoparasitic process. Phytopathology **83,** 1062-1071.

Benhamou, N. and Chet, I. 1997. Cellular and molecular mechanisms involved in the interaction between *Trichoderma harzianum* and *Pythium ultimum*. Applied and Environmental Microbiology **63,** 2095-2099.

Bertagnolli, B.L., Dal Soglio, F.K. and Sinclair, J.B. 1996. Extracellular enzyme profiles of the fungal pathogen *Rhizoctonia solani* isolate 2B-12 and 2 antagonists, *Bacillus megaterium* strain B153-2-2 and *Trichoderma harzianum* isolate Th008.1 possible correlations with inhibition of growth and biocontrol. Physiological and Molecular Plant Pathology **48,** 145-160.

Campbell, R. 1989. Biological control of microbial plant pathogens. Cambridge University Press, Cambridge, UK.

Chet, I. and Baker, R. 1980. Induction of suppressiveness to *Rhizoctonia solani* in soil. Phytopathology **70,** 994-998.

Chet, I. Harman, G.E. and Baker, R. 1981. *Trichoderma hamatum*: its hyphal interaction with *Rhizoctonia solani* and *Pythium* spp. Microbial Ecology **7,** 29-38.

Chet, I. 1987. *Trichoderma* application, mode of action and potential as a biocontrol agent of soil-borne plant pathogenic fungi. In: I. Chet (Ed.). Innovative approach to plant disease control. John Wiley and Sons, New York, USA. Pp. 137-160.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens. APS Press, St Paul, Minnesota, USA.

Denis, C. and Webster, J. 1971. Antagonistic properties of species group of *Trichoderma* III hyphal interactions. Transactions of The British Mycological Society **57,** 363-369.

Elad, Y., Barak, R. and Chet, I. 1983a. Possible role of lectins in mycoparasitism. Journal of Bacteriology **154,** 1431-1435.

Elad, Y., Chet, I. and Henis, Y. 1982. Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian Journal of Microbiology **28,** 719-725.

Elad, Y., Chet, I., Boyle, P. and Henis, Y. 1983b. Parasitism of *Trichoderma* spp. on *Rhizoctonia solani* and *Sclerotium rolfsii* scanning electron microscopy and fluorescence microscopy. Phytopathology **73,** 85-88.

Fravel, D.R. 1988. Role of antibiosis in the biocontrol of plant diseases. Annual Review of Phytopathology **26,** 75-91.

Gams, W. and Meyer, W. 1998. What exactly is *Trichoderma harzianum*? Mycologia **90,** 904-915.

Ghisalberti, E.L. and Sivasinthamparan, K. 1991. Antifungal antibiotics produced by *Trichoderma* spp. Soil Biology and Biochemistry **23,** 1011-1020.

Gupta, V.P., Tewari, K., Govindha, A.K. and Bajpai, A.K. 1999. Ultrastructure of mycoparasitism of *Trichoderma*, *Gliocladium* and *Laetisaria* species on *Botryodiplodia theobromae*. Journal of Phytopathology **147,** 19-24.

Howell, C.R. 2002. Cotton seedling pre-emergence damping-off incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. Phytopathology **92,** 177-180.

Inbar, J., Abramsky, M., Cohen, D. and Chet, I. 1994. Plant growth enhancement and disease control by *Trichoderma harzianum* in vegetable seedlings grown under commercial conditions. European Journal of Plant Pathology **100,** 337-346.

Lorito, M., Harman, G.E., Hayer, C.K., Broadway, R.M., Tronsmo, A., Woo, S.L. and di Pietro, A. 1993. Chitinolytic enzymes produced by *Trichoderma harzianum*: antifungal activity of purified endochitinase and chitobiosidase. Phytopathology **83,** 302-307.

Migheli, Q., Gonzalez-Kandelas, L., Dealessi, L., Camponogara, A. and Ramon-Vidal, D. 1998. Transformants of *Trichoderma longibrachiatum* overexpressing the β-1,4-endoglucanase gene *egl1* show enhanced biocontrol of *Pythium ultimum* on cucumber. Phytopathology **88**, 673-677.

Omarjee, J. 2002. Studies on the application of biocontrol agents for the control of seedling diseases. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Sivan, A. and Chet, I. 1989. The possible role of competition between *Trichoderma harzianum* and *Fusarium oxysporum* on rhizosphere colonization. Phytopathology **79,** 198-203.

Thrane, C., Tronsmo, A. and Jensen, D.F. 1997. Endo-1,3-β-glucanases and cellulases from *Trichoderma harzianum*: purification and partial characterization, induction of biological activity against plant pathogenic *Pythium* spp. European Journal of Plant Pathology **103,** 331-344.

Tronsmo A. and Hjeljord, L.G. 1998. Biological control with *Trichoderma* species. In: G.S. Boland and L.D. Kuykendall (Eds.). Plant-microbe interactions and biological control. Marcel Dekker, New York, USA. Pp. 111-126.

Whipps, J.M., Lewis, K. and Cooke, R.C. 1988. Mycoparasitism and plant disease control. In: M.N. Burge (Ed.). Fungi in biological control systems. Manchester University Press, Manchester, UK. Pp.161-187.

CHAPTER 4

EVALUATION OF BIOCONTROL AGENTS FOR CONTROLLING SEEDLING DISEASES OF SORGHUM AND TEF CAUSED BY *RHIZOCTONIA* AND *PYTHIUM* UNDER CONTROLLED GREENHOUSE CONDITIONS

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Abstract

A greenhouse trial was conducted to determine the effectiveness of seed treatments with antagonistic *Bacillus* spp. (strains B81, H44 and H51) and *Trichoderma* (*T. harzianum* Eco-T and *Trichoderma* spp. SY3 and SY4), applied as a seed coating or drenching, and fungicides for the reduction of damping-off of sorghum and tef caused by *Rhizoctonia solani* and *Pythium* sp. Damping-off caused by *R. solani* and a *Pythium* sp. affected stand and growth of seedlings of the two crops severely. With the exceptions of H51, applications of all isolates reduced damping-off incited by *R. solani* in both crops. Application of Eco-T, H44 and SY3 to sorghum controlled *Pythium* sp. effectively, by yielding similar results to that of Previcur®. On tef, biological treatments with Eco-T and SY4 reduced seedling damping-off of *R. solani* and the *Pythium* sp., respectively, by providing seedling results similar to the standard fungicides, Benlate® and Previcur®. Most other treatments gave substantial control of the two pathogens on tef. Overall, H44 and Eco-T were the best biocontrol agents from their respective groups in reducing damping-off by these two pathogens. In all instances, effects of application method on disease control activities of biocontrol agents and the adhesive on germination of seeds were not significant.

4.1 INTRODUCTION

Species of *Pythium* and *Rhizoctonia* cause seed rot and seedling damping-off in many agricultural crops growing under greenhouse and field conditions. Cultural practices and chemical treatments are commonly used to reduce initial inoculum of these pathogens. However, none of these measures can prevent later infections and development of seedling diseases in the season (Kiewnick *et al.*, 2001). In addition, because of the detrimental effects of chemicals and the development of pathogen resistance to these chemicals, most efforts have been directed to wards developing biological control as an alternative approach for more effective disease management (Mao *et al.*, 1997; Zaki *et al.*, 1998; Lewis and Lumsden, 2001; Howell, 2002).

Trichoderma (Hadar *et al.*, 1984; Koch, 1999) and *Bacillus* (Handelsman *et al.*, 1990; Berger *et al.*, 1996) have been used extensively as biocontrol agents against many soil-borne fungi, especially *Pythium* and *Rhizoctonia.* Introduction of *Trichoderma* (Elad *et al.*, 1982) and *Bacillus* (Kim *et al.*, 1997) into the soil in greenhouse trials has reduced damping-off caused by *R. solani* and *Pythium* spp. on a number of plant spp.

Success in biocontrol strategy depends on appropriate application of one or more antagonists to the appropriate ecological niche at the right time (Harman, 1992; Lewis *et al.*, 1995; Mao *et al.*, 1998a). Its efficiency in controlling disease relies on the ability of antagonists to grow fast and to colonize the rhizosphere and spermatosphere quickly, produce high levels of inoculum and survive in adverse environmental conditions (Lewis and Papavizas, 1984; Papavizas, 1985; Mahaffee and Backman, 1993; Kim *et al.*, 1997).

Several application methods of biocontrol fungi (Bae and Knudsen, 2001) and bacteria (Mao *et al.*, 1998b; Zaki *et al.*, 1998) have been developed, with the goal of enhancing proliferation and establishment of these agents. Drenching the seed, roots, or soil with microbes (Mao *et al.*, 1998a; Georgakopoulos *et al.*, 2002), coating of microbes onto the surface of the seed and mixing of microbial formulations directly with the soil are some of the common ways of introducing beneficial organisms into the soil (Lewis and Lumsden, 2001).

In dual culture tests, selected isolates of *T. harzianum* Eco-T (Omarjee, 2002) and *Trichoderma* spp. SY3 and SY4 (K.S. Yobo[1], personal communication) and *Bacillus* strains B81 (B. Kubheka[2], personal communication), H44 and H51 (Chapter 2) inhibited growth of *R. solani* and a *Pythium* sp. It is however unknown whether these isolates can provide control of seedling diseases incited by species of *Rhizoctonia* and *Pythium*. Although several bacteria and fungal biocontrol agents have been investigated for control of damping-off on many crops, only one report was accessed on the use of *Pseudomonas* on damping-off control of grain sorghum (El-Meligi, 1989). To date, there is no information on the effectiveness of biocontrol agents in reducing seed and seedling diseases of *Eragrostis tef*.

This study presents the first research results using isolates of *Trichoderma* and *Bacillus* for control of damping-off caused by *R. solani* and a *Pythium* sp on grain sorghum and tef. The objectives were (i) investigate the efficiency of selected bacterial and fungal antagonists on control of seedling damping-off, (ii) compare microbial application methods, and (iii) determine the effect of coating seeds with carboxylmethyl cellulose (CMC) as a sticking agent for the biocontrol agents on germination.

4.2 MATERIALS AND METHODS

4.2.1 Source of materials

Grain sorghum (cv. PAN8446) obtained from Pannar[3] Seeds and *Eragrostis tef* (cv. SA Brown) were obtained from McDonalds[4] Seeds. *Bacillus* spp. H44 and H51 were originally isolated from a soil collected from a sorghum field at Cedara, KwaZulu-Natal, South Africa. Isolate B81 was provided by the Discipline of Plant Pathology[5], University of Natal. *Trichoderma harzianum* (Eco-T) and *Trichoderma* spp. SY3 and SY4 were supplied by Plant

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[3] Pannar Seeds (Pty), P.O.Box 19, Greytown 3250, South Africa
[4] McDonalds Seeds, 61 Boshoff Street, P.O.Box 238, Pietermaritzburg, South Africa
[5] Discipline of Plant Pathology, School of Applied Environmental Sciences, University of Natal, Private Bag X01, Scottsville, 3209, South Africa

Health Products[6] in formulation forms consisting of 10^8 colony forming units per gram (c.f.u g^{-1}). *Pythium* sp. and *R. solani* were obtained from C.C. Clark[7]. Except for the *Trichoderma* spp. that were in a formulation form, all microbes were maintained on agar slants and subcultured on V-8 agar when needed.

4.2.2 Preparation of antagonistic bacteria and fungi

All isolates of *Bacillus* were grown on Potato Dextose Agar (PDA) for two days and inoculated into conical flasks (250ml) containing 100ml of Tryptone Soy Broth (TSB). The flasks were placed in a rotary shaker (120rpm) for 48 h at 23 $\pm$ 2°C. The resultant suspension was centrifuged for 15min at 9,000rpm. The broth was decanted and the pellet of cells resuspended in sterilized distilled water. Counting of bacterial c.f.u. was done after 24h of incubation on Tryptone Soy Agar (TSA) at 25-28°C. The concentrations of *Bacillus* and *Trichoderma* were adjusted to 10^9 and 10^5 c.f.u. ml^{-1} of sterile distilled water, respectively.

4.2.3 Biological treatments

- **Seed coating**

A microbial-sticker mixture was prepared by mixing 2g of CMC with 98ml microbial suspension of the specified concentrations into a conical flask (250ml). Mixtures of microbial-sticker were dissolved by stirring vigorously and allowed to stand for 1h to obtain a uniform mixture. Then 10g of seeds were immersed into 10ml of the mixture for 12-15min. All treated seeds were placed on filter paper and air-dried overnight. Coated seeds were stored at 4°C and planted no longer than five days after treatment (Mao *et al.*, 1998a). Speedling® 24 trays containing coated seeds were left in the planting room overnight and only watered the following day to avoid removal of microbes before their establishment. At the first sign of germination, Speedling® 24 trays were transferred to a greenhouse opertating at temperature of 26-28 °C and relative humuidty of 75-85%.

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- **Drenching**

Untreated seeds of sorghum and tef were planted into Speedling® 24 trays. Before seeds were covered, 1ml of *Trichoderma* (10^5 c.f.u. ml^{-1}) or *Bacillus* (10^9 c.f.u. ml^{-1}) suspension was drenched directly onto seeds in their respective trays. All Speedling® 24 trays were left in the planting room overnight, and only watered the following day to allow establishment of microbes on the target seed. At the first sign of germination, Speedling® 24 trays were transferred to a greenhouse operating at temperatures of 26-28 °C and relative humidity 75-85%.

4.2.4 Other treatments

Control treatments consisted of clean (seeds without CMC) or untreated (seeds coated only with CMC) seeds were planted in infested (with pathogen) or non-infested (without pathogen) soils. For the chemical treatments, untreated seeds were planted into Speedling® 24 trays and covered with composted pine bark and drenched with 3ml of Benlate®[8] at 1.0g l^{-1} and Previcur®[9] at 1.2ml l^{-1} during planting to control *R. solani* and *Pythium* sp., respectively. Sprayed trays were left in the planting room overnight and watered the next day to avoid immediate leaching of chemicals. At the first sign of germination, the Speedling® 24 trays were transferred to the greenhouse.

4.2.5 Artificial inoculation of the media with pathogens

Initially, a thin layer of composted pine bark was placed in Speedling® 24 trays. A 4mm square agar plug, containing the pathogen of a five day-old culture was placed on each cell above the composted pine bark and slightly covered with additional composted pine bark. The media was then gently watered. One seed was placed on the top of the pine bark before treatments were applied, resulting in 24 seeds per tray and 96 seeds per treatment.

[8] El du Ponte de Nemours and Co., Wilmington, Delaware. 19898, USA
[9] AgroEvo South Africa (Pty), P.O.Box 6065, Halfway House 1685, South Africa

4.2.6 Variables of seedlings

Assessment of pre- and post-emergence damping-off caused by a *Pythium* sp. and *R. solani* was conducted by counting the number of emerged seedlings two weeks after planting and the percentage of seedlings that survived four (for tef) and six (for sorghum) weeks of growth. All surviving seedlings in each tray were cut at their base, oven dried for two days at 70°C and then weighed.

4.2.7 Statistical analysis

Each treatment had four replicates, each plot having 24 seedlings hence, 96 seedlings per treatment. Treatments were arranged in a randomized complete block design. The experiment was conducted twice. Data obtained from the two trials were analysed three times using Genstat® Statistical Analysis Software (Genstat, 2002). Firstly, data of the two trials were analyzed separately to determine Analysis of Variance (ANOVA), to make contrasts between infected and uninfected controls, to determine the effect of the sticker on seedling emergence and to separated treatment means using Fisher's Protected LSD. Secondly, data of the two trials were combined and analysed together to determine the effect of season on treatments. Thirdly, data of the two trials containing only results of BCAs were combined together and analysed to determine interactive effects between season, application method and BCAs and to compare the two application methods. In each analysis, transformation of data using square root transformations was performed where CV of the original data was >20% and the statistical results generated from the transformed data were used to meet the objectives.

4.3 RESULTS

4.3.1 Effects of treatments on damping-off caused by *Rhizoctonia solani*

Significant differences were obtained in percentage emergence and final seedling stand of sorghum and tef (Table 4.1 and 4.2). Less than 55% of the untreated seeds of sorghum and tef germinated when planted on soils infested with *R. solani* compared with 88 and 82%, respectively, in non-infested soils (Tables 4.1 and 4.2). At the end of the trial, percentage of

seedling stands recorded in the pathogen-infested control were 52% and 51% for sorghum and tef, respectively. This means that stands of sorghum and tef were reduced by >50%. On both crops most BCAs provided a higher percentage of emergence and stand of seedlings when compared to the plot treated only with *R. solani* (Tables 4.1 and 4.2).

Treatment effect was also statistically significant ($P<0.001$) on plot weight of sorghum and tef (Table 4.5). With sorghum, mean plot weights of untreated controls declined up to 44%, when soils were inoculated with *R. solani*. On average, treatments with H44, Eco-T, SY3 and SY4 and Benlate resulted in increased plot weight of sorghum when compared to the pathogen-infested control (Table 4.1). Compared to the non-infested control, significant reduction ($P<0.05$) in plot weights (up to 45%) were observed in tef as a result of inoculation by *R. solani*. Treatments with H44, H51, Eco-T, SY3, SY4 and Benlate® significantly increased dry weight of plots inoculated with *R. solani* in at least one season (Table 4.2).

Stand reduction, as a result of post-emergence damping-off caused by *R. solani,* was in the range of 5-15% for both crops. For plots containing *R. solani*, it appears that plot weights were the product of final seedling stand. Overall performance of antagonistic fungi were relatively better than that of bacteria (Tables 4.1 and 4.2).

Table 4.1 Percentage seedling emergence, final stand and plot weights of greenhouse grown sorghum seedlings inoculated with *Rhizoctonia solani* and treated with biocontrol agents applied as seed coating or drenching

Treatment	Application method	Season 1			Season 2		
		Emergence (%)	Final stand (%)	Plot weight (g)	Emergence (%)	Final stand (%)	Plot weight (g)
Bacillus sp. H44	Seed coating	74.0 ab	72.9 ab	7.7 (2.78) b	82.3 a	78.1 a	5.5 (2.33) ab
Bacillus sp. H44	Drenching	62.5 bc	60.4 bc	8.5 (2.92) ab	79.2 ab	74.0 ab	5.4 (2.31) ab
Bacillus sp. H51	Seed coating	62.5 bc	60.4 bc	5.4 2.28) c	82.3 a	77.1 a	5.2 (2.28) ab
Bacillus sp. H51	Drenching	60.4 bc	59.4 bc	5.0 (2.20) c	83.3 a	82.3 a	5.9 (2.41) ab
Bacillus sp. B81	Seed coating	54.1 bc	50.0 bc	5.1 (2.22) c	64.6 b	60.4 b	5.9 (2.40) ab
Bacillus sp. B81	Drenching	56.3 bc	60.4 bc	6.6 2.55) c	74.0 ab	74.0 ab	5.9 (2.42) a
T. harzianum Eco-T	Seed coating	62.5 bc	53.1 bc	8.4 (2.89) b	74.0 ab	68.8 ab	5.2 (2.27) ab
T. harzianum Eco-T	Drenching	63.5 bc	62.5 bc	7.2 (2.68) bc	76.0 ab	74.0 ab	4.9 (2.21) ab
Trichoderma sp. SY3	Seed coating	71.9 ab	71.9 ab	8.9 2.98) ab	84.4 a	81.3 a	5.7 (2.38) ab
Trichoderma sp. SY3	Drenching	69.8 b	67.7 ab	7.7 (2.77) b	83.3 a	83.3 a	5.1 (2.23) ab
Trichoderma sp. SY4	Seed coating	53.1 c	51.0 bc	8.5 (2.91) ab	81.3 a	77.1 a	6.2 (2.46) a
Trichoderma sp. SY4	Drenching	59.4 bc	59.4 bc	6.4 (2.53) bc	63.5 b	58.3 b	4.1 (2.01) b
Benlate®	Nil	69.8 ab	65.6 b	8.4 (2.89) b	79.2 ab	67.7 ab	5.0 (2.23) ab
R.solani only	Nil	50.0 c	45.8 c	5.2 (2.24) c	60.4 b	57.3 b	4.2 (2.05) b
Control (only sticker)	Nil	89.6 a	89.6 a	9.7 (3.09) ab	82.3 a	81.3 a	6.3 (2.51) a
Control (only water)	Nil	92.7 a	92.7 a	11.0 (3.31) a	83.3 a	82.3 a	5.9 (2.41) ab
Effects		**P-values**	**P-values**	**P-values**	**P-values**	**P-values**	**P-values**
Treatment		<.001***	<.001***	<.001***	0.029*	0.013*	0.360
Pathogen vs. control		<.001***	<.001***	<.001***	0.005**	0.003**	0.056
Sticker		0.704	0.733	0.322	0.893	0.897	0.596
% CV		17.6	20.2	11.7	14.1	15.4	11.4
MSE		133.5	166.0	0.099	118.3	128.8	0.069
LSD		16.43	18.32	0.45	15.46	16.13	0.37

1. * = significant at P<0.05; **= significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

Table 4.2 Percentage seedling emergence, final stand and plot weights of greenhouse grown tef seedlings inoculated with *Rhizoctonia solani* and treated with biocontrol agents applied as seed coating or drenching

Treatment	Application method	Season 1			Season 2		
		Emergence (%)	Final stand (%)	Plot weight (g)	Emergence (%)	Final stand (%)	Plot weight (g)
Bacillus sp. H44	Seed coating	55.2 (7.40) b	45.8 (6.75) b	1.2 (1.11) b	53.1 ab	53.1 ab	1.1 (1.05) b
Bacillus sp. H44	Drenching	69.8 (8.31) ab	56.3 (7.45) b	1.5 (1.01) b	61.5 ab	61.5 ab	1.5 (1.22) ab
Bacillus sp. H51	Seed coating	61.4 (7.74) b	49.0 (6.91) b	1.3 (1.06) b	66.7 a	66.7 a	1.2 (1.08) ab
Bacillus sp. H51	Drenching	57.3 (7.56) b	46.8 (6.84) b	1.0 (1.01) b	51.0 b	51.0 b	1.3 (1.14) ab
Bacillus sp. B81	Seed coating	59.4 (7.67) b	48.9 (6.97) b	1.1 (1.21) ab	61.5 ab	60.4 ab	1.4 (1.17) ab
Bacillus sp. B81	Drenching	54.2 (7.35) b	43.7 (6.60) b	1.0 (1.36) a	67.7 a	66.7 a	1.2 (1.12) ab
T. harzianum Eco-T	Seed coating	59.4 (7.68) b	50.0 (7.04) b	1.5 (1.23) ab	62.5 ab	62.5 ab	1.4 (1.20) ab
T. harzianum Eco-T	Drenching	77.1 (8.72) ab	62.5 (7.86) ab	1.9 (1.26) ab	56.3 ab	56.3 ab	1.3 (1.15) ab
Trichoderma sp. SY3	Seed coating	72.9 (8.49) ab	61.5 (7.79) ab	1.5 (1.21) ab	50.0 b	50.0 b	1.4 (1.20) ab
Trichoderma sp. SY3	Drenching	69.8 (8.35) ab	58.3 (7.63) ab	1.6 (1.31) ab	59.4 ab	59.4 ab	1.4 (1.16) ab
Trichoderma sp. SY4	Seed coating	74.0 (8.53) ab	62.5 (7.84) ab	1.5 (1.23) ab	57.3 ab	56.3 ab	1.1 (1.04) b
Trichoderma sp. SY4	Drenching	79.2 (8.84) ab	66.6 (8.12) ab	1.7 (1.13) ab	59.4 ab	59.4 ab	1.4 (1.13) ab
Benlate®	Nil	64.6 (7.97) b	53.1 (7.25) b	1.3 (1.07) b	64.6 a	62.5 ab	1.5 (1.23) ab
R. solani. only	Nil	55.2 (7.41) b	46.8 (6.82) b	1.1 (1.04) b	54.2b	54.2 b	1.1 (1.06) b
Control (only sticker)	Nil	82.3 (9.06) ab	69.7 (8.35) ab	1.4 (1.16) ab	72.9 a	72.9 a	1.6 (1.26) a
Control (only water)	Nil	89.6 (9.45) a	76.0 (8.71) a	1.7 (1.29) ab	76.0 a	75.0 a	1.9 (1.37) a
Effects		**P-values**	**P-values**	**P-values**	**P-values**	**P-values**	**P-values**
Treatment		0.027*	0.011*	0.051	0.025*	0.035*	0.221
Pathogen vs. control		0.002**	0.002**	0.035*	0.013*	0.012*	0.005**
Sticker		0.540	0.533	0.287	0.668	0.774	0.278
% CV		11.1	11.0	13.7	16.8	16.9	12.9
MSE		0.817	0.665	0.026	104.8	104.1	0.022
LSD		1.29	1.16	0.23	14.56	14.50	0.21

1. * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

4.3.2 Effects of treatments on damping-off caused by *Pythium* sp.

Effects of treatments on seedling emergence and final stand were significant on sorghum but not on tef at $P<0.05$. On sorghum, *Pythium* sp. reduced final stand of seedlings remarkably ($P<0.05$). In contrast, effects of this pathogen on percentage emergence and final stand of tef seedlings were not significant (Table 4.5). On both crops, none of the control measures used could provide a significant increase in terms of seedling emergence or stand over the control inoculated *Pythium* infested soils (Tables 4.3 and 4.4). However, although only on the second season, Eco-T and SY3 significantly increased seedling final stand of tef seedlings inoculated with *Pythium* sp. Stand reduction due to post-emergence damping-off ranged from 1-5% in sorghum and 1-10% in tef.

Significant variation ($P<0.05$) was obtained on plot weights of sorghum and tef as a result of treatments. On both crops, *Pythium* sp. caused significant reduction ($P<0.001$) in plot weight of control (Tables 4.7 and 4.8). Biological treatments with SY3 (on sorghum and tef), SY4 (sorghum) and Eco-T (on tef) significantly increased plot weight of controls inoculated by (Tables 4.3 and 4.4). Although statistically non-significant, most of biological treatments also gave comparable results to that of control and caused increased plot weight relative to the *Pythium* infested control.

Table 4.3 Percentage seedling emergence, final stand and plot weights of greenhouse grown sorghum seedlings inoculated with *Pythium* sp. and treated with biocontrol agents applied as seed coating or drenching

Treatment	Application method	Season 2			Season 2		
		Emergence (%)	Final stand (%)	Plot weight (g)	Emergence (%)	Final stand (%)	Plot weight (g)
Bacillus sp. H44	Seed coating	77.1 ab	76.0 ab	7.6 (2.74) ab	74.0 b	70.8 b	5.7 b
Bacillus sp. H44	Drenching	78.1 ab	77.18 ab	6.8 (2.60) ab	80.2 ab	78.1 b	6.2 ab
Bacillus sp. H51	Seed coating	78.1 ab	77.1 ab	7.2 (2.67) ab	75.0 b	70.8 b	4.3 b
Bacillus sp. H51	Drenching	81.3 ab	81.2 ab	7.7 (2.75) ab	80.2 ab	79.2 ab	5.7 b
Bacillus sp. B81	Seed coating	77.1 ab	73.9 ab	7.0 (2.64) ab	77.2 b	71.9 b	5.3 b
Bacillus sp. B81	Drenching	79.2 ab	79.2 ab	7.6 (2.72) ab	79.2 ab	77.1 b	5.8 b
T. harzianum Eco-T	Seed coating	79.2 ab	79.2 ab	6.6 (2.56) ab	81.3 ab	79.2 ab	6.5 ab
T. harzianum Eco-T	Drenching	78.1 ab	75.0 ab	7.2 (2.67) ab	72.9 b	72.9 b	5.8 b
Trichoderma sp. SY3	Seed coating	74.06 ab	70.8 b	6.2 (2.48) ab	82.2 ab	80.2 ab	7.0 ab
Trichoderma sp. SY3	Drenching	75.0 ab	72.9 ab	7.0 (2.63) ab	77.0 b	76.0 b	7.6 a
Trichoderma sp. SY4	Seed coating	71.9 b	70.8 b	7.5 (2.73) ab	79.1 ab	74.0 b	7.1 ab
Trichoderma sp. SY4	Drenching	77.1 ab	76.0 ab	7.3 (2.69) ab	78.1 ab	78.1 b	6.7 ab
Previcur®	Nil	78.1 ab	77.1 ab	8.1 (2.83) a	84.3 ab	83.3 ab	6.1 ab
Pythium sp. only	Nil	76.0 ab	74.0 ab	5.6 (2.37) b	83.3 ab	81.2 ab	5.3 b
Control (only sticker)	Nil	85.4 a	87.5 a	8.8 (2.96) a	87.5 a	87.5 ab	7.0 b
Control (only water)	Nil	88.5 a	84.4 a	9.3 (3.04) a	88.5 a	89.6 a	6.8 ab
Effects		**P-values**	**P-values**	**P-values**	**P-values**	**P-values**	**P-values**
Treatment		0.469	0.388	0.310	0.151	0.040*	0.029*
Pathogen vs. control		0.108	0.096	0.003**	0.322	0.143	0.071
Sticker		0.588	0.613	0.723	0.842	0.711	0.806
% CV		10.3	11.3	11.1	9.2	10.1	19.2
MSE		65.56	75.50	0.08914	54.06	62.48	1.407
LSD		11.51	12.35	0.42	10.45	11.24	1.67

1. * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

Table 4.4 Percentage seedling emergence, final stand and plot weights of tunnel grown tef seedlings inoculated with *Pythium* sp. and treated with biocontrol agents applied as seed coating or drenching

Treatment	Application method	Season 2			Season 2		
		Emergence (%)	Final stand (%)	Plot weight (g)	Emergence (%)	Final stand (%)	Plot weight (g)
Bacillus sp. H44	Seed coating	80.2 a	77.1a	1.4 (1.18) ab	78.1 ab	71.9 ab	1.5 ab
Bacillus sp. H44	Drenching	72.9 ab	70.8 a	1.4 (1.19) ab	78.1 ab	76.1 b	1.7 ab
Bacillus sp. H51	Seed coating	72.9 ab	68.7 a	1.5 (1.21) ab	71.9 b	67.7 b	1.3 b
Bacillus sp. H51	Drenching	70.8 b	69.7 a	1.3 (1.15) ab	77.1 ab	74.0 ab	1.4 b
Bacillus sp. B81	Seed coating	72.9 ab	69.7 a	1.5 (1.21) ab	79.2 ab	77.1 ab	1.4 b
Bacillus sp. B81	Drenching	76.0 ab	72.9 a	1.4 (1.15) ab	75.0 ab	71.9 ab	1.6 ab
T. harzianum Eco-T	Seed coating	75.0 ab	68.7 a	1.6 (1.28) a	82.3 ab	79.2 ab	1.7 ab
T. harzianum Eco-T	Drenching	71.9 ab	68.7 a	1.6 (1.25) a	77.1 ab	74.0 ab	1.6 ab
Trichoderma sp. SY3	Seed coating	77.1 a	73.9 a	1.0 (1.01) b	82.3 ab	81.3 ab	1.6 ab
Trichoderma sp. SY3	Drenching	80.2 a	69.7 a	1.3 (1.13) ab	77.1 ab	75.0 ab	1.6 ab
Trichoderma sp. SY4	Seed coating	74.0 ab	71.8 a	1.3 (1.13) ab	83.3 a	78.1 a	1.6 ab
Trichoderma sp. SY4	Drenching	74.0 ab	76.0 a	1.3 (1.13) ab	81.2 ab	78.1 ab	1.6 ab
Previcur®	Nil	79.2 a	77.0 a	1.5 (1.21) ab	72.9 b	70.8 b	1.4 ab
Pythium sp. only	Nil	74.0 ab	68.7 a	1.0 (1.01) b	75.0 ab	68.7 b	1.4 b
Control (only sticker)	Nil	75.0 ab	73.9 a	1.7 (1.31) a	78.1 ab	77.1 ab	1.7 a
Control (only water)	Nil	75.0 ab	75.0 a	1.7 (1.30) a	74.0 ab	72.9 ab	1.8 ab
Effects		**P-values**	**P-values**	**P-values**	**P-values**	**P-values**	**P-values**
Treatment		0.517	0.671	0.142	0.401	0.597	0.281
Pathogen vs. control		0.801	0.292	0.006**	0.824	0.475	0.033*
Sticker		1.000	0.832	0.946	0.376	0.475	0.720
% CV		7.7	9.6	12.0	8.5	11.0	17.1
MSE		33.73	47.84	0.020	43.49	66.91	0.070
LSD		8.26	9.83	0.20	9.38	11.63	0.38

1. * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

4.3.3 Effects of season and application methods on biological control

On both crops, the effects of season on percentage emergence and final stand obtained form various treatments were not significant in controlling damping-off caused by *R. solani* or *Pythium* sp. There was also no significant variation P<0.05) in activities of BCAs controlling *Pythium* sp. (on both crops) and *R. solani* (only on tef). Moreover, there was no direct relationship between season and application method of BCAs. However, there was significant variation on plot weight of sorghum inoculated with *R. solani* and treated with various treatments (P<0.05). On sorghum, the activities of BCAs in controlling damping-off caused by *R. solani*, as expressed on plot weight, varied significantly due to season (P<0.001). Moreover, there was significant effect of season on plot weight of sorghum harvested from control treatments inoculated with *R. solani* (Tables 4.5-4.8).

There was no significant difference between the two microbial application methods, seed coating and drenching, and with respect to both crops, effect of application technique on performance of BCAs was not significant at P<0.05. Moreover, activities of BCAs were not affected by season regardless of application method. Contrast analysis between seeds coated only with the sticker and clean seeds gave insignificant differences in all seedling variables at P<0.05, indicating that the sticker, CMC, had no detrimental effect on germination of seeds and survival of seedlings as well as plot weight (Tables 4.5-4.8).

Table 4.5 ANOVA table showing factorial interactions between season and treatment; season, application methd and biocontrol agents and season and pathogen on emergence, final stand and plot weight of tunnel grown seedlings of sorghum inoculated with *Rhizoctonia solani* and treated with biocontrol agents applied as seed coating or drenching.

	Emergence (%)	Final stand (%)	Plot weight
Effects	**P-value**	**P-value**	**P-value**
Season	<.001***	<.001***	<.001***
Treatment	<.001***	<.001***	<.001***
Season x Treatment	0.061	0.132	<.001***
Season	<.001***	<.001***	<.001***
Application	0.912	0.650	0.185
BCA	0.004**	0.004**	0.038*
Season x Application	0.579	0.807	0.861
Season x BCA	0.836	0.893	<.001***
Application x BCA	0.342	0.175	0.075
Season x Application x BCA	0.437	0.307	0.788
Season	<.001***	<.001***	<.001***
Pathogen	<.001***	<.001***	<.001***
Season x Pathogen	0.080	0.077	0.018

* = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001

Table 4.6 ANOVA table showing factorial interactions between season and treatment; season, application methd and biocontrol agents and season and pathogen on emergence, final stand and plot weight of tunnel grown seedlings of tef inoculated with *Rhizoctonia solani* and treated with biocontrol agents applied as seed coating or drenching.

	Emergence (%)	Final stand (%)	Plot weight
Effects	**P-value**	**P-value**	**P-value**
Season	0.004	0.031	0.794
Treatment	<.001	<.001	0.002
Season x Treatment	0.158	0.120	0.208
Season	0.018	0.065	0.313
Application	0.360	0.426	0.266
BCA	0.207	0.152	0.604
Season x Application	0.522	0.669	0.789
Season x BCA	0.216	0.156	0.017
Application x BCA	0.402	0.468	0.495
Season x Application x BCA	0.339	0.331	0.789
Season	0.004	0.031	0.794
Pathogen	<.001	<.001	<.001
Season x Pathogen	0.331	0.461	0.651

* = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001

Table 4.7 ANOVA table showing factorial interactions between season and treatment; season, application methd and biocontrol agents and season and pathogen on emergence, final stand and plot weight of tunnel grown seedlings of sorghum inoculated with *Pythium* sp. and treated with biocontrol agents applied as seed coating or drenching.

	Emergence (%)	Final stand (%)	Plot weight
Effects	**P-value**	**P-value**	**P-value**
Season	0.226	0.446	<.001***
Treatment	0.005**	0.002**	0.003**
Season x Treatment	0.786	0.646	0.068
Season	0.983	0.974	0.442
Application	0.599	0.201	0.395
BCA	0.752	0.627	0.080
Season x Application	0.594	0.413	0.826
Season x BCA	0.505	0.668	0.001
Application x BCA	0.873	0.936	0.839
Season x Application x BCA	0.416	0.944	0.672
Season	0.226	0.446	<.001***
Pathogen	0.021*	0.009**	<.001***
Season x Pathogen	0.337	0.526	0.188

* = significant at P<0.05, ** = significant at P<0.01, *** = significant at P<0.001

Table 4.8 ANOVA table showing factorial interactions between season and treatment; season, application methd and biocontrol agents and season and pathogen on emergence, final stand and plot weight of tunnel grown seedlings of tef inoculated with *Pythium* sp. and treated with biocontrol agents applied as seed coating or drenching.

	Emergence (%)	Final stand (%)	Plot weight
Effects	**P-value**	**P-value**	**P-value**
Season	0.018*	0.055	0.011*
Treatment	0.287	0.278	0.003
Season x Treatment	0.343	0.570	0.337
Season	0.005**	0.024*	0.017*
Application	0.260	0.676	0.701
BCA	0.488	0.753	0.106
Season x Application	0.740	0.834	0.656
Season x BCA	0.157	0.313	0.407
Application x BCA	0.430	0.659	0.561
Season x Application x BCA	0.698	0.719	0.987
Season	0.018*	0.055	0.011*
Pathogen	1.000	0.162	<.001***
Season x Pathogen	0.735	0.779	0.364

* = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001

4.4 DISCUSSION

Controlled environmental conditions and restriction in the use of chemical pesticides make greenhouses a favourable place for the implementation of biological control as an effective disease control strategy (Lewis and Lumsden, 2001). Percentage emergence, final seedling stand and plot weight of seedlings were used in this study as criteria for screening potential antagonists for their ability to protect plants against seed and seedling diseases in a greenhouse. These parameters provided useful and quick information for testing many candidates.

Tables of the statistical analysis indicated that the CV percentage of most treatments and LSD differences between non-infested and infested controls were high. These two values caused some results to be non-significant even if the trends were clear. Therefore, it was difficult to make an overall conclusion. Nevertheless, based on the trends presented on Tables 4.1-4.4, the following points are discussed.

4.4.1 Control of damping-off caused by *Rhizoctonia solani*

Percentage of seedlings emerging within two weeks after planting was low in treatments containing *R. solani*, indicating severe pre-emergence damping-off (Tables 4.5 and 4.6). In contrast to pre-emergence damping-off, post-emergence damping-off was relatively limited. In some trays of sorghum, *R. solani* reduced the stand by more than 50%. Similar effects were also observed on seedlings of tef. The results demonstrated the potential of *R. solani* to cause extensive loss in stand if conditions are favourable for its development. Such reduction in stand can impact upon grain yield by a similar level.

Effect of Season on aggressiveness of *R. solani* was significant on plot weight of sorghum. In the second season, loss in plot weight of sorghum due to *R. solani* was declined by 34%. Season has also caused improvements in plot weight of tef inoculated with *R. solani* by 6%. However, results in Table 4.1 and 4.2 indicate that percentage of emergence, final stand and plot weight of most treatments were relatively lower in the second trial even if they were not

inoculated with the pathogen. This could have been related with variability in growth conditions of the tunnel. The amount of irrigation, fertilization and light intensity in the tunnel may affect emergence and growth of seedlings. With relative humidity fluctuating from 75 to >90%, any interruption in frequency or distribution of irrigation could have been responsible for this difference. Jarvie (1994) pointed out that pathogenicity of *R. solani* is affected by availability of nutrients that comes either the from seed or soil solution. Based on that information, it was assumed that the amount of fertilization might have been lower in the second trial, which resulted in lower seedling growth and reduced disease severity.

In both cereals, significant results were obtained in percentage emergence and final stand of seedlings and plot weights regardless of season (Table 4.1). These were clearly due to differences in performances of the treatments. Most treatments minimized disease by providing a relatively higher stand and plot weight (Fig. 4.1 and 4.2). Biological treatments with B81, H44 and all the *Trichoderma* isolated used in this study caused improved stand and weight seedlings in plots inoculated with *R. solani* and similar yields to plots treated with the standard fungicide, Benlate®. According to this investigation, with the exception of H51, all the above used BCAs can provide substantial to satisfactory control of seedling damping-off of sorghum and tef caused by *R. solani*. In this particular study, plot weights of these two crops appeared to be the direct outcome of final stand.

4.4.2 Control of damping-off caused by *Pythium* sp.

Interactive effects between season and *Pythium* (Table 4.8) showed that there was no significant difference in severity of damping-off caused by *Pythium*. This means that aggressiveness of *Pythium* remained the same regardless of the season. For both crops, the effects of *Pythium* sp. on seedling emergence and stand were relatively less than that of *R. solani*. Treatment effects were significant on percentage emergence and final stand of sorghum but not tef. Inoculation of *Pythium* sp. onto sorghum caused significant reduction of stand of the unprotected plots. The effects of *Pythium* on emergence and stand of tef was negligible.

In contrast to the effect on emergence and stand, in both crops, *Pythium* sp. significantly affected the growth of seedlings, resulting in lower plot weight. For example, in some plots of tef, higher seedling stand was harvested from treatments containing *Pythium* than the uninfected control. Interestingly, mean plot weights of these treatments were the lowest of most treatments tested. This suggests that where *Pythium* is a major pathogen, stand difference alone could not account for yield response. Davis and Bockus (2001) made similar findings. In their study, they pointed out that the main effect of *Pythium* was on growth of seedlings rather than final stand. According to McLaren (1987), seedling stunting is the result of mesocotyl rot, primary rot, or both. Under natural field conditions, final stand can be compensated through increased growth and tillering of plants if the reduction in stand is not too high, provided they have a relatively healthy root system (Davis and Bockus, 2001). This indicates that healthy root systems are equally important to producing good seedling stand. This is because any damage to the root system is manifested by impairment of the physiological activities of the plant, such as water absorption and nutrient uptake from the soil.

Effects of *Pythium* on growth of the two crops was effectively controlled by most of the treatments by yielding plot weights comparable to that of the standard fungicide, Previcur® and similar to that of the non-infested control.

In general, application of biocontrol agents had a beneficial effect in controlling seedling damping-off on sorghum and tef. Although the overall effects of chemical treatments was better than that of biological treatments, in some instances, performance of certain isolates was superior to these two chemicals. Based on the above tests, the overall performance of fungal antagonists was better than that of bacterial antagonists. The most effective antagonists were H44 and Eco-T.

In Chapter 2, H51 showed strong inhibition of *in vitro* growth of *Pythium* sp. and *R. solani.* Nevertheless, in this trial, its performance was poor compared to most biological treatments. This finding correlates with the argument that *in vitro* and *in vivo* biocontrol activities not well correlated. However, results obtained from most other isolates still support the theory that an

antagonist capable of inhibiting *in vitro* growth of a pathogen is a promising potential agent. There are several explanations for relatively poor performances of some of the antagonists used to control damping-off in this trial.

One of the possible reasons can be attributed to the timing of antagonistic activity in relation to infection. For example, *Pythium* zoospores invade host tissues very fast when stimulated by seed and root exudates (Georgakopoulos *et al.*, 2002). Therefore, to provide protection to the target, the antagonist must colonize the spermatosphere prior to infection by the pathogen. An alternative reason could be the concentration of the antagonists applied onto the seed. Too low or too high concentration may cause negative effects on the germination of seeds and seedling health. For instance, Hadar *et al.* (1984) and Adekunle *et al.* (2001) noted that the highest concentration of *Trichoderma* resulted in less effectiveness in disease protection. According to their investigations, the detrimental effects could be the result of self-inhibition of the antagonist or intrinsic pathogenicity.

Lack of compatibility between the antagonists and the crop could also be a factor (Papavizas, 1985; Koch, 1999). For instance, a biocontrol agent that provides a significant control of one pathogen in one crop may fail to control the same pathogen in different crops or different varieties of the same crop (El-Meligi, 1989). Alternatively, the disease severity in the control might not have been sufficient enough to demonstrate convincing differences of treatments (Elad *et al.*, 1982).

Statistical analysis on Tables 4.5-4.8 indicated that there was no significant difference in applying BCAs as a seed coat or drench. This means that the two application methods performed equally well or badly regardless of other effects. In controlling damping-off incited by *R. solani* and *Pythium* sp., there was no significant effect of application method on activities on BCAs (Tables 4.5-4.8). This means that none of the combinations of BCAs and application is better or worse than the others. Moreover, in all instances, interactive effects of season and application on activities of BCAs (season x application x BCAs) gave non-significant effects. This indicates that activities of all BCAs remained the constant irrespective of season and how they were applied.

In practical field conditions, utilization of these alternatives may depend on the biology of the beneficial agent to be used, efficiency of the delivery machine and the amount of biocontrol agent applied. Some biocontrol agents are less rhizosphere competent and lack the ability to spread throughout the soil to kill active or dormant pathogens. In such cases, application of antagonists in a liquid form may facilitate active colonization of the root zone compared to seed coating, where antagonists are coated onto the surface of the seed. In addition, application of biocontrol agents onto a dry soil in a liquid suspension can maintain their hydrated state at least for a few hours. However, this delivery method lacks uniform application of biocontrol agents throughout the field and takes time and labour. A user may spray the suspension directly into a furrow evenly with a machine (Harman, 1991). Nevertheless, this system of application cannot save extra application of the BCA. In addition, antagonists that do not adhere to the seed are prone to be washed-off by irrigation water or rain before they colonize the spermatosphere.

In contrast, beneficial agents that are coated onto the surface of the seed have the chance to be the first colonizers of spermatosphere (Hadar *et al.*, 1984; Chao *et al.*, 1986). If they can grow fast and dominate the spermatosphere, they can have a high potential to protect the germinating seed simply by pre-empting the energy supply used by the pathogen. Application of biocontrol agents as a seed coating is simpler and needs fewer microbes to treat a large quantity of seed to provide similar efficiency provided by drenching (Chao *et al.*, 1986; Harman, 1991). According to Tronsmo and Hjeljord (1998), introduction of biocontrol agent as seed coatings allows any farmer to apply it without changing his planting equipment or procedures.

One of the probable drawbacks of a seed coating technique may be the inability of seeds to be stored after treatment. There are recommendations indicating that viability of antagonists can be reduced if coated seeds are not sown within 4-5days after treatment (Mao *et al.*, 1998a). However, deterioration in viability depends on the formulation and the biocontrol agent. For instance, *Bacillus* and *Trichoderma* can be stored for long periods without deterioration. Thus, the issue of storage of treated seeds may not be a limiting factor for these two antagonists. There is also a fear that certain adhesives that are normally used as seed coatings can cause

inhibition of seed germination (El-Meligi, 1989). In spite of that, the coating material used in this study had no detrimental effects on seedling emergence.

Overall the data obtained from this chapter indicate that control of damping-off caused by *R. solani* and *Pythium* sp. under greenhouse condition was possible with some of the BCAs. Most isolates consistently improved stand and growth of sorghum and tef seedling inoculated with these two pathogens regardless of application method (Table 4.1-4.4). However, activities of BCAs was slightly varied especially in controlling *R. solani* on sorghum when reflected in plot weight of seedlings. This is not surprising as any fluctuation in environmental condition in the tunnel can affect the plant, pathogen and BCAs, which in turn affects activities of antagonists.

The result also suggested that there is a need to test the effects of the sticker CMC on germination of seeds and emergence of seedlings on different crops both in greenhouse and field conditions. There is also a need to determine contribution of the adhesive to disease levels to accurately assess effects of the main treatments. Moreover, the study recommends further investigations on some of the above tested BCAs to control damping-off and other diseases caused by various pathogens on different crops under different environmental conditions.

4.5 REFERENCES

Adekunle, A.T., Kardwell, K.F., Florini, D.A. and Ikotun, T. 2001. Seed treatment with *Trichoderma* species for control of damping-off of cowpea caused by *Macrophominia phaseolina*. Biocontrol Science and Technology **11**, 449-457.

Bae, Y.S. and Knudsen, G.R. 2001. Influence of fungal-feeding nematode on growth and biocontrol efficacy of *Trichoderma harzianum*. Phytopathology **91**, 301-306.

Berger, F., Li, H., White, D., Frazer, R. and Leifert, C. 1996. Effect of pathogen inoculum, antagonist density and plant species on biological control of *Phytophthora* and *Pythium* damping-off by *Bacillus subtilis* Cot1 in high-humidity fogging glasshouses. Phytopathology **86**, 428-433.

Chao, W.L., Nelson, E.B., Harman, G.E. and Hoch, H.C. 1986. Colonization of the rhizosphere by biological control agents applied to seeds. Phytopathology **76,** 60-65.

Davis, M.A. and Bockus, W.W. 2001. Evidence for *Pythium* as a yield reducer under a continuous grain sorghum. Plant Disease **85,** 780-784.

Elad, Y., Kaflon, A. and Chet, I. 1982. Control of *Rhizoctonia solani* in cotton by seed coating with *Trichoderma* spores. Plant and Soil **66,** 279-281.

El-Meligi, A.M. 1989. Effect of soil *Pseudomonas* isolates applied to corn, sorghum and wheat seeds on seedling growth and corn yield. Canadian Journal of Plant Sciences **69,** 101-108.

Genstat. 2002. Genstat Statistical Analysis Software. 6th ed. Lawes Agricultural Trust, Oxford, UK.

Georgakopoulos, D.G., Fiddaman, P., Leifert, C. and Malathrakis, N.E. 2002. Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists. Journal of Applied Microbiology **92,** 1078-1086.

Hadar, Y., Harman, G.A. and Taylor, A.C. 1984. Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soils for biological control of seed rot caused by *Pythium* spp. Phytopathology **74,** 6.

Handelsman, J., Raffel, S., Mester, E.H., Wunderlich, L. and Grau, C.R. 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Applied and Environmental Microbiology **56,** 713-718.

Harman, G.E. 1991. Seed treatment for biological control of root diseases. Crop Protection **10,** 795-798.

Harman, G.E. 1992. Development and benefits of rhizosphere competent fungi for biological control of plant pathogens. Journal of Plant Nutrition **15,** 835-843.

Howell, C.R. 2002. Cotton seedling pre-emergence damping-off incited by *Rhizopus oryzae* and *Pythium spp.* and its biological control with *Trichoderma spp.* Phytopathology **92,** 177-180.

Inglis, G.D. and Kawchuk, L.M. 2002. Comparative degradation of oomycete, ascomycete and basidomycete cell walls by mycoparasitic and biocontrol fungi. Canadian Journal of Microbiology **48,** 60-70.

Kiewnick, S., Jacobsen, B.J., Braun-Kiewnick, A., Eckhoff, J.L.A. and Bergman, J.W. 2001. Integrated control of *Rhizoctonia* crown and root rot of sugar beet with fungicide and antagonistic bacteria. Plant Disease **85,** 718-722.

Kim, D.S., Cook, R.J and Weller, D.M. 1997. *Bacillus* sp. L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology **87,** 551-558.

Koch, E. 1999. Evaluation of commercial products for microbial control of soil-borne plant diseases. Crop Protection **18,** 119-125.

Lewis, J.A. and Lumsden, R.D. 2001. Biocontrol of damping-off of greenhouse-grown crops caused by *Rhizoctonia solani* with formulation of *Trichoderma* spp. Crop Protection **20,** 49-56.

Lewis, J.A. and Papavizas, G.C. 1984. A new approach to stimulate population proliferation of *Trichoderma* species and other potential biocontrol fungi introduced into natural soils. Phytopathology **74,** 1240-1244.

Lewis, J.A, Fravel, A., Lumsden, R.D. and Shasha, B.S. 1995. Application of biocontrol fungi in granular formulations of pregelatinized starch-flow to control damping-off diseases caused by *Rhizoctonia solani.* Biological Control **5,** 397-404.

Mahaffee, W.F. and Backman, P. A. 1993. Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. Phytopathology **83,** 1120-1125.

Mao, W., Lewis, J.A., Hebbar, P.K. and Lumsden, R.D. 1997. Seed treatment with a fungal or bacterial antagonist for reducing corn damping-off caused by species of *Pythium* and *Fusarium.* Plant Disease **81,** 450-454.

Mao, W., Lewis, J.A., Lumsden, R.D. and Hebbar, P.K. 1998a. Biocontrol of selected soil-borne diseases of tomato and pepper plants. Crop Protection **17,** 535-542.

Mao, W., Lumsden, R.D., Lewis, J.A. and Hebbar, P.K. 1998b. Seed treatment using pre-infiltration and biocontrol agents to reduce damping-off of corn caused by species of *Pythium* and *Fusarium.* Plant Disease **82,** 294-299.

McLaren, N.W. 1987. Studies on pre-and post-emergence damping-off and seedling blight of sorghum. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Omarjee, J. 2002. Studies on the application of biocontrol agents for the control of seedling diseases. M.Sc. thesis, University of Natal, Pietermaritzburg, South Africa.

Papavizas, G.C. 1985. *Trichoderma* and *Gliocladium*: Biology, ecology and potential for biocontrol. Annual Review of Microbiology **23,** 23-54.

Tronsmo, A. and Hjeljord, L.G. 1998. Biological control with *Trichoderma* species. In: G.S. Boland and L.D. Kuykendall (Eds.). Plant-microbe interactions and biological control. Marcel Dekker, New York, USA. Pp. 111-126.

Zaki, K., Misaghi, A. and Shatla, M.N. 1998. Control of cotton seedling damping-off in the field by *Burkholderia (Pseudomonas) cepacia*. Plant Disease **82,** 291-293.

CHAPTER 5

FIELD EVALUATIONS OF BIOLOGICAL AND CHEMICAL TREATMENTS IN CONTROLLING DAMPING-OFF AND GROWTH STIMULATION OF SORGHUM AND TEF

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Abstract

A field trial was conducted at Ukulinga Research Farm to determine the efficacy of biological and chemical treatments on growth promotion and reduction of damping-off incited by *R. solani* and *Pythium* sp. on sorghum and tef and to evaluate the effects of a sticker on seedling emergence and disease incidence. Seeds of sorghum and tef were treated with suspensions of antagonistic *Bacillus* sp. H44 or *Trichoderma harzianum* Eco-T, or sprayed with fungicides, Benlate® or Previcur®. Application of Benlate® and Previcur® during planting significantly increased final stand and growth of sorghum seedlings ($P<0.05$). Although statistically not significant, seed treatments with H44 and Eco-T improved stand of srghum seedlings and increased plot weight of both cops incocuted with Pythium sp. However, both H44 and Eco-T had negative effects in growth stimulation and control of *R. solani*. The coating material, carboxylmethyl cellulose (CMC), had no significant effects on germination and disease levels as presented on final stand (only for sorghum) and plot weights of both crops ($P<0.05$). Water stress and weeds had massive effects in both crops regardless of treatments. The result showed that *Bacillus* H44 and *T. harzianum* Eco-T can be used as biocontrol agents against *Pythium* sp. However, repeated trials and a better understanding of the interactions between the antagonist, pathogen, the crop and their environment are needed to enhance control efficiency and growth promotion of theses potential antagonists.

5.1 INTRODUCTION

Damping-off, caused by complex soil-borne pathogens, is a common problem in almost all field and greenhouse crops (Bruehl, 1987; Georgakopoulos *et al.*, 2002). Damping-off occurs in seedlings before and after emergence, and is induced by various species of *Pythium*, *Phytophthora*, *Fusarium*, *Aphanomyces* and *Rhizoctonia* (Agrios, 1997). Seed treatments with antagonistic microorganisms have been successfully used to eliminate or supplement the use of chemical fungicides to protect germinating embryos and seedlings from soil-borne diseases (Mao *et al.*, 1998a; Nasby *et al.*, 2000; Adekunle *et al.*, 2001). Since protection of seeds or seedlings is needed only for few days or weeks after planting, biological control of damping-off is relatively simple compared to other soil-borne diseases (Georgakopoulos *et al.*, 2002). Many beneficial fungi and bacteria with a wide range of activity have been developed to control damping-off and improve stand establishment and seedling vigour (Kim *et al.*, 1997a; Handelsman *et al.*, 1990).

In field conditions, seed treatments with antagonists have increased growth and yield of a number of plants by stimulating their growth (Cook, 1985; Kim *et al.*, 1997b; Mao *et al.*, 1998b). This is one of advantages of biocontrol agents over chemical pesticides that frequently cause phytotoxicity (Elad *et al.*, 1980). However, because of their variable responses, the use of biological control agents in the field has been limited compared to fungicides (Berger *et al.*, 1996). This is partly as a result of many other factors that govern the ability of the introduced microbe to be established in the rhizosphere (Hadar *et al.*, 1984). There are several biotic and abiotic factors, including temperature, moisture, nutrients and microorganinsms, that may affect the survival, growth and activity of beneficial organisms in soil (Bae and Knudsen, 2001; Benizri *et al.*, 2001). With increased investigations, these problems have been solved by adequate knowledge of the host, pathogen and antagonists (Ayers and Adams, 1981; Cook and Baker 1983; Paulitz, 2000).

Compared to other microbial delivery techniques, seed treatment has been recommended by many authors as the best technique because it requires a very small volume of the antagonist to treat a large number of seeds (Hadar *et al.*, 1984; Harman, 1991; Mao *et al.*, 1998b). Using

this technique, microbes are coated onto seeds using adhesives such as carboxylmethyl cellulose (CMC). There is a concern, however, that certain adhesives may have inhibitory effects on seed germination (El-Meligi, 1989), or, they may exacerbate seed infection by stimulating germination of the pathogen since they have a high nutrient value (Adekunle *et al.*, 2001). Inglis and Kawchuk (2002) discovered that several fungi (including *Trichoderma* spp.) produce the enzyme, carboxylmethyl cellulase, to degrade and utilize CMC as a source of food.

The aims of this study was firstly to determine the efficacy of selected biocontrol agents (*Bacillus* sp. H44 and *T. harzianum* Eco-T) and chemical treatments in suppressing seedling diseases of grain sorghum (*Sorghum bicolor*) and tef (*Eragrostis tef)* caused by *R. solani* and *Pythium* sp. Secondly, to investigate effects of CMC on emergence and susceptibility of seeds toward soil-borne pathogens.

5.2 MATERIALS AND METHODS

5.2.1 Source of materials

Seeds of sorghum (cv. PAN8446) and tef (cv. SA Brown) were supplied by Pannar Seeds[1] and McDonald[2] Seeds, respectively. *Trichoderma harzianum* Eco-T, was obtained from Plant Health Products[3] in a formulation containing 10^8 colony forming units (c.f.u) g^{-1}. *Rhizoctonia solani* and *Pythium* sp. were provided by C. Clark[4] and *Bacillus* sp. H44 was isolated from a soil sample collected from Cedara, KwaZulu-Natal, South Africa.

5.2.2 Preparation of microbes

- **Production of antagonists:** *Bacillus* sp. H44 was grown on V8 agar for two days and then inoculated into conical flasks (250ml) containing 100ml of Tryptone Soy Broth (TSB).

[1] Pannar Seeds (Pty), P.O.Box 19, Greytown 3250, South Africa
[2] McDonalds Seeds, 61 Boshoff Street, P.O.Box 238, Pietermaritzburg, South Africa
[3] Dr. M. Morris, Plant Health Products, P.O.Box 207, Nottingham Road, South Africa
[4] C. Clark, Discipline of Plant Pathology, School of Applied Environmental Sciences, University of Natal, Private Bag X01, Scottsville, 3209, South Africa

The flask was placed in a rotary shaker (120rpm) for 48h at 28 ± 2°C. The resultant suspension was centrifuged for 20min at 9,000rpm. The broth was then decanted and the pellet of cells was resuspended in sterilized distilled water. Counting of bacterial c.f.u.s was done by incubating a serially diluted suspension for 24h on Tryptone Soy Agar (TSA) at 25-28°C. The concentration of bacterial cells was adjusted to 10^8 c.f.u. ml^{-1} of sterile distilled water. For *Trichoderma,* the microbial suspension was prepared at concentration of 10^5 c.f.u. ml^{-1} of sterile distilled water.

- **Production of pathogen inoculum:** *Pythium* sp. and *R. solani* were grown on V8 agar. Seeds of barley (100g) were put into 1ℓ Erlenmeyer flasks and autoclaved at 121°C for 15min and allowed to cool. Six mycelial agar plugs (8mm in diameter, cut from the edge of a five days old mycelial mat on V8 agar) of the pathogen were inoculated into the flasks. Flasks were incubated at room temperature for one week by shaking daily to obtain uniform growth of inoculum over all the seeds. Seeds infested with the pathogen were dried for one day and stored at 4°C until further use.

5.2.3 Trial site and land preparation

The trials were conducted at Ukulinga University Reseasrch Farm located at 29°40' E and 30°24' S, 715m above sea level, in the Southern Tall Grassveld of South Africa (Morris, 2002) on heavy, deep soil of Bonheimer clays (Dr. Melis, personal communication[5]). The trial site was previously planted by maize (*Zea mays*). Two trials were conducted during February-May 2003. However, in the second trial a commulative rainfall of 134mm within one week (i.e., between 18th–25th of March) caused waterlogging and cross contamination on the field. Hence, the results from the second trial were discarded. After running the experiment for more than two months it was not possible to repeat it any more because of the expense, arrival of winter night frosts and the end of the project. Rainfal data of the trial site for the period of January to July was obtained form Dr. Melis and temperature data recorded at the University of Natal by the Department of Agrometrology[6] (7km from the trial site). Climatic data is

[5] Dr. R. Melis, Pro-seed C.C., P.O.Box 101477, Scottsville 3209, South Africa
[6] Prof. M. Savage, Department of Agrometrology, School of Applied Environmental Sciences, Univesity of Natal, Private Bag X01, Scottsville 3209, South Africa

included in the appendix of this chapter. One week before sowing, the land was ploughed with disc plow and then irrigated. Irrigation frequency was once every week.

5.2.4 Sowing and other practices

The field was irrigated for a second time one week after ploughing. The following day, all treated/untreated seeds were planted by hand. Different treatments were applied to both crops as described below. The sorghum and tef trials were planted in two areas as discrete trials. For sorghum, each treatment consisted of 40 seeds in rows 65 cm apart with 20 cm within-row spacing. Tef seeds (15kg ha^{-1}) were uniformly sown in rows 0.25 m apart. Each plot of tef had an area of 1 m^2. Seeding depth for sorghum and tef were approximately 3-5 cm and 1-1.5 cm, respectively. Due to lack of facilities irrigation was once every week. Planting of the first trial was conducted on 26th of February and harvested on 4th (for tef) and 11th (for sorghum)of April 2003. The second trial planted on 12-13th of April, was discontinued due to weathe rdifficulties. Hand weeding was held twice, after two and four weeks after sowing.

5.2.5 Seed and soil treatments

- **Biological seed treatment:** A microbial-sticker suspension was prepared by thoroughly dissolving 2g of CMC in 98ml of microbial suspension. The mixtures of microbial-sticker were allowed to stand for 1h, to get a uniform mixture. Seeds (10g) were immersed into 10ml of the microbial-sticker suspension. The seeds were left until the solution was fully imbibed by the seeds. Treated seed were placed on filter paper, air-dried overnight and stored at 4°C until sowing. Treated seeds were sown within five days after treatment.
- **Chemical treatment:** Benlate®[7] (at 1g l^{-1}) and Previcur®[8] (at 1.5 ml l^{-1}) were sprayed during sowing as chemical treatments for species of *R. solani* and *Pythium* sp., respectively.
- **Control:** Control treatments consisted of seeds without sticker (clean seeds) or coated only with the sticker (untreated seeds) and planted in plots artificially inoculated (infested

[7] El du Pont de Nemours and Co., Wilmington, Delaware. 19898. USA
[8] AgroEvo South Africa (Pty), P.O.Box 6065, Halfway House 1685, South Africa

control) or uninfected (non-infested control) with pathogen. Due to space limits the same plots were used as controls for both th growth stimulation and biocontrol treatments.

- **Soil infestation:** To ensure uniform infestation of the field with *R. solani* and *Pythium* sp., inoculation of the soil was done by placing two infested barley seeds on two sides of each seed of sorghum, approximately 1cm apart. In tef fields, infestation was done by spreading 6g of infested barley per m^2.

5.2.6 Data collection

- **Assay systems**: In sorghum, assessments on growth stimulation and damping-off caused by *Pythium* sp. and *R. solani* were done by taking final stand and plot weights. In the case of tef, only plot weights were considered. Data on final stand and growth of sorghum were taken by counting the number of seedlings which survived six weeks after planting and cut at their base. In tef, seedlings were grown for five weeks and the central two rows were harvested leaving 25cm at each end. Harvested seedlings of the two crops were weighed directly to obtain their total wet weight. After oven drying at 70°C for two days, the dry weights of the plant material were recorded. Dry weigts of the two crops were calculated in terms of g/m^2.

- **Statistical analysis**: The trials were conducted twice. The treatments, replicated three times, were arranged in a randomized complete block design. Transformations of data with square root transformation techniques (where CV was >20%) and Analysis of Variance (ANOVA) were performed using factorial treatment structure. Interactions between treatments and contrasts among controls (clean vs. coated seeds and inoculated vs. non-inoculated) were performed using Genstat® Statistical Analysis Software (Genstat, 2002). Treatment means were separated with Fisher's protected least significant difference (LSD).

5.3 RESULTS

5.3.1 Effects of biological and chemical treatments on seedling growth

Significant differences were obtained on stand and growth of sorghum at P<0.05. Best results on seedling stands and plot weights were obtained by treatments with Benlate® and Previcur®. Application of Benlate® increased final stand and dry weights of the plots containing untreated clean seeds by 15 and 15%, respectively. Similarly, treatments with Previcur® showed increased growth of sorghum seedlings ranging from 29-32% over the control containing untreated clean seeds. In contrast, treatments with biological control agents gave the worst results (Table 5.1).

Effects of treatments were not significant on growth of tef as represented by dry weight of plots. Application of Benlate® increased dry weight of tef by 13% when compared to the control. In contrast, plots treated with Previcur®, *Bacillus* and *Trichoderma* yielded plot weights slightly lower than that of untreated controls (Table 5.2).

Table 5.1 Effects of biological and chemical treatments on final stand and growth of sorghum seedlings after six weeks of growth

Treatments	**Final stand (%)**	**Plot weight (g m^{-2})**
Control 1 (only water)	45.8 a	151.6 (12.26) ab
Control 2 (water + CMC)	36.7 b	141.5 (11.86) b
Bacillus sp. H44	15.0 c	42.0 (6.47) c
T. harzianum Eco-T	32.5 b	103.1 (10.15) bc
Benlate®	52.5 a	174.6 (13.14) ab
Previcur®	41.7 b	195.9 (13.94) a
Effects (F- tests)	**P-value**	**P-value**
Treatment	<.001***	<.001***
Contrast Control 1 Vs. Control 2	0.069	0.675
MSE	30.47	1.304.
%CV	14.8	10.1
LSD	10.043	2.077

1. * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

Table 5.2 Effects of biological and chemical treatments on growth of tef seedlings after five weeks of growth

Treatments	Plot weight (g m^{-2})
Control 1 (only water)	489.6 (21.96) a
Control 2 (water + CMC)	542.1 (23.09) a
Bacillus sp. H44	470.4 (24.50) a
T. harzianum Eco-T	488.3 (19.62) a
Benlate®	610.8 (21.45) a
Previcur®	396.1 (21.99) a
Effects (F- tests)	**P-value**
Treatment	0.659
Contrast Control 1 Vs. Control 2	0.703
MSE	12.05
%CV	15.7
LSD	6.314

1. * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

5.3.2 Effects of biological and chemical treatments on damping-off caused by *Rhizoctonia solani* and *Pythium* sp.

Treatment effect was only significant (P<0.05) on final stand of sorghum seedlings (Table 5.3 and 5.4). Significant variations due to the presence of the two pathogens was also observed on plot weight of sorghum at P<0.05. Reductions in seedling stand (by 21 and 37%) and plot weight (by 23 and 48%) of the control were recorded as a result of inoculation with *R. solani* and *Pythium* sp., respectively. In some plots, severe root rots of *Pythium* resulted in lodging and lightening of shoots. Seed treatment with *Bacillus* and *Trichoderma* improved plot weights of clean seeds (without CMC) infested with *Pythium* by 105 and 87%, respectively. Moreover, application of Previcur during planting yielded seedling stand and plot weight similar to that of biocontrol agents. In controlling *R. solani*, none of the treatments provided statistically better results over the pathogen infested-control (Table 5.3).

On tef, reductions in plot weight of the control was <10 % when plots containing clean seeds were infested with *R. solani* or *Pythium*. In contrast, when *Pythium* was infested into plots containing CMC, it caused plot weight reduction of 37%. Nevertheless, treatment effect was

not significant on seedlings of tef at P<0.05 and comparisons between the infected and uninfected controls were not significant. Moreover, none of the treatments intended to control the two pathogens gave better results over the untreated control in pathogen-infested soil. However, application of Previcur® and seed treatment with both *Bacillus* and *Trichoderma* gave improved plot weight of seedlings infested with *Pythium* sp. and application of Benlate® during sowing gave substantial control of *R. solani* (Table 5.4).

Table 5.3 Effects of biological and chemical treatments on damping-off caused by *Rhizoctonia solani* and *Pythium* sp. on sorghum seedlings after six weeks of growth

Treatments	**Pathogen**	**Final stand (%)**	**Plot weight (g m^{-2})**
Control 1 (only water)	Nil	45.8 (6.74) ab	151.6 (12.26) a
Control 1 (only water)	*R. solani*	35.8 (5.96) ab	123.5 (11.09) ab
Control 1 (only water)	*Pythium* sp.	28.8 (5.37) b	79.2 (8.86) ab
Control 2 (only CMC)	Nil	36.7 (6.05) ab	141.5 (11.86) a
Control 2 (only CMC)	*R. solani*	36.0 (5.40) b	133.3 (11.28) a
Control 2 (only CMC)	*Pythium* sp.	30.0 (5.97) ab	137.7 (11.53) a
Bacillus sp. H44	*R. solani*	14.2 (3.67) c	49.6 (6.87) b
Bacillus sp. H44	*Pythium* sp.	50.8 (7.11) a	162.7 (12.69) a
T. harzianum Eco-T	*R. solani*	26.7 (5.14) bc	93.1 (9.54) ab
T. harzianum Eco-T	*Pythium* sp.	43.3 (6.58) ab	148.3 (12.11) a
Fungicide (Benlate®)	*R. solani*	24.2 (4.61) bc	104.6 (9.37) c
Fungicide (Previcur®)	*Pythium* sp.	34.2 (5.72) ab	137.3 (11.42) a
		Treat = 0.019, MSE = 0.967 %CV = 17.3, LSD = 1.67	Treat = 0.202, MSE = 5.934 %CV = 22.7, LSD = 4.13
Effects		**P-value**	**P-value**
Pathogen		0.025*	0.377
Treatment		0.823	0.812
Sticker		0.913	0.363
Pathogen x Treatment		0.070	0.296
Pathogen x Sticker		0.295	0.427
MSE		106.9	6.935
%CV		30.0	24.4

1. * = significant at P<0.05; ** = significant at P<0.01; *** = significant at P<0.001
2. Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P<0.05)
3. Values in brackets are means of data transformed using square root transformations

Table 5.4 Effects of biological and chemical treatments on damping-off caused by *Rhizoctonia solani* and *Pythium* sp. on tef seedlings after five weeks of growth

Treatments	Pathogen	Plot weight ($g\ m^{-2}$)
Control 1 (only water)	Nil	489.6 (21.98) ab
Control 1 (only water)	*R. solani*	447.8 (21.10) ab
Control 1 (only water)	*Pythium* sp.	454.5 (21.24) ab
Control 2 (only CMC)	Nil	542.1 (23.09) ab
Control 2 (only CMC)	*R. solani.*	521.8 (22.54) ab
Control 2 (only CMC)	*Pythium* sp.	343.2 (18.51) b
Bacillus sp. H44	*R. solani*	362.1 (19.03) ab
Bacillus sp. H44	*Pythium* sp.	487.5 (21.92) b
T. harzianum Eco-T	*R. solani*	383.4 (19.48) b
T. harzianum Eco-T	*Pythium* sp.	425.0 (20.54) ab
Fungicide (Benlate®)	*R. solani.*	588.8 (24.16) a
Fungicide (Previcur®)	*Pythium* sp.	604.4 (24.30) a
		Treat= 0.140, MSE = 6.298
		%CV = 11.7, LSD = 4.249
Effects	**P-value**	**P-value**
Pathogen		0.377
Treatment		0.812
Pathogen x Treatment		0.296
Sticker		0.363
Pathogen x Sticker		0.427
		% CV = 24.4, MSE = 6.935

Means within column followed by a common letter were not significantly different according to Fisher's protected least significant difference (P=0.05)

Values in brackets are means of data transformed using square root transformations

5.3.3 Effects of adhesive on germination and disease level

For both crops, there were no significant differences in seedling variables when contrasts were made between plots containing either clean seeds or seeds coated only with the sticker (CMC). Moreover, there was no significant difference in disease level when clean or seeds coated only with CMC were planted in plots artificially infested with either *R. solani* or *Pythium* (Tables 5.3 and 5.4). Effects of the sticker on growth of seedlings and disease severity in relation to that of clean seeds are summarized on Tables 5.5 and 5.6.

Table 5.5 Effects of the sticker, carboxylmethyl cellulose, on final stand and growth of sorghum seedlings after six weeks of growth in non-infested or infested soils

Description	Final stand		Dry weight	
	Increase (%)	Reduction (%)	Increase (%)	Reduction (%)
No pathogen	-	20	-	7
R. solani	-	16	8	-
Pythium	25	-	74	-

Effects of CMC on percentage increase or decrease of seedling variables of plots were calculated by comparing the results of seeds coated only with CMC against that of clean seeds.

Table 5.6 Effects of the sticker, carboxylmethyl cellulose, on growth of tef seedlings after five weeks of growth in non-infested or infested soils

Description	Dry weight	
	Increase	Reduction
No pathogen	11	-
R. solani	17	-
Pythium	-	24

Effects of CMC on percentage increase or decrease of plot weights were calculated by comparing the results of seeds coated only with CMC against that of clean seeds.

5.4 DISCUSSION

On sorghum, seedling stand and dry matter production were taken to assess damping-off caused by *R. solani* and *Pythium* sp. However on tef, because of its high plant population, it was only practicable to use dry matter production.

Germination percentage of sorghum under laboratory conditions was more than 85% (data not given). However, emergence and survival of seedlings were low in the field even when the soil was not infested with pathogens. This indicates that seedling emergence under field conditions was reduced by a complex of factors. Possible reasons for the low percentage of seedling emergence and final stand in the field could have been ascribed to the following reasons.

Firstly, insufficient irrigation facilities: watering was only done once per week and at the time of sowing day temperatures reached up to 35-37°C. These two factors caused rapid drying of the soil less than one day after irrigation. In addition, weeds emerged soon after sowing the target crop. Control of these weeds with herbicide was not convenient for two reasons: (1) there was uncertainty on the effects of herbicides on the survival and performance of the biological control agents and target pathogens, (2) there was a fear that drift from the herbicides could cause damage to seedlings, leading to infection by several soil-borne pathogens. For these two reasons, weeds were removed manually when large enough. Allowing growth of weeds for this period (i.e. until weeding) might have exacerbated moisture stress to the plants as weeds could utilize the available water.

Secondly, since the soil is a complex ecosystem with many microorganisms, several inherent soil-borne pathogens might have caused pre- and post-emergence damping-off, resulting in lower emergence and survival of seedlings.

It is not known if seeds rotted in the soil before they germinated, or died shortly after germination because they were not strong enough to emerge above the drying soil surface. However, non-uniform emergence and continuous blighting of seedlings were observed until the end of the experiment.

- **Effects of treatments on growth stimulation of seedlings**

A significant variation on seedling stand and growth of sorghum was observed when treated seeds were planted in non-infested soils. Chemical treatments with Benlate® and Previcur® increased the survival of seedlings compared to the controls, implying that the untreated controls might have been infected by certain soil-borne pathogens. The increase in plot weights obtained from the chemical treatments over the other treatments could have been associated with the activities of these two fungicides against several soil-borne diseases.

Seed treatments with *Bacillus* or *Trichoderma* negatively affected growth of seedling of the two crops. Many microbial treatments of plants in the past have been shown to have positive or negative influence on plant growth (Lynch, *et al.*, 1991). For example, in controlled environmental conditions, seed treatments with *Pseudomonas* increased growth of sorghum seedlings (El-Meligi, 1989). In contrast, Nesby *et al.* (2000) observed slight inhibitory effects of *Trichoderma* on pea germination and growth. According to their investigations, Nesby *et al.* (2000) ascribed the negative effects to the production of volatile pentyl and pentenyl-pyrones by *Trichoderma*, which besides being fungistatic, could have phytotoxic side effects at high doses. Another possible explanation could be related to the secretion of plant growth hormones by *Bacillus* and *Trichoderma*. In large populations, it might be possible that the production of growth hormones by these two antagonists in large concentrations would result in negative responses. It should also be noted that these two antagonists were initially selected for their biocontrol activities rather than growth stimulation. Indeed, the approach would have been more fruitful if growth stimulation had been included as an *in vitro* screening criteria together with assessment of biocontrol activity.

Effects of treatments on growth of tef were not statistically different. However, trends indicates that performance of each treatment on tef resembled that of sorghum except that Previcur® negatively affected emergence of tef seedlings. This might be associated with its phytotoxic effects on the germination and emergence of this crop as opposed to sorghum.

- **Effects of treatments on damping-off caused by *Rhizoctonia solani* and *Pythium* sp.**

The main effect of Treatment was significant only in final stand of sorghum, because of the variations in disease level caused by *R. solani* and *Pythium* sp. Although both pathogens caused reductions in final stand and plot weight of the control, *R. solani* was more aggressive than *Pythium* on sorghum. Nevertheless, in controlling *R. solani* and *Pythium* sp., the treatment effect was non-significant in either crop (Tables 5.3 and 5.4). The main reason for the failure might have been linked to the lower germination and survival of seedlings on sorghum and relatively mild effects of the two pathogens on untreated plots of tef.

Since several soil-borne pathogens cause damping-off with similar symptoms, it was difficult to determine if *R. solani* and *Pythium* were the sole pathogens responsible for stand reduction on sorghum. Indeed, the contribution of environmental and soil factors was much bigger than that of these two pathogens. In addition, because the final stand of sorghum at harvest was low, it was difficult to make viable comparisons between treatments. Destructive effects of these two pathogens on sorghum could be noticed by comparing the results of individual treatments of this test with that of growth stimulation. Nevertheless, none of the control measures employed to control damping-off provided satisfactory results over the untreated seeds in pathogen-inoculated soils. Despite the statistical outcomes, if one looks at the results (Tables 5.3 and 5.4), many of the trends are similar for these two crops. Looking at the parameters of each plot, control activity of individual treatments can be noted. For example, in controlling *R. solani* in both crops, best results were obtained by application of Benlate® during sowing and worst results were obtained by seed treatment with *Bacillus* and *Trichoderma*. Both biocontrol agents gave results that were even worse than that of untreated seeds grown in soil inoculated with *R. solani*. It is not known if these organisms inhibit germination of seeds or not. However, beneficial microorganisms can have deleterious effects under specific conditions (Lynch, 2000).

For both crops, all treatments gave relatively better results compared to the untreated seeds in *Pythium*-infested soils. In the case of sorghum, highest stand was obtained by treating seeds with *Bacillus*. Seed treatment with *Bacillus* yielded plot weights of the two plants better than that of the non-infested controls and comparable to that of the fungicide, Previcur®. Similarly, *Trichoderma* was effective in controlling *Pythium* damping-off on both crops.

It was thought that the antagonists might have obtained their food (nutrient) requirements from the pathogen instead of from the seeds which would lead to inhibition of seed germination. Alternatively, some, or all, of the secondary metabolites that could have been produced and observed to cause negative effects on germination of seeds might have been used against the pathogen or diluted or inhibited due to the presence of *Pythium*. Similar explanations can also be given for the good performance of *Trichoderma* in controlling *Pythium*. A report by Kommedhal and Windels (1981) supports this theory. Working with

barley, the investigators observed that seed treatment with *Gliocladium roseum* Bain. inhibited germination of seeds by depleting their oxygen supply. However, coating of seeds of the same crop with *Azotobacter chroococcum* Beijer. overcame the inhibition of seed germination that occurred with *G. roseum* alone.

Application of Previcur® during sowing provided substantial control of *Pythium* on sorghum and excellent results on tef. The biocontrol agents were better in controlling *Pythium* than *R. solani*. This was partly due to the differences in disease severity caused by these two pathogens. Generally, *R. solani* was more destructive than *Pythium* on both plants. If the disease level caused by the target fungus or combination of several pathogens is higher than a certain level, contribution of each control measure becomes minimal and comparison between treatments usually results in non-significant variations. In contrast, if the damage caused by the target pathogen is minimal compared to the non-infested control, efficiency of various treatments in controlling the disease remains undetected. Cook and Baker (1983) have previously reported such cases.

Significant control of diseases and subsequent increases in plant development and yield have been obtained in a number of field studies. The major problem, however, is the failure to repeat these results consistently on different soils or different years in naturally contaminated fields and to make biological control of soil-borne pathogens competitive to chemical control (Schippers, 1988). Unlike glasshouse trials, where most growth requirements are controlled, field results are the outcome of several factors operating independently or in combination with each other. Possible challenges for field results are mostly associated with the knowledge of the target crop, the pathogen and the antagonist. For instance, the biocontrol efficiency of most antagonists varies depending on the level of damage caused by a target pathogen on a specific crop or cultivar. In addition, any biotic or abiotic characteristic of the soil that affects survival of the antagonist affects its biocontrol activity. For instance, Bae and Knudsen (2001) found that a fungivorous nematode, *Aphalenchoides* spp. reduced growth and biocontrol efficacy of a *T. harzianum* introduced into a soil.

Co-application of the antagonists and chemicals used in this study might have provided enhanced performance on biocontrol activity and growth stimulation of the plants. In a number of studies, synergistic effects have been observed by combining antagonists with chemical fungicides to control damping-off caused by various soil-borne fungi (Kiewnick *et al.*, 2001). The additive effects were likely due to a combination of mechanisms that affect the fungus as opposed to the efficacy provided by the fungicide or antagonist alone.

Based on the results obtained, no definitive conclusions can be made in terms of the use of *Trichoderma* and *Bacillus* for growth stimulation or control of damping-off incited by *R. solani*. However, both antagonists could provide better results in these two crops when tested against *Pythium* sp. Therefore, more trials with larger plot sizes and a better understanding of the interactions between the crop, the pathogen and the antagonists in a natural soil environment could give conclusive results. Moreover, since disease suppression and growth stimulation activities of an introduced beneficial organism depend on the amount of inoculum applied the soil, further trials are needed to determine optimum levels that gives positive results.

- **The effects of adhesive on germination and disease level**

Although certain adhesives have been reported to inhibit the germination of seeds (El-Meligi, 1989) and increase disease levels (Inglis and Kawchuk, 2002), the adhesive (CMC) used in this trial had no significant effects on emergence and survival of seedlings and disease severity. Despite the statistical information, the trends of Tables 5.5 and 5.6 show that effects of the adhesive on the two crops was variable. For instance, seed coating with CMC has beneficial effects on seedlings of sorghum infested with *Pythium*. In addition, it increased seedling growth of tef grown in non-infested soils or soils infested with *R. solani*. However, CMC had negative effects on the survival and growth of sorghum seedlings grown in non-infested soils or soils infested with *Pythium*. Moreover, it reduced seedling growth of tef grown in plots containing *R. solani*.

The trial returned non-significant results for sticker. Hence, no clear conclusions could be made on the effects of the adhesive on growth and survival of seedlings as well as disease severity. Therefore, repeated trials on a number of crops with larger plots using various adhesives at different levels of concentrations are needed to determine the contribution of the adhesive on growth of seedlings and disease incidence.

5.4 REFERENCES

Adekunle, A.T., Kardwell, K.F., Florini, D.A. and Ikotun, T. 2001. Seed treatment with *Trichoderma* species for control of damping-off of cowpea caused by *Macrophominia phaseolina*. Biocontrol Science and Technology **11,** 449-457.

Agrios, G.N. 1997. Plant pathology. 4th ed. Academic Press, London, UK.

Ayers, W.A. and Adams, P.B. 1981. Mycoparasitism and its applications to biological control of plant diseases. In: G.C. Papavizas (Ed.). Biological control in crop production. Granada, London, UK. Pp. 91-103.

Bae, Y.S. and Knudsen, G.R. 2001. Influence of fungal-feeding nematode on growth and biocontrol efficacy of *Trichoderma harzianum*. Phytopathology **91,** 301-306.

Benizri, E., Baudoin, E. and Guckert, A. 2001. Root colonization by inoculated plant growth-promoting rhizobacteria. Biocontrol Science and Technology **11,** 557-574.

Berger, F., Li, H., White, D., Frazer, R. and Leifert, C. 1996. Effect of pathogen inoculum, antagonist density and plant species on biological control of *Phytophthora* and *Pythium* damping-off by *Bacillus subtilis* Cot1 in high-humidity fogging glasshouses. Phytopathology **86,** 428-433.

Bruehl, G.W. 1987. Soil-borne plant pathogens. MacMillan, New York, USA.

Cook, R.J. 1985. Biological control of plant pathogens: theory to application. Phytopathology **75,** 25-29.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens. APS Press, St Paul, Minnesota, USA.

Elad, Y., Chet, I. and Katan, J. 1980. *Trichoderma harzianum*: A biocontrol agent effective against *Sclerotium rolfsii* and *Rhizoctonia solani*. Phytopathology **70,** 119-121.

El-Meligi, A.M. 1989. Effect of soil *Pseudomonas* isolates applied to corn, sorghum and wheat seeds on seedling growth and corn yield. Canadian Journal of Plant Sciences **69,** 101-108.

Genstat. 2002. Genstat Statistical Analysis Software. 6th ed. Lawes Agricultural Trust, Oxford, UK.

Georgakopoulos, D.G., Fiddaman, P., Leifert, C. and Malathrakis, N.E. 2002. Biological control of cucumber and sugar beet damping-off caused by *Pythium ultimum* with bacterial and fungal antagonists. Journal of Applied Microbiology **92,** 1078-1086.

Hadar, Y., Harman, G. E. and Taylor, A.G. 1984. Evaluation of *Trichoderma koningii* and *T. harzianum* from New York soils for biological control of seed rot caused by *Pythium* spp. Phytopathology **74,** 106-110.

Handelsman, J., Raffel, S., Mester, E.H., Wunderlich, L. and Grau, C.R. 1990. Biological control of damping-off of alfalfa seedlings with *Bacillus cereus* UW85. Applied and Environmental Microbiology **56,** 713-718.

Harman, G.E. 1991. Seed treatment for biological control of root diseases. Crop Protection **10,** 795-798.

Inglis, G.D. and Kawchuk, L.M. 2002. Comparative degradation of oomycete, ascomycete and basidomycete cell walls by mycoparasitic and biocontrol fungi. Canadian Journal of Microbiology **48,** 60-70.

Kiewnick, S., Jacobsen, B.J., Braun-Kiewnick, A., Eckhoff, J.L.A. and Bergman, J.W. 2001. Integrated control of *Rhizoctonia* crown and root rot of sugar beet with fungicide and antagonistic bacteria. Plant Disease **85,** 718-722.

Kim, D.S., Cook, R.J. and Weller, D.M. 1997a. *Bacillus sp.* L324-92 for biological control of three root diseases of wheat grown with reduced tillage. Phytopathology **87,** 551-558.

Kim, D.S., Weller, D.M. and Cook, R.J. 1997b. Population dynamic of *Bacillus sp.* L324-92R_{12} and *Pseudomonas fluorescens* 2-79RN_{10} in the rhizosphere of wheat. Phytopathology **87,** 599-564.

Kommedahl, T. and Windels, C. 1981. Introduction of microbial antagonists to specific courts of infection: seeds, seedlings and wounds. In: G.C. Papavizas (Ed.). Biological control in crop production. Granada, London, UK. Pp. 227-248.

Lynch, J.M., Wilson, K.L., Ousley, M.A. and Whipps, J.M. 1991. Response of lettuce to *Trichoderma* treatments. Letters in Applied Microbiology **12,** 56-61.

Mao, W., Lewis, J.A., Lumsden, R.D. and Hebbar, P.K. 1998a. Biocontrol of selected soil-borne diseases of tomato and pepper plants. Crop Protection **17,** 535-542.

Mao, W., Lumsden, R.D., Lewis, J.A. and Hebbar, P.K. 1998b. Seed treatment using pre-infiltration and biocontrol agents to reduce damping-off of corn caused by species of *Pythium* and *Fusarium*. Plant Disease **82,** 294-299.

Morris, C.D. 2002. Patch grazing of Tall Grassveld by cattle after spring burn. African Journal of Range and Forage Science **19**, 188-194.

Nesby, D.C., Pascual, J.A. and Lynch, J.M. 2000. Effects of biocontrol strain of *Trichoderma* on plant growth, *Pythium ultimum* populations, soil microbial communities and soil enzyme activities. Journal of Applied Microbiology **88,** 161-169.

Paulitz, T.C. 2000. Population dynamics of biocontrol agents and pathogens in the soil and rhizosphere. European Journal of Plant Pathology **106,** 401-413.

Schippers, B. 1988. Biological control of pathogens with rhizobacteria. In: R.K.S. Wood and M.J. Way (Eds.). Biological control of pests, pathogens and weeds: developments and prospects. Royal society, London, UK. Pp. 283-290.

Appendices

Appendix 1

Table 1. Rainfall (mm) figure of Ukulinga Research Farm for period January to June
Lat:-29°40' E and Lon:30°24' S Height: 715m

DAY	Months					
	January	February	March	April	May	June
1						
2						
3						
4			15	3		
5	18					
6		15.5				
7						
8						
9	8	5.5				
10	5.5					
11						
12						
13				58		
14				7.5		
15						
16						
17						
18			70			
19		6	12			
20		13				
21	8.5					
22						
23	22		10	14		
24	6.5	6				
25		5	42			
26						
27						
28						
29						
30						
31						
Total	68.5	51	149	82.5	0	0

Appendix 2

Average daily temperature (in °C) of Pietermaritzburg for the periods of January ro July 2003
Lat:-29.6330 Lon:30.4000 Height:672 m

	Months					
DAY	January	February	March	April	May	June
1	25.3	25.1	23.7	22.9	19.8	15.4
2	29.8	26.8	26.1	23.8	19.6	18.4
3	26	27.3	27.8	25.8	19.5	14.8
4	23.4	30.4	25.6	20.9	16.1	11.8
5	22.6	26.8	25	20.6	17	12.6
6	28	26.2	21.9	22.9	15.1	10.8
7	24.5	27.1	23.8	23.5	17.4	12.2
8	26.9	27.8	22.3	21.9	19.3	11.4
9	18.9	21.9	23.6	20.9	19.4	11.2
10	17	22.3	23.1	16.9	16.9	9.7
11	21.1	23.1	25.6	22.9	18.9	12.1
12	22.9	27.3	25.1	26.4	15.4	12.9
13	26.1	26.1	25.6	23.8	15.5	12.8
14	23	27.4	25.1	20.4	13.2	14.8
15	27.3	27.9	26.1	19.3	17.3	12.3
16	30.9	27.9	21.8	19.9	17.9	16.9
17	22.1	26.5	25.9	17.8	19.5	13.1
18	25.8	27.4	28.4	19.1	14.9	15.2
19	28.4	27.4	19.1	20.6	16.8	16.1
20	26.3	23.1	17.9	20.4	16.4	14.1
21	21.1	23.4	22.4	22.8	16.1	15.6
22	27.3	21.6	23.4	19.3	17	14
23	24.4	25.9	26.1	18.9	17.2	15.6
24	21.8	24.6	24.1	19.5	17.2	13.8
25	24.5	22.1	18.4	20.9	16.4	13.9
26	22.9	24.4	17.7	21.3	15.9	11.9
27	23.6	26.2	19.5	19.4	11.9	12.3
28	25.5	22.6	20.9	20.3	11.6	12.1
29	26.1		22.5	22.6	12.6	13.6
30	26.5		24.4	19.1	13.1	13.2
31	22.1		23.9		15.6	
Average	24.6	25.6	23.4	21.1	16.4	13.5

CHAPTER 6

GENERAL OVERVIEW

Over the past three decades, research has repeatedly demonstrated that several microorganisms can act as natural antagonists of various plant pathogens (Cook, 2000). Increased reports on the success of a biocontrol strategy in disease management, both under greenhouse and field conditions, is strong evidence for the reliability of this approach. Success with this strategy depends on the development of effective biocontrol products, which needs investigation under various environmental conditions. Experiments conducted in this research were aimed at selecting and utilizing biocontrol agents and are summarized below.

6.1 ISOLATION AND SCREENING OF POTENTIAL BIOCONTROL AGENTS

Bacillus and *Trichoderma* are ubiquitous in nature and can be isolated from soil or plant roots by heat treatment for a few minutes (for *Bacillus*) or plating directly onto basic or selective media (for *Trichoderma*). Biocontrol efficiency of antagonists is increased if they are isolated from the soil in which they are going to be re-inoculated (Weller, 1988). Cook and Baker (1983) recommend that suppressive soils are ideal sources of effective antagonists.

In the process of biocontrol development, screening is the most critical step. This is because the success of all subsequent stages depends on the ability of a screening procedure to identify an appropriate candidate (Whipps *et al.*, 1988). Biocontrol agents are usually screened based on their interaction with the test-pathogen. Antagonists used in this thesis were screened based on their ability to inhibit *in vitro* growth of the test-pathogens (*R. solani* and *Pythium* sp.). The bacterial antagonists were selected for their reproducible antifungal activities and the presence of endospores. For *Trichoderma,* isolates were screened based on dual culture tests together with ESEM observations. In controlling *in vitro* growth of *Pythium*, *T. harzianum* Eco-T employed antibiosis and mycoparasitism. *Trichoderma* spp. SY3 and SY4 inhibited the growth of *Pythium* sp. through mycoparasitism. The ability of Eco-T to use more than one mode of action makes it superior to SY3 and SY4.

In vitro screening methods usually identify biocontrol agents that act through antibiosis and parasitism and rarely through competition. For that reason, the actual disease control mechanisms under field conditions are not fully understood. In laboratory conditions, screening for competition can be done by assessing the potential of the antagonist to colonize sterilized soil quickly and to exclude other microorganisms by preempting available nutrients.

Numerous isolates can inhibit *in vitro* growth of pathogens, fewer can suppress plant diseases under diverse growth conditions and very few of them still have a broad-spectrum of activity against multiple pathogens (Weller, 1988). This is because the relationship between *in vitro* biocontrol activity and disease suppression under natural conditions is limited (Deacon, 1991). Nonetheless, intensive *in vitro* screenings have initiated the development of a number of commercial biocontrol products.

Although considerable progress has been made in the use of beneficial microbes for growth stimulation of plants, little work has been done in screening beneficial microbes for this purpose. Most of the growth promoters currently in development are believed to have initially been screened for their biocontrol activities rather than growth stimulation. The reasons being that the assessment for biocontrol activities are quicker than that for growth promotion. In my research, failure of the bacterial and fungal isolates to enhance plant growth in the field was assumed to result from the initial screening objectives. Based on the findings of this thesis it is recommended that screening for growth promotion must follow a separate approach. It can be done by comparing growth of a plant inoculated with a potential growth-promoting organism against an uninoculated control.

6.2 UTILIZATION OF BIOCONTROL AGENTS IN GREENHOUSE CONDITIONS

The controlled environmental conditions in a greenhouse make biological control of plant diseases relatively simple and effective. This is because most environmental factors such as temperature, light, moisture and soil composition that govern the survival and activities of biocontrol agents can be partially regulated.

In controlling *R. solani* on sorghum, Eco-T, SY3 and H44 were as effective as the standard fungicide Benlate®. Control efficiency of most biocontrol agents was also comparable to Previcur® in controlling damping-off of sorghum incited by *Pythium* sp. On tef, *T. harzianum* Eco-T controlled damping-off caused by *Pythium* by yielding plot weight similar to that of the uninfected control. However, efficiency of all antagonists was poor in controlling *Rhizoctonia* on tef. In general, sorghum was more responsive to biological and chemical treatments than tef.

The potential of biocontrol agents used in controlling damping-off incited by *R. solani* and *Pythium* might have been improved by mixing different antagonists (preferably *Bacillus* with *Trichoderma*) or co-application with chemical fungicides. Use of appropriate formulations may also enhance their performance.

6.3 METHOD OF APPLICATIONS OF BIOCONTROL AGENTS

Success with biocontrol strategy is obtained by the application of an effective antagonist in an appropriate delivery method. Biocontrol agents are normally introduced into the soil with the plant material, seed, or directly by mixing them with the soil. Different application methods may be preferred based on the ability of the antagonist to colonize a given area over a desired period of time, the biology of the target pathogen and the amount of biocontrol agent to be applied. Compared to other microbial delivery methods, application of biocontrol agents as seed coatings is easy and economical. In my research, it was found that biocontrol efficiencies of all antagonists were similar whether they were applied as a drench or seed coating. However, different adhesives used for coating the beneficial microbes onto seeds have been found to have some detrimental effects on the emergence of seeds and the survival of seedlings (El-Meligi, 1989). Nevertheless, effects of the adhesive material carboxylmethyl cellulose (CMC) used in this study had no detrimental effects on sorghum or tef in terms of emergence of seedlings and disease severity.

6.4 FIELD EVALUATION OF BIOCONTROL AGENTS

The biological control of plant diseases involves the complex interaction between the host, pathogen, antagonist and environment. Unlike a greenhouse, field conditions are variable and unpredictable. Fluctuating abiotic factors, such as soil moisture, temperature and pH, day length, microclimate, rainfall, as well as biotic factors including other soil inhabitants all impact on the prospective BCA (Megan, 2001). Hence, disease control efficiency of an introduced antagonist into the soil is the result of all the above interactions.

In field trials of this study, *Bacillus* sp. H44 and *T. harzianum* Eco-T showed effective control of *Pythium* on sorghum and substantial control on tef. However, they had negative effects on seedling emergence and the final stand of the two plants when they were tested for their growth stimulation and biocontrol activity against *R. solani*. The detrimental effects of these antagonists were thought to have been linked to the concentration of the biocontrol agents applied and the nature of the compounds they release. Application of biocontrol agents at a higher concentration may facilitate the secretion of various compounds that may have phytotoxic effects on the seed or seedlings other than controlling minor and major soil-pathogens. The appropriate concentrations of these isolates that can provide effective disease control with minimal or no damage to the plant at a reasonable cost and the nature of the compounds that were assumed to be secreted by these antagonists, remains to be uncovered in the future.

Under natural field conditions disease is rarely caused by a single pathogen. However, with the exception of a few, most antagonists are limited in ther spectrum of action. In addition, weeds and insects contribute to considerable yield loss if they are not managed properly. Therefore, effective crop protection management needs the application of several control measures with varying modes of action. This calls for the use of integrated pest management (IPM) as an effective disease management strategy.

6.5 FUTURE PERSPECTIVES

A number of microbes with the potential for disease control and plant growth promotion have been isolated and screened by the Department of Plant Pathology, University of Natal, South Africa. More research is being undertaken in this field and it seems likely that more efforts will be needed to diversify the potential applications of beneficial microbes. There is a possibility of utilizing molecular technologies to promote biological control that will be effective in disease control strategy.

There is a need to investigate the interactions among pathogens, the plant and the antagonists in different agricultural systems (Kerry, 2000). It is by means of thorough research that one will be able to determine whether biocontrol will replace or supplement existing crop protection strategies. Moreover, although a market exists for biocontrol products, considerable progress is still needed on the technical, agronomic, socio-economic and political issues. Still more studies on the practical aspects of mass-production and formulation need to be undertaken to make new biocontrol products stable, effective, safe and more cost-effective. In addition, the successful utilization of a biocontrol product requires adequate knowledge on how, when and where to apply it. For this purpose, the user (farmer) must be trained or be made fully aware of storage requirements and the safe and effective use of the product.

Pesticide bans (Whipps and Lumsden, 2001) and grower's interest in alternative disease control measures (Finch, 1992) suggests that the market potential of biocontrol products will increase in coming years. However, the future success of the biological control industry will entirely depend on innovative business management, product marketing, extension education and research (Mathre *et al.*, 1999).

Based on the findings of the research and the foundations laid out in various parts of this thesis, a forecast on the research needs in various areas of the field is as follows:

6.5.1 Isolation of beneficial microorganisms from the soil

- Initially, microbes must be isolated from an area where they are going to be re-inoculated, e.g., from a sorghum rhizosphere if sorghum is the target crop;
- Where isolation of antagonists with a wide-spectrum of action is needed, suppressive soils are ideal sources and therefore, isolation from such soils can ease the screening procedures;
- Heat treatment that was used in this thesis may be a quick method to select only endospore forming-*Bacillus* sp. However, spores in a natural environment may posses different heat sensitivities from those which have been cultured following such treatment. Therefore, a more precise technique is needed to isolate antagonists without affecting their characteristics. This may be done by the use of selective media.

6.5.2 Screening of biocontrol agents

- Screening is needed for competence and induction of host resistance;
- Where screening for biocontrol agents is required to be exerted via hyphal interactions, it should be carried out under nutrient conditions that at least approximate the existing environment in which biocontrol is to be used;
- There is a need to develop an *in vitro* screening protocol that includes interactions of the pathogen, antagonist and the host;
- When assessing the effect of the antagonist on the target pathogen, information should be sought on the susceptibility of the biocontrol agent to attack by major components of the soil microbial populations;
- There is a need to develop a discrete screening approach for microorganisms intended for growth stimulation.

6.5.3 Biocontrol trials in greenhouse

- Good disease control potential was observed when *Bacillus* and *Trichoderma* were tested against *R. solani* and *Pythium* sp. especially on sorghum;
- I therefore, suggest diversified trials against a number of pathogens on various crops in other controlled growth conditions such as hydroponics and growth chambers;
- Research is needed to investigate the possibility of mixing antagonists or with chemicals for disease control and growth stimulation under controlled environmental conditions.

6.5.4 Biocontrol trials in the field

- Detailed information on the history of the field, especially on cropping sequences and on physical and chemical characteristics of the soil are needed before attempting trials;
- Knowledge is required on the behaviour of the biocontrol agent in the field. In particular, the short- and long-term population dynamics of the antagonist and the pathogen;
- Assessment is needed on levels of pathogen inoculum and concentrations of the antagonist that should be applied in the field to demonstrate disease control efficiency;
- More fieldwork is needed to demonstrate efficiency and cost effectiveness of biocontrol agents in disease management;
- Consideration should be given for possible integration of biocontrol strategies with existing crop management programs;
- Following appropriate cultural practices (soil preparation, time of planting, irrigation regime, fertilization, etc) often has a significant impact on disease severity and control efficiency of the antagonist. Therefore, consideration should be given to cultural practices to maximize biocontrol efficiencies of introduced antagonists.

6.5.5 Production and marketing of biocontrol products

Before developing an effective biocontrol product the following points must be fulfilled.

- Crops are grown under a variety of climatic and environmental conditions including temperature, rainfall and soil type. Additionally, crop variety and pathogens change from

farm to farm or sometimes even within one field. Therefore, for effective field performance, formulations must have the ability to tolerate a wide range of climatic and biotic factors;

- Assessments on different formulations, packaging and storage conditions are needed to stabilize and extend the shelf-life of the product;
- Production costs need to be reduced to market the product at a price competitive with standard fungicides;
- Many failures in the use of biocontrol products for disease control have been linked to strong pressure to market biocontrol agents before they are fully tested for their efficacy. It is therefore recommended that biocontrol agents be adequately investigated before they are released. It would be advisable if growers test the product and use their feedback so that unexpected problems and failures can be avoided;
- Users need to understand the limitations of biocontrol agents. It is important to realize that biocontrol agents are not like chemicals, and the user should not expect fast, complete disease control, and that accepting certain levels of crop damage is a given;
- Scientific, regulatory and marketing issues must all be assessed effectively for a biocontrol product to be successful in the market.

6.6 CONCLUSION

The result of this study demonstrated that seedling diseases caused by *R. solani* and *Pythium* are major problems to the final stand of sorghum and tef if they are not properly managed. Most of the antagonists used in various parts of this thesis seemed to have the potential biocontrol agents against damping-off incited by *R. solani* and *Pythium* sp. Under laboratory conditions, all the selected bacterial and fungal antagonists inhibited *in vitro* growth of the two pathogens by antibiosis, mycoparasitism, or both. Biocontrol mechanisms other than antibiosis and mycoparasitism remain to be uncovered in the future. Under greenhouse conditions, biocontrol activities of some biocontrol agents were comparable to that of standard fungicides. Although field performance of the best isolates was good in controlling *Pythium*, they had negative effects when they were tested for their growth promotion and biocontrol activity against R *solani*. Under greenhouse and field conditions, the response of sorghum to various

treatments was better than that of tef. Therefore, there is a need for multiple trials and a thorough understanding on the interactions among the pathogens, the plant and the antagonist under a variety of conditions. It is after a thorough research that we can conclude whether these selected biocontrol agents can effectively control soil-borne diseases responsible for root and seedling diseases of plants. However, biological control should not be seen as a control method that will completely replace chemical control. It should be used in combination with other control measures including chemicals. In order to serve agriculture as well as the environment and human health, we should harvest the best from both to develop effective IPM methods (Kerry, 1992).

6.7 REFERENCES

Cook, R. J. 2000. Advances in plant health management in the 20th century. Annual Review of Phytopathology **38,** 95-116.

Cook, R.J. and Baker, K.F. 1983. The nature and practice of biological control of plant pathogens. APS Press, St Paul, Minnesota, USA.

Deacon, J.W. 1991. Significance of ecology in the development of biocontrol agents against soil-borne plant pathogens. Biocontrol Science and Technology **1,** 5-20.

El-Meligi, A.M. 1989. Effect of soil *Pseudomonas* isolates applied to corn, sorghum and wheat seeds on seedling growth and corn yield. Canadian Journal of Plant Sciences **69,** 101-108.

Finch, S. 1992. Appraisal of integrated pest management systems for controlling insects of vegetable crops. In: J.C. van Lateren, A.K. Minks and O.M.B. de Ponti (Eds.). Biological control and integrated crop protection: towards environmentally safer agriculture. Podac Scientific, Wageningen, The Netherlands. Pp. 49-60.

Kerry, B.R. 1992. The biological control of soil-borne pests and diseases. In: J.C. van Lateren, A.K. Minks and O.M.B. de Ponti (Eds.). Biological control and integrated crop protection: towards environmentally safer agriculture. Podac Scientific, Wageningen, The Netherlands. Pp. 117-124.

Kerry, B.R. 2000. Rhizosphere interactions and the exploitation of microbial agents for the biological control of plant-pathogenic fungi. Annual Review of Phytopathology **38,** 423-441.

Mathre, D.E., Cook, R.J. and Callan N. W. 1999. From discovery to use: traversing the world of commercializing biocontrol agents for plant disease control. Plant Disease **83,** 972-983.

Megan, N. 2001. Physiological approaches to improving the ecological fitness of fungal biocontrol agents. In: T.M. Butt, C.W. Jackson and N. Megan (Eds.). Fungi as biocontrol agents: progress, problems and potential. CABI, Wallingford, UK. Pp. 239-251.

Weller, D.M. 1988. Biological control of soil-borne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology **26,** 379-407.

Whipps, J.M. and Lumsden, R. 2001. Commercial use of fungi as plant disease biological control agent: status and prospects. In: T.M. Butt, C.W. Jackson and N. Megan (Eds.). Fungi as biocontrol agents: progress, problems and potential. CABI, Wallingford, UK. Pp. 9-22.

Whipps, J.M., Lewis, K. and Cook, R.C. 1988. Mycoparasitism and plant disease control. In: M.N. Burge (Ed.). Fungi in biological control systems. Manchester University Press, Manchester, UK. Pp. 161-187.