UNIVERSITY OF
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BIOCONTROL OF THREE FUSARIAL DISEASES

BY

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ABSTRACT

Over the past one hundred years, research has repeatedly demonstrated that phylogenetically diverse microorganisms can act as natural antagonists of various plant pathogens. Interest in biological control research continues reflecting the desire of multiple constituencies to develop sustainable methods for controlling plant diseases.

The review of the literature comprises information on the epidemiology, economic importance and the different control options available against Fusarial diseases of cabbage, maize and pine, and the safety of microorganisms intended for use as biocontrol agents, their management and strategy of control.

Trichoderma and *Bacillus* isolates used as biocontrol agents were obtained mainly from the rhizosphere of cabbage, maize and pine with a view that they would be adapted to those habitats where they would eventually to be used as innundative biocontrol agents.

Preliminary selection was made based on *in vitro* antagonism of those isolates towards *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) W.C. Snyder & H.N. Hans. Ultrastructural studies of mycoparsitism of *Trichoderma* Isolates ET23, ET13 and *Trichoderma harzianum* Eco-T® which caused significant reduction in disease incidence and severity on later study under greenhouse conditions, were investigated on the vascular pathogen, *F. oxysporum* f. sp. *conglutinans*. Although the mode of action of the three isolates was not fully elucidated, certain mechanisms such as mycoparasitism and antibiosis or production of antimicrobial substances, which cause cell wall degradation and lysis, have been identified.

Twenty *Trichoderma* and 18 *Bacillus* isolates which showed antagonism towards *F. oxysporum* f. sp. *conglutinans* were tested against the same pathogen on cabbage under greenhouse conditions. *Trichoderma* isolates were delivered to the soil in two different ways, i.e., seed treatment and drenching, while *Bacillus* isolates were only drenched as spore suspensions. More than two-third of the biocontrol isolates caused significant reductions in disease incidence and severity of the vascular wilt disease. Application of

Trichoderma isolates by drenching resulted in better control of the disease than when applied as a seed treatment.

Of the 38 *Bacillus* and *Trichoderma* isolates tested against the cabbage yellows fungus, three *Trichoderma* and four *Bacillus* isolates were selected for further testing against *Fusarium* sp. and *Rhizoctonia solani* Kühn on maize and *Fusarium circinatum* on *Pinus patula* seedlings. Since none of the *Fusarium* isolates obtained from diseased kernels and cobs of maize were pathogenic to the two maize cultivars, yellow maize and PAN 6479, provided by Pannar® seed company, biocontrol experiments on *Fusarium* diseases of maize could not be conducted. Only *Trichoderma* Isolate ET23 and *T. harzianum* Eco-T® were found to significantly control Rhizoctonia preemergency damping-off on maize while none of the *Bacillus* isolates caused significant increase in seedling emergence. In the test against *F. circinatum* on pine, in most cases, significant reduction in seedling mortality was observed in the first 4 to 8wk, however, after 12wk they were no longer effective.

Improvement in the survival of pine seedlings were observed when *T. harzianum* Eco-T® was applied prior to the introduction of *F. circinatum*. There was almost a direct relationship between the inoculation time and percentage of survival of seedlings. Prior inoculation gives the biocontrol agent time to colonize the potential infection courts for the pathogen in the root area and to be established in the rhizosphere of the pine seedlings.

It has been reported that the inconsistent and poor performance of biocontrol agents in the field can be improved with the use of mixtures of biocontrol agents to mimic the naturally suppressive soils which comprise numerous saprophytic microorganisms. However, these organisms have co-evolved for many years that they are adapted to live together in the same soil ecosystem. Therefore, when combinations of biocontrol organisms are used, the compatibility between these isolates is important.
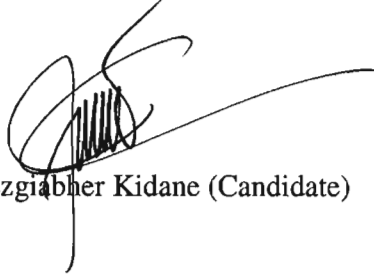
Compatibility tests between and among *Bacillus* and *Trichoderma* isolates were carried out *in vitro*. The tests revealed that the *Bacillus* and *Trichoderma* isolates are not all compatible. *Trichoderma* Isolate ET13 showed antagonism towards Isolates ET23 and *T. harzianum* Eco-T®; *Bacillus* Isolates B81 and BFO11 were slightly antagonistic to Isolates EXR and JRO1, and Isolate JRO1 was slightly antagonistic to Isolate EXR.

Comparisons of single versus mixtures of *Bacillus* or *Trichoderma* isolates showed that mixtures of *Bacillus* or *Trichoderma* isolates did not result in significantly greater reduction in disease incidence and severity of cabbage yellows.

DECLARATION

The work presented in this thesis represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to another university. Where use has been made of the work of others, it is acknowledged in the text.

Signed:

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ACKNOWLEDGEMENT

DEDICATION

To my mother, Hiwet F're, and my brothers and sisters

FOREWORD

Biocontrol of plant pathogens, insects and weeds is currently the most prominent research area at the Discipline of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg. All laboratory, electron microscopy and greenhouse studies in this thesis were carried out at the University of KwaZulu-Natal, Pietermaritzburg, Republic of South Africa.

Few areas of research and education within plant pathology have attracted more interest during the past three decades than the use of introduced microorganisms for biological control of plant pathogens. Biological control is now an established sub-discipline in the science of plant pathology. The heightened scientific interest in biological control of plant pathogens is a response, in part, to growing public concern over chemical pesticides. However, there is an equally great, or greater, need for biological control of pathogens, such as many *Fusarium* spp., that presently go uncontrolled or only partially controlled.

The main objectives of the research in this thesis were to produce solutions to the problems incited by soilborne diseases, particularly Fusarial diseases, by isolating beneficial resident antagonists and screening them against different pathogens on different crops to find the best possible candidates with broad spectrum activity against different soilborne pathogens and improve their efficacy of control, which has usually been characterized as inconsistent compared to chemical fungicides.

The scope of this research is broad, traversing seven chapters.

Chapter One is a review of the literature on the epidemiology, economic importance and control options available against different soilborne pathogens, with particular reference to Fusarial diseases, and the safety of microorganisms intended for use as biocontrol agents, their management and strategy.

Chapter Two covers on the isolation and *in vitro* screening of different *Trichoderma* and *Bacillus* isolates, and ultrastructural studies on *Trichoderma* isolates which showed antagonism during whole plant tests in the greenhouse.

Chapter Three reports on greenhouse evaluation of *Trichoderma* and *Bacillus* isolates selected from *in vitro* screening tests against *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) W.C. Snyder & H.N. Hans. on cabbage.

Chapter Four encompasses isolation and pathogenicity tests of *Fusarium* spp. on maize and biocontrol studies on *Rhizoctonia solani* Kühn on maize using *Trichoderma* and *Bacillus* isolates selected from previous greenhouse screening tests against *F. oxysporum* f. sp. *conglutinans* on cabbage.

Chapter Five contains evaluation of selected *Trichoderma* and *Bacillus* isolates against *F. circinatum* on pine seedlings and effect of application time in improving biocontrol efficacy of *Trichoderma harzianum* Eco-T® Rifai.

Chapters Six reports on the comparison of mixtures of *Trichoderma* or *Bacillus* isolates versus their single application against *F. oxysporum* f. sp. *conglutinans* on cabbage.

Chapter Seven reviews the experimental results, conclusions and recommendations for future research on the isolation, screening and improving control efficacy of biocontrol microorganisms.

CHAPTER ONE

LITERATURE REVIEW

1.1 The Genus *Fusarium*

The genus *Fusarium* may have been one of the earlier fungi to become established on earth. *Fusarium* is well adapted to habitats on earth (Snyder, 1981), and occurs in such diverse environments as the arctic and deserts (Nelson, 1981). Fusaria are among the most common soilborne fungi. Populations of Fusaria in agricultural fields can be greater than 100, 000 g^{-1} of soil (Smith, 1971).

The importance of *Fusarium* to world food production has greatly increased in recent decades. The genus has emerged as a leading threat to most of the principal food crops of the world, as well as to oil, fibre, ornamental and forest plantings. It seems evident, then, that *Fusarium* is a growing threat to the world crops, more serious now with the shortage of food than at any time before (Snyder, 1981). Essentially, *Fusarium* causes two types of plant diseases: cortical rots and vascular wilts. Vascular wilts have been the most destructive for they jeopardized, during the first part of the twentieth century, many agricultural industries in the world. Currently, *Fusarium* cortical and root rot has become more important and two of the most prominent of this category are the *Fusarium* disease of maize, which is a staple crop for mankind outside the lowland tropics (Toussoun, 1981) and *Fusarium* pitch canker of pine (Wingfield *et al.*, 2002).

1.1.1 *Fusarium* Wilt of Cabbage (Cabbage Yellows)

1.1.1.1 Introduction

Fusarium wilts are widespread, destructive plant diseases, which can cause severe losses on most vegetables and flowers, several field and plantation crops, and a few shade trees. The vascular wilt pathogen in this genus is *F. oxysporum*. Different host plants are attacked by special forms or races of the fungus (Agrios, 1997). According to the Snyder & Hansen (1940) taxonomic system, *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) W.C. Snyder & H.N. Hans belongs to the section Elegans. *Fusarium oxysporum* may be further

subdivided into races based on the pathogenicity of an isolate to certain cultivars of the host plants. Yellows of cabbage, *Brassica oleracea* L. var. *capitata* L., is caused by Races 1 and 2 of *F. oxysporum* f. sp. *conglutinans* (Bosland & Williams, 1986). Fusarium wilts of Cruciferae and Chenopodiacea have been common in many parts of the world (Snyder & Smith, 1981). The most widely distributed of these diseases is perhaps cabbage yellows, which occurs on all continents (Booth, 1971).

1.1.1.2 Symptoms

Plants can be affected at any age (Walker, 1957) but the fungus first manifest itself in the field on very young seedlings (Fowler & Pound, 1953; Walker, 1971; Agrios, 1997). A lifeless yellow-green colour on the lower foliage indicates initial symptom of cabbage yellows. Sometimes the yellowing is uniform, but more often unilateral, i.e., more intense on one side of the leaf and plant, causing a lateral warping or curling of the leaves and stem. Yellowing starts with the lower leaves and progresses upward. Old yellowed tissue turns brown and becomes dead and brittle (Walker, 1957). Fowler & Pound (1953) described that the stem at first remains turgid and, when the entire plant is dead, the stiffened stem often stands erect with the leaves attached but drooping. Premature leaf drop and retarded plant growth becomes distinct. The vascular ring or a single vascular band of the root, stem, hypocotyls, and leaf petiole become discoloured (Fowler & Pound, 1953; Walker, 1971; Agrios, 1997). The vascular system turns yellow to dark brown (Smith & Walker, 1930a). The disease may be confused with black rot since it resembles black rot in some respects. However, in the case of black rot the veins become black rather than brown as in the case of cabbage yellows and the smaller veins of diseased leaves are much more generally discoloured (Walker, 1957).

1.1.1.3 Disease Cycle and Epidemiology

Like most *formae speciales* of *F. oxysporum*, *F. oxysporum* f. sp. *conglutinans,* survives unfavourable conditions as mycelia and in all its spore forms, but most commonly as chlamydospores in the soil and in decaying host tissue, until stimulated to germinate (Smith & Snyder, 1975; Phipps & Stipes, 1976; Nelson, 1981).

Many *formae speciales* of *F. oxysporum* penetrate host tissue directly and do not require a wound. *Fusarium oxysporum* f. sp. *conglutinans* Race 1 penetrates cabbage tissues intercellularly in the apical meristematic regions of the root, the zone of elongation, and

occasionally through root hairs (Smith & Walker, 1930a). As described by Anderson & Walker (1935), direct intercellular and intracellular penetration occurred equally often, both in the young root and in the hypocotyls, whereas in the root cap, penetration was generally intercellular. In injured roots, the fungus can enter through both meristematic and permanent tissue (Nelson, 1981).

Smith & Walker (1930a) showed that in the primary and secondary roots of susceptible cabbage plants, *F. oxysporum* f. sp. *conglutinans* Race 1 grew into the vascular system of young roots. Most often the fungus was confined to the xylem vessel elements and xylem parenchyma cells, but in a few secondary roots the fungus was in the cortex. Only a few vascular bundles of the stem were infected.

In advanced stages of disease development, the fungus grows out of the vascular system and adjacent parenchyma tissue and produces conidia and chlamydospores (Kommedahl *et al.*, 1970, *cited by* Nelson, 1981; Phipps & Stipes, 1976). The chlamydospores eventually find their way to the soil when the plant dies, and persist in plant debris where they may remain dormant and viable for several years (Kommedahl *et al.*, 1970, Stover, 1970, *cited by* Nelson, 1981). *Fusarium* wilt of cabbage can also survive for a long time in the absence of susceptible host plants by invasion and colonization of plants other than cabbage. Various wild and cultivated plants are symptomless carriers of several soil pathogens (Armstrong *et al.*, 1942; Armstrong & Armstrong, 1948; Hendrix & Nielsen, 1958; Katan, 1971; Smith & Snyder, 1975). The life cycle is repeated when the chlamydospores germinate and growth occurs either saprophytically or by the invasion of a suitable host plant (Nelson, 1981).

Dissemination of *Fusarium* wilt pathogens occurs in a variety of ways. Man (Kommedahl *et al.*, 1970), irrigation or flood-waters, animals and wind (Kommedahl *et al.*, 1970, Stover, 1970, *cited by* Nelson, 1981) move infested soil and plant parts from one area to another. Ooka (1975), *cited by* Nelson (1981), found randomly isolated fungal colonies from the soil from North Dakota which settled on snow deposited during a blizzard in January 1975 in St. Paul, Minnesota, of which 11% were *F. oxysporum*.

1.1.1.4 *Fusarium* Nematode Interaction

Many disease complexes involve a *Fusarium* wilt pathogen and nematodes, particularly root-knot nematodes (Powell, 1971). Root-knot nematodes and other nematodes increased the incidence of disease development in tomato, tobacco, pea, cotton, etc. (Holdeman & Graham, 1954; Jenkins & Coursen, 1957; Davis & Jenkins, 1963; Porter & Powell, 1967; Mitchell & Powell, 1972). However, root-knot nematodes did not affect the incidence of *Fusarium* wilt on some plants. Fassuliotis & Rau (1969), *cited by* Nelson (1981), found that none of the three inoculum level combinations of *Meloidogyne incognita-acrita* Chitwood and *F. oxysporum* f. sp. *conglutinans* Race 1 affected the incidence of cabbage yellows in a moderately resistant cultivar, Marion Market.

1.1.1.5 Economic Importance

Of all the diseases caused by *Fusarium,* probably the most important are the vascular wilt diseases caused by *formae speciales* of *F. oxysporum* (Nelson, 1981). Vascular wilt diseases are highly destructive, whether they occur in cultivated crops or in indigenous species. Losses are often such that it is no longer profitable, sometimes even impossible, to continue to grow the crop without effective control of the disease (Green, 1981).

Cabbage yellows disease was important in the Middle West of the U.S. for many years (Anderson, 1933). Though a resistant variety, "Wisconsin Hollander", was introduced for general use in 1916, the disease was unusually destructive in the same year (Jones *et al.*, 1920, *cited by* Anderson, 1933). Several fields of Wisconsin Hollander showed a high incidence of the disease averaging to 24.3% (Anderson, 1933). *Fusarium oxysporum* f. sp. *conglutinans* reportedly caused severe losses on white cabbage in the Volga-Akhtubinsk floodlands of the USSR (Zaitseve-Katarzhina *et al.*, 1973, Vladimershaya *et al.*, 1975, *cited by* Snyder & Smith, 1981) and on mustard in the Uttar Pradish Province of India (Rai & Singh, 1973).

1.1.2   *Fusarium* Pitch Canker of Pine

1.1.2.1 Introduction

Species of *Fusarium* have world wide distribution and pathogenic species have been recorded from a large number of hosts. They are well-known pathogens in forest nurseries in many parts of the world (Bloomberg, 1981; Viljoen *et al.*, 1992). This group of fungi are

associated with diseases such as seed decay, damping-off, root rot, and stem cankers (Rathbun, 1922; Pawuk, 1978; Barnard & Blakeslee, 1980; Sutherland & van Eeden, 1980; Morgan, 1983; Huang & Kuhlman, 1990). *Fusarium* spp. are particularly damaging to conifer seedlings such as Douglas-fir and pine (Bloomberg, 1981; Huang & Kuhlman, 1990; James *et al.*, 1991) and are mainly associated with root diseases.

Pitch canker of pine, caused by *Fusarium circinatum* Nirenberg and O'Donnell (= *F. subglutinans* [Wollenw. and Reinking] Nelson *et al.* f. sp. *pini* Correll *et al.*), was first reported in North Carolina in 1946 (Hepting & Roth, 1946, *cited by* Dwinell *et al.*, 1985), where it remains a chronic problem in plantations, seed orchards, and nurseries (Dwinell *et al.*, 1985). The disease was reported from California in 1986 (McCain *et al.*, 1987, *cited by* Gordon *et al.*, 1998), mainly from *Pinus radiata* D. Don planted in landscape settings (Correll *et al.*, 1991) and has since been reported in Mexico (Rodriquez, 1989, *cited by* Gordon *et al.*, 1998), Japan (Muramoto and Dwinell, 1990) and South Africa (Viljoen *et al.*, 1994).

1.1.2.2 Symptoms

The earliest typical symptoms of pitch canker on pines are usually the wilting and fading of the needles near the tip of young branches (Gordon *et al.*, 1998). In nurseries, diseased pine seedlings show chlorotic or reddish brown needles and wilting. Pitch-soaked lesions usually occur at or near the soil line but occasionally are found in the region of the cotyledonary node (Barnard & Blakeslee, 1980).

A more advanced symptom of the disease is the appearance of bleeding, formation of resinous canker on the trunk, terminals, or large branches (Hepting & Roth, 1946, *cited by* Dwinell *et al.*, 1985). The canker is usually sunken and the bark is retained, while the wood beneath the canker is deeply pitch-soaked (Dwinell *et al.*, 1985). Foliage becomes yellow, then red and falls from the branch (Dwinell *et al.*, 1985). Repeated infection of the plant leads to extensive die-back in the canopy (Gordon *et al.*, 2001). Infected pine cones tend to be mishappen and smaller than normal, and scales on green cones at harvest have purple discoloration (Dwinell *et al.*, 1985).

1.1.2.3 Disease Cycle and Epidemiology

Fusarium circinatum is well known to survive in and on pine seeds (Barrows-Broaddus & Dwinell, 1985; Storer *et al.*, 1998). Seed appears a likely medium of transport. Wingfield *et al.* (1999) proposed that pitch canker of pine may have reached South Africa on infected pine seeds from Mexico. Insects also are suspected to be vectors or wounding agents. For instance, in California, most infections appear to result from feeding of various insect associates of conifers that carry the pitch canker pathogen. The principal insect vectors include: engraver beetles, *Ips* spp. (Fox *et al.*, 1991), twig beetles, *Pityophthorus* spp. (Fox *et al.*, 1990), the cone beetle, *Conophthorus radiatae* Hopkins, and the dry twig and cone beetle, *Ernius punctulatus* Fall (Hoover *et al.*, 1995, 1996). In nurseries inoculum could originate from sources such as wind and irrigation (Bloomberg, 1981) or from reused containers (James *et al.*, 1991). Gordon *et al.* (2001) noted that the availability of infected but presymptomatic trees at a "choose and cut" Christmas tree farm provided an ideal opportunity to move the pathogen to other locations in California.

1.1.2.4 Economic Importance

Pitch canker of pine causes heavy losses to the forest industry in the world. It is currently regarded as the most serious disease of pines in the world (Wingfield *et al.*, 2002). Damage includes tree mortality, reduced lumber quality because of stem deformation, reduced growth, seed contamination in seed orchards, and seedling mortality in nurseries (Dwinell *et al.*, 1985). Seedlings that have been morphologically or physiologically impaired in the nursery and that are shipped for outplanting may die or grow poorly (Smith, 1975; Johnson *et al.*, 1989).

Heavy losses from suspected seedborne *Fusarium* spp. have been observed in container-grown longleaf pine (*P. palustris* Mill.) in Louisiana and nursery bed sugar pine (*P. lambertiana* Dougl.) in northern California (Graham & Linderman, 1983). During 1990, a major forest nursery in the Mpumalanga Province of South Africa reported devastating losses of containerized *P. patula* Schlechtend. & Cham. seedlings (Viljoen *et al.*, 1994).

Pitch canker may destroy the aesthetic value of urban forests. Repeated infections, which eventually include older branches and the main stem, make trees unattractive and prone to premature death. In urban forests these necessitate a costly process of removal and replacement (Gordon *et al.*, 1998). For example, in California thousands of infected trees

have been killed and thousands more have sustained so much damage as to have entirely lost their aesthetic value (Gordon *et al.*, 1998).

1.1.3 *Fusarium* Root and Stalk Rot of Maize

1.1.3.1 Introduction

Fusarium verticillioides (Sacc.) Nirenberg (teleomorph *Gibberella moniliformis* Wineland) (= *F. moniliforme* J. Sheldon, the conidial stage of *G. fujikuroi* (Saw.) Wr.,) is often found in maize, and constitutes an important source of inoculum in the soil (Pamphile *et al.*, 2004). It commonly infects a wide range of crops throughout the world and is considered a major parasite of the Poaceae, particularly in tropical and subtropical regions (Webster & Gunnell, 1992).

On maize (*Zea mays* L.) *F. verticillioides* causes a number of diseases including stalk rot, root rot, cob rot, ear and kernel rot, damping-off and seedling blight (Styer & Cantliffe, 1984; Frederiksen, 1986; Headrick & Pataky, 1989; Drepper & Renfro, 1990), and has been listed as one of the major factors limiting the utilization of *Opaque-2* (high-lysine) maize (Ullstrup, 1971; Loesch *et al.*, 1976; Warren, 1978; Gulya *et al.*, 1980) as well as sweet corn hybrids with the recessive mutant gene *Shrunken-2* (*sh2*) (Andregg & Guthrie, 1981; Headrick & Pataky, 1989, 1991; Headrick *et al.*, 1990). Economically, stalk rots have been considered more important than any other diseases of maize, and *F. verticillioides* is regarded as one of the major causal organisms (De Leon & Pandey, 1989; Drepper & Renfro, 1990).

1.1.3.2 Symptoms

Seedling blight. Because of its ability to cause symptomless infection, *F. verticillioides* is one of the most common contaminants of seed (Edward, 1936, *cited by* Burgess *et al.*, 1981; Gelderblom *et al.*, 2001). Seedborne inoculum can cause severe seedling blight when climatic conditions are unfavourable for germination and seedling growth (Burgess *et al.*, 1981).

Ear rot. *Fusarium verticillioides* causes a pronounced reddish discolouration of the rotted grain and husk tissues and produces a growth of pinkish-white to red mycelia on the surface of the colonized grain. The fungus commonly infects the grain internally at the

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embryo end without causing visible symptoms (Edward, 1936, *cited by* Burgess *et al.*, 1981; Pont, 1963).

Root and stalk rot. Symptoms associated with root and stalk rot of maize, caused by *F. verticillioides,* have been described by Dodd (1980). Typically the first sign of stalk rot is permanent wilting of the plant. Within a day, all leaves become grey and the leaves and ears droop. The outer rind of the lower stalk is yellow-green, turning yellow-brown within a week. At this point, pith tissue in the lowest internode rots and pulls away from the rind, greatly weakening the stalk, whose structure changes from a solid rod to a tube. Severe stalk rot causes premature senescence of the stalks, which turn light brown externally while the internal tissue becomes shredded and often develops a pink or red colour (Pont, 1963).

Symptoms do not occur uniformly within a field but are scattered, with wilted plants found adjacent to healthy ones. Rot generally begins at the lowest internode, and development probably increases with rising temperatures. Affected plants usually have rotted roots and are easily pulled from the ground. Plants affected by stalk rot are very susceptible to lodging by wind or during harvesting (Pont, 1963; Dodd, 1980).

1.1.3.3 Disease Cycle and Epidemiology

Fusarium verticillioides, one of the most cosmopolitan of plant pathogens, is found in most soils where maize can be grown. The fungus persists on plant residues and organic matter in or on the soil and may invade plants whenever the environment becomes favourable (Zummo & Scott, 1992; White, 1999). *Fusarium* stalk rot seems to be more important in warm, dry areas, even though the causal agents may be found wherever maize is grown. The pathogens can be isolated from both rotted and healthy stalks near the end of the growing season. *Fusarium verticillioides* may be present throughout the life cycle of the plant, originating from infected seed (White, 1999).

Fusarium verticillioides, the incitant of *Fusarium* ear rot, enters the ear through the silk channel (Edward, 1936, *cited by* Burgess *et al.*, 1981; Koehler, 1942) or from adjacent cob tissue (Edward, 1936, *cited by* Burgess *et al.*, 1981), spreads within the ear on the silk, and infects isolated single kernels or groups of kernels in localized areas of the ear. Growth cracks in the pericarp or other damage enhance the infection of kernels by the pathogen, but the fungus can penetrate the pedicel of intact kernels (Koehler, 1942).

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1.1.3.4 Economic Importance

Fusarium verticillioides has a wide host range throughout the world. It is particularly important in the family Gramineae, especially in tropical and subtropical regions, resulting in severe economic losses estimated at up to 50% (Webster & Gunnel, 1992). There were also 40% losses reported on maize in two countries in the 1970s (Snyder, 1981).

Root and stalk rots have been shown to be responsible for yield decline in continuously grown maize in the United States (U.S.) and Europe (Koehler, 1959, 1960; Williams & Schmitthenner, 1963; Stucky *et al.*, 1986; Scholte, 1987; Sumner *et al.*, 1990). Stalk rot, whether or not it is associated with lodging, is without doubt, the most important maize disease in Western Europe, and probably the only one that is really damaging to early maize. Stalk rots are the most serious and widespread diseases of field maize in Nebraska and in the corn belt (Wysong & Kerr, 1969). In France losses are about 10% per year (Barriè, 1979, *cited by* Cassini, 1981; Cassini, 1973, *cited by* Cassini, 1981). In Great Britain, Cook (1978) estimated that between 1973 and 1975 the median loss in seed weight ranged from 18.7 % to 11.2% in 39 fields under observation. Losses of 20 to 25% have been regularly recorded in the U.S., and these are further aggravated when stalk rot is accompanied by lodging, as many ears fall to the ground. Furthermore, lodging resulting from maize stalk rot causes difficulty and slow machine harvesting, e.g., in the U.S. maize belt, this is crucial time when farmers are concerned about harvesting before winter (Dodd, 1980).

Pont (*unpublished data*, Burgess *et al.*, 1981) considered that root rot caused by *F. verticillioides* could be responsible for significant yield reductions in the Atherton Tablelands of northern Queensland. Many of the same disease organisms are known to be responsible for the widespread occurrence of root and stalk rots in South Africa (Du Toit, 1968; Krüger, 1970a,b; Channon & Farina, 1991) and it is not unreasonable to assume that yield stability has been similarly affected in this country (Channon & Farina, 1991). In Kenya, maize grain losses caused by ear rot vary from negligible to significant (13-70%) (Anon, 1986, *cited by* Kedera *et al.*, 1994) and diseased ears have been observed under field conditions in all cultivars grown in the region (Kedera *et al.*, 1994).

Stalk and root rot of maize causes problems for farmers somewhere every year. Potential grain yields are lost because kernels on rotted plants are lightweight and ears are missed

during harvest (Dodd, 1980). This disease is responsible for direct yield reduction due to premature dying of infected plants and a softening of the rind resulting in loss of strength of the stalk. Lodged maize is difficult to harvest and ears in contact with moist soil soon deteriorate (Wysong & Kerr, 1969).

Fusarium verticillioides is known to produce a number of mycotoxins, including moniliformins, fumonisins, and zearalenones which have been implicated in animal and human disorders including oesophageal cancers, infertility and kidney disorders (Marasas *et al.*, 1981, 1984; Gelderblom, *et al.*, 1992; Rheeder, 1992; Thiel *et al.*, 1992; Julian *et al.*, 1995).

Equine leukoencephalomalacia and hepatosis have a long record of association with maize contaminated with *Fusarium* spp. from the *Leseola* section (Butler, 1902, Biester & Schwarte, 1939, Biester *et al.*, 1940, Badiali *et al.*, 1968, Buck *et al.*, 1979, and Brownie & Cullen, 1986, *all cited by* Leslie *et al.*, 1992; Schwarte *et al.*, 1937; Kellerman *et al.*, 1972; Marasas *et al.*, 1976; Kriek *et al.*, 1981; Pienaar *et al.*, 1981; Haschek & Haliburton, 1986; Kellerman *et al.*, 1990). Recently, leukoencephalomalacia has been found in deer that were believed to have consumed maize contaminated with *F. verticillioides* (Howerth *et al.*, 1989, *cited by* Leslie *et al.*, 1992). This fungus has been correlated with pulmonary edema syndrome in swine (Kriek *et al.*, 1981; Harrison *et al.*, 1990, *cited by* Leslie *et al.*, 1992; Ross *et al.*, 1990). Fumonisin B_1 has induced equine leukoencephalomalacia when administered intravenously (Marasas *et al.*, 1988) or orally (Kellerman *et al.*, 1990), and fumonisin B_1 has induced pulmonary edema in swine (Harrison *et al.*, 1990, *cited by* Leslie *et al.*, 1992). The presence of this compound also has been correlated with cancer-promoting activities in rats (Gelderblom *et al.*, 1988).

Fusarium verticillioides also produces fusaric acid, a toxin, which has been implicated in reproductive and birth defects in rats (Porter *et al.*, 1995, *cited by* MacDonald & Chapman, 1997). It is also a non-specific phytotoxin, which has been reported to be mutagenic in plant tissue cultures (Nguen-Khong-Min & Smirnov, 1992, *cited by* MacDonald & Chapman, 1997). High levels of fusaric acid cause root stunting in maize (Nielsen *et al.*, 1978, *cited by* MacDonald & Chapman, 1997), and seed infected with such isolates may fail to grow, which would result in poor establishment of the crop (MacDonald & Chapman, 1997).

Members of *Fusarium* section *Liseola* are widely distributed in commercial feeds and fields, and many of the strains in this section, but not in other *Fusarium* sections (Thiel *et al.*, 1991), have the potential to produce significant levels of fumonisin B_1. Strains of *F. verticillioides* recovered from feeds associated with equine leukoencephalomalacia produced significant quantities of fumonisin B_1 (Ross *et al.*, 1990). Geographically diverse strains of *F. verticillioides* collected from various substrates in Africa, Asia, and North America produced significant levels of fumonisin B_1 (Nelson *et al.*, 1991). The nearly universal distribution of these fungi in maize and sorghum (Marasas *et al.*, 1979; Thomas & Buddenhagen, 1980; Leslie *et al.*, 1990) and their ability to be internally seedborne in symptomless, apparently healthy grain (Foley, 1962; Marasas *et al.*, 1979; Thomas & Buddenhagen, 1980) suggest a significant potential for widespread contamination of human foods and animal feeds. In a survey made by Leslie *et al.* (1990), isolates belonging to *Fusarium* spp. in the *Liseola* section were recovered from all maize and sorghum fields sampled; *F. verticillioides* and *F. proliferatum* predominated.

Fungal contamination of maize grain clearly remains a serious problem for subsistence and small farmers. Discoloured and lightweight grains may be removed, however, asymptomatic infections often caused by *F. verticillioides* can easily be overlooked and the mycotoxins associated with these pathogens pose a serious health risk to the consumer. It has been suggested that the high incidence of *F. verticillioides* in the region around Lake Victoria (Kedera *et al.*, 1994) may account for the increasing incidence of oesophageal cancer in this region (Ochor *et al.*, 1989, *cited by* MacDonald & Chapman, 1997).

Asymptomatic infection of seed is also significant for seed health. *Fusarium verticillioides* is one of the most important contaminants of commercial maize seed (Edwards, 1936, *cited by* Burgess *et al.*, 1981). The pathogen may cause seedling blights (Burgess *et al.*, 1981; Shurtleff, 1980), and farmers who sow seed saved from their previous crops may thus aggravate the problem of infection.

1.2   Control Options

1.2.1   Use of Resistant Cultivars

1.2.1.1 *Fusarium* Wilt of Cabbage

Fungal wilt resistance incorporated into many vegetable cultivars and hybrids is one of the amazing accomplishments of modern vegetable breeding. Thus, it has been possible to reduce yield and quality losses due to wilt organisms, to decrease the cost of production, and to expand or stabilize vegetable production areas (Goth & Webb, 1981).

Resistance obtained some time ago (Walker *et al.*, 1957) is holding well (Goth & Webb, 1981; Snyder & Smith, 1981). However, pathogenic forms of *Fusarium* may still arise as recombinants or mutations from within the diverse and malleable population of *F. oxysporum*, which forms normal part of the soil microflora (Beckman, 1987). Though such occurrences are apparently rare, they pose a continuing threat. Thus, *F. oxysporum* remains a serious and constant threat to crop production wherever and however these crops are grown, and consequently wilt diseases continue to be studied intensively for practical reasons. In addition, new resistant varieties are not and should not be expected to be permanent (Walker, 1959).

Jones & Gilman (1915) and Jones *et al.* (1920), *cited by* Goth & Webb (1981) released the yellows-tolerant cabbage cultivars Wisconsin Hollander, and Wisconsin All Season and Wisconsin Brunswick, respectively. These three cultivars were selected from progenies resulting from natural cross-pollinations among plants that survived in fields with severe natural infestation of *F. oxysporum* f. sp. *conglutinans.* These cultivars were not immune to *Fusarium* wilt and in warm seasons they frequently developed symptoms (Walker, 1957). When Jones & Gilman (1915), *cited by* Walker (1959), released their first yellows resistant cabbage in 1916 under the name 'Wisconsin Hollander', they were careful not to make any undue claims as to the nature and stability of this resistant character. In that very year the disease was unusually destructive (Jones *et al.*, 1920, *cited by* Anderson, 1933). Blank (1934) collected *Fusarium* isolates from widespread locations but they were all uniformly pathogenic. Although it is still a highly successful variety in the field and is still used

widely more than 85 years after its introduction, resistance in Wisconsin Hollander is unstable (Walker, 1957).

Walker and his colleagues (Tisdale, 1923; Tims, 1926; Walker, 1930; Smith & Walker, 1930b; Blank, 1932; Anderson, 1933) studied the effect of soil temperature on disease development and the inheritance of resistance in cabbage. They described two types of resistance, a monogenic dominant (Type A) and a polygenic (Type B) resistance. Type A resistance was found to be stable at soil temperatures up to 28 °C. Type A and B have been deployed for over 70 years in many economically important cabbage cultivars. They are still effective in most cabbage growing areas of the U.S., including Wisconsin where they were first introduced (Bosland *et al.*, 1988). However, Walker (1957) warned that a virulence breakthrough might occur at any time and anywhere. Monogenic resistance, although it usually approaches a high level of immunity, has decided limitations if the pathogen is extremely variable in selective pathogenicity. It is interesting that the new race appeared in Southern California, where cabbage is rarely if ever attacked by the yellows fungus, and that the disease appeared in a field where no previous occurrences of Fusarium yellows of crucifers had been reported.

The new pathotype of cabbage yellows, found in California in 1985, originally designated as *F. oxysporum* f. sp. *conglutinans* Race 5 but later renamed as Race 2, was pathogenic to cultivars with Type A resistance (Ramirez-Villupadua *et al.*, 1985). A preliminary study of the effect of soil temperature on disease expression by *F. oxysporum* f. sp. *conglutinans* Race 1 and Race 2 indicated that *F. oxysporum* f. sp. *conglutinans* Race 2, the new California isolate, was virulent at 14°C, whereas Race 1 was avirulent at this temperature (Bosland & Williams, 1984, *cited by* Bosland *et al.*, 1988). Bosland *et al.* (1988) found that in the cabbage cultivar Golden Acre, monogenic dominant (Type A) resistance was highly effective against Race 1, but was progressively less effective against *F. oxysporum* f. sp. *conglutinans* Race 2 as soil temperature increased from 14-20°C, and was ineffective at 22 and 24°C. Further, the polygenic (Type B) resistance was highly effective against Race 1 at 20°C and below, but was only effective against Race 2 at 10 and 12°C.

Polygenic resistance is suppressed by various modifications of host nutrition (Walker & Hooker, 1945) and much more by environmental conditions, and was expressed much less effectively in young seedlings than in older plants (Anderson, 1933). Bell & Mace (1981)

generalized that temperature has a greater effect on *Fusarium* wilt severity than host genotype and that resistance to cabbage yellows, whether mono-or polygenic, breaks down at temperatures above 24°C.

When resistance is multigenic in inheritance, as a rule this type of resistance is relatively less complete, is seldom completely fixed, and is usually influenced considerably by the environment (Walker, 1957). A variety resistant under field conditions may show various degrees of susceptibility as temperature and inoculum load vary (Wingard, 1941; Gorenz *et al.*, 1949). For this reason breeders and pathologist tend to sidetrack multiple-gene resistance in favour of monogenic resistance if there is a choice to be made. It may well be questioned whether this is the safest procedure. When monogenic resistance, controlled by a single gene pair, is relied upon, it takes only a step mutation in the pathogen to bring out a race towards which the resistance gene pair is not effective (Walker, 1957).

1.2.1.2 *Fusarium* Root and Stalk Rot of Maize

There are no economically feasible control measures available for root and stalk rots of maize. However, some hybrids have gained a reputation for standability under conditions which favour these diseases (Burgess *et al.*, 1981).

1.2.1.3 *Fusarium* Pitch Canker of Pine

Pines are not indigenous to South Africa. Three of the most important commercial species grown in South Africa are *P. patula* (45%), native to Mexico, and *P. elliottii* Engelm. (27%) and *P. radiata* (9%), naturally occurring in southern U.S. and California, respectively (Critchfield & Little, 1966; Hinze, 1993). These three species constitute 80% of all pines grown in South Africa and almost half of the total commercial forest investment (Hinze, 1993). Sadly, Viljoen *et al.* (1995) have shown that *P. patula* and *P. radiata* are highly susceptible to the pitch canker pathogen, whereas *P. elliottii* is only moderately susceptible. This brings to light the need to look for other control options such as biocontrol.

1.2.2 Cultural Practices

1.2.2.1 *Fusarium* Wilt of Cabbage

Steam pasteurisation or sterilization of soil in glasshouse culture of intensively grown crops is a normal practice to destroy inoculum prior to planting. This prevents *Fusarium* wilt diseases as well as the root diseases caused by other soilborne pathogens (Erwin, 1981). Baker & Roistacher (1975), *cited by* Erwin (1981), showed that soil could be sterilized by maintaining a temperature of 80°C for 30min with steam. Steam-air mixtures have also been used in some situations (Baker, 1970, *cited by* Erwin, 1981). However, in the field, steam is an expensive method of pasteurisation of soil. Moreover, due to condensation of water, the depth of penetration of soil by steam is limited (Erwin, 1981). Similarly, soil solarization is another safe, nonchemical, and inexpensive method of controlling soilborne diseases. Its use, however, is limited to regions where the climate is suitable and the soil is free of a crop for about one month before mulching (Katan, 1980).

High losses may force abandonment of the affected area (Katan, 1980). Movement of crop cultures to new, noninfested land has also been used when the incidence of vascular wilt disease reached a level that prevented profitable production of crops (Erwin, 1981). For example, in the Florida tomato growing industry, there appears to be a situation similar to that of the banana industry in Central America before the 1950s (Stover, 1955) in which the grower moved to new lands to escape *Fusarium* wilt, *Verticillium* wilt, or the dagger nematode disease (Jones *et al.*, 1966). However, with the growing world population, new land is becoming scarcer and scarcer.

Another sensible alternative is rotation to nonsusceptible crops. However, the crop may be less profitable (Katan, 1980) and propagules of many of the vascular wilt pathogens are able to persist in soil for longer periods of time. It is well known that *F. oxysporum* persists in the soil due to the production of chlamydospores (Smith & Snyder, 1975). They reported quantitative data on the variation in the number of propagules of *F. oxysporum* f. sp. *vasinfectum* (G.F. Atk.) W.C. Snyder & H.N. Hansen in the soil in a field subjected to different crop rotations. They showed that the actual number of pathogenic propagules did not decrease over a period of years even when a nonsusceptible crop such as barley was grown. This is certainly the reason why growers with severe *Fusarium* wilt problems must

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seek new land unless they can rejuvenate the old land by an economically and ecologically effective method (Erwin, 1977).

1.2.2.2 *Fusarium* Pitch Canker of Pine

Cultural methods generally have not been effective in controlling the disease. Soil amendments which showed promise in controlling certain *Fusarium* diseases of agricultural crops have not yet proved to be adequately effective in the forest nursery (Smith, 1975). For example, attempts to control root diseases caused by *Fusarium* spp. in ponderosa pine (*P. ponderosa* Douglas ex Lawson & C. Lawson) and lodgepole pine (*P. contorta* Dougl. ex Loud.) seedbeds at the Bend Pine Nursery, Bend, Oregon, U.S. using soil fumigation, soil acidification and addition of organic matter and fertilizers have not consistently controlled the disease (Wright *et al.*, 1963, *cited by* Johnson & Zak, 1977; Lu, 1968; Johnson & Zak, 1977).

1.2.2.3 *Fusarium* Root and Stalk Rot of Maize

The value of cultural practices such as crop rotation is uncertain for the fungus has usually been isolated from sorghum, wheat, maize and pasture soils in areas where these crops are grown (Wearing, 1976, *cited by* Burgess *et al.*, 1981; Wearing & Burgess, 1977; Webster & Gunnell, 1992; MacDonald & Chapman, 1997).

Since *F. verticillioides* is known to persist in soil in infested crop residue (Nyvall & Kommedahl, 1968; Burgess, 1981), stubble burning has been a common practice and eliminates the inoculum in the aboveground part of the stubble. However, stubble retention is becoming a popular practice to reduce the risks of soil erosion and to increase water infiltration and storage (Harte & Armstrong, 1983, *cited by* Summerell *et al.*, 1989). Besides *F. verticillioides* could infect the stalk directly from airborne inocula (Ooka & Kommedahl, 1977; Burgess, 1981) and also is known to be seedborne (Burgess *et al.*, 1981).

1.2.3 Chemical Control

When crop production is at at risk because of a heavy inoculum load of a given pathogen in the soil, the use of selective fungicides to reduce the inoculum load can be effective (Katan, 1980; Beckman, 1987). Fungicides have provided great advantages to agricultural

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producers for many years (Gamaliel & Stapleton, 1995), but are limited by safety considerations, the need for complicated equipment, highly trained personnel, high cost, pesticide residues, phytotoxicity, and reinfestation of soil resulting from drastic reductions in antagonistic microbes (Katan, 1980). Although about half a million tons of pesticides are used annually, one-third of all crop production is still lost (Campbell, 1989).

As described by Beckman (1987), use of fungicides is economically justified only under conditions in which the soil mass to be treated is limited or the crop value and increased yields warrant the considerable expense. In many field plantations cost can be prohibitive. To maintain a low inoculum load by a continuous supply of synthetic fungicides, systemic chemicals or biocides alone is not practical to control soilborne diseases. The cost of treatment that results from the high degree of adsorption in soils and the need for a sustained dosage level has prevented their use except under special circumstances. Erwin (1981) indicated that chemical control with systemic benzimidazole chemicals, and general biocides such as methyl bromide, chloropicrin, mixtures of these chemicals, and vapam is limited to intensively grown crops because of the great expense of the chemicals, inefficiency of uptake by roots, and the tarping of the land with polyethylene sheeting.

Pesticides may influence populations of organisms affecting interactions among species within ecosystems, or they may destabilize these systems (USDA, 1980, *cited by* Pimental & Edwards, 1982). One such non-target effect of pesticides is suppression of mycorrhizal fungi (Kleinschmit & Gerdemann, 1972; Johnson & Zak, 1977). Fumigation with methyl bromide:chloropicrin (45:55) for control of *Verticillium* wilt in cotton had this effect (Erwin, 1981). The best-documented examples of nontarget effects are the agroecosystems. For example, more than 500,000tons of pesticides are produced annually for application in US agroecosystems to control insect pests, plant pathogens, and weeds (USDA, 1980, *cited by* Pimental & Edwards, 1982). However, as little as 1% of this may actually hit the target organisms (PSAC, 1965, *cited by* Pimental & Edwards, 1982). Instead, most reaches nontarget sectors of agroecosystems and/or is spread to surrounding ecosystems as chemical pollutants (Pimental & Edwards, 1982). Due to their high vapour pressure, most chemical fumigants have caused negative effects on the environment or human beings.

Although the large costs (approx. $400 ha^{-1}) of broadcast fumigant chemical treatments have resulted in their limited use on broad-acre crops, health hazards and environmental

problems, including the contamination of ground water have been widely reported. As a result, a number of products have been withdrawn from the market (Thomason, 1987). Some of the early pesticides were potent toxins (e.g., mercury and organochlorine insecticides). They persisted in the environment, accumulated in predators at the top of food chains and were shown to have long-term effects on nontarget organisms. The use of such chemicals has now been discouraged or prohibited in many countries (Campbell, 1989).

Modern pesticides have to pass very stringent tests for safety and for lack of any environmental hazard. The fact remains that they are toxins and occasional examples of misuse or unexpected side effects do occur. It is estimated that there are about 3000 hospitalisations and 200 fatalities per year due to pesticides, apart from the problems, which are unrecognised or not considered serious enough to warrant medical attention (Pimental et al,. 1983). Though normal precautions are employed in the use of fungicides within the knowledge currently available, additional information, however, sometimes comes to light that many cast suspicion on the safety of the continued use of some even well-established materials. For example, benomyl and related compounds prevent completion of mitosis (Seiler, 1973; Styles, 1973); and it has been reported that some of the benzimidazolecarbamates and related compounds have deleterious cytogenetic effects on a variety of organisms (Seiler, 1973; Styles, 1973; Styler & Robert, 1974).

In 2000, the Antarctic ozone hole reached approximately 28m sq. km in size and present trends indicate that the size is unlikely to start to decline until 2015 (Weatherhead, 2000, *cited by* Porter & Mercado, 2001). Under the Montreal Protocol of 1991, methyl bromide was defined as a chemical that contributes to depletion of the Earth's ozone layer. The definition was based on scientific data. Accordingly, the manufacture and importation of methyl bromide will be completely phased out in 2005 and 2015 in developed countries and developing countries, respectively (Vick, 2002). Buschena et al. (1995) indicated that with the loss of methyl bromide as a soil fumigant, reduction of seedling growth and vigour, as well as increased seedling mortality is anticipated. Despite the comprehensive research worldwide, the Methyl Bromide Technical Option Committee still reports that no alternative has been found (Bachelor, 2000, *cited by* Porter & Mercado, 2001).

Fungicides are discovered by an essentially random process of testing as many chemicals as possible for their effect on fungi. A large agrochemical company may test several tens of thousands of chemicals each year with and hope was that one in about 5000 might lead to a product. However, it has recently become clear that it is now more difficult to find new compounds; perhaps only 1 in 100,000 tested now becomes a new product. This is partly because new compounds have to pass more stringent tests than previously required, but there is also the suspicion that perhaps most of the effective chemicals have already been found (Delp 1977; Lewis, 1977; Campbell 1989). After discovery of a new disease-control agent, it may take 5 to 10yr to develop the necessary data on efficacy, residue, and toxicology to register the compound for sale (Delp, 1977). Moreover, the cost of development of each new pesticide is now estimated at about US \$30m (Chet, 1990) to US \$180m (M. D. Laing, 2004, pers. comm.[1]) per compound. An additional investment of millions of dollars is often required for a full-scale manufacturing plant for a new compound.

Another interesting negative side of fungicides is the issue of development of resistance. Fungi develop resistance to fungicides, therefore, new chemicals are constantly needed, but they are becoming more difficult, and therefore, more expensive to find (Delp, 1977; Lewis, 1977; Campbell, 1989). Benomyl resistance in cucumber powdery mildew was reported within one year after introduction of this fungicide (Schroeder & Provvidenti, 1969). Bollen & Scholten (1971) showed that *Botrytis cinerea* Pers. isolated from diseased plant tolerated much higher concentrations of benomyl than the wild type fungus. Even at 1000ppm of benomyl there was not a complete inhibition of growth, while the wild type fungus was eliminated at 0.5ppm of benomyl in the medium. Paper disc bioassay tests by Magie & Wilfret (1974) reported that isolations of *F. oxysporum* f. sp. *gladioli* (Massey) W.C. Snyder & H.N. Hansen from benomyl-treated corms tolerated 50 to 200 times more benomyl than those from nontolerant corms. Bioassay data indicated that the growth of nontolerant strains was slightly restricted by concentrations of benomyl on the paper disc as low as 2-5mg^{-1}. Nakanishi & Oku (1969) also reported *F. oxysporum* f. sp. *niveum* (E.F. Sm.) W.C. Snyder & H.N. Hansen and f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen tolerant to quintozene (pentachloronitrobenzene).

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1.2.4 Biological Control

Soilborne plant pathogens survive in the soil for long periods of time and cause extensive damage to many crops. None of the available methods used to control soilborne diseases are effective against all pathogens or can be used in all instances. Thus, the search for innovative, effective, nonhazardous, and economically feasible methods for effective control of soilborne diseases is a never-ending task (Gamaliel & Stapleton, 1995).

Biological control of soilborne plant pathogens has recently received considerable attention throughout the world because of the growing public concern regarding the extensive use of hazardous chemicals in pest control (Mulder, 1979). Soilborne pests and diseases are essentially difficult to control and integrated control strategies using several methods have been used traditionally for their management. They have been identified by certain commercial companies as promising targets for biological control either due to the unsuitability or inefficiency of chemical treatments (Kerry, 1992). Presently there are over 80 products for biocontrol of pathogens worldwide (Whipps & Davies, 2000). Most of these products are formulations either of the fungi *Gliocladium-Trichoderma* or the bacteria *Pseudomonas* and *Bacillus* (Paulitz & Bélanger, 2001). In South Africa there are about six biocontrol microorganisms registered for use as biocontrol agents of soilborne plant pathogens (M. D. Laing, 2004, pers. comm.[2]).

Biocontrol of plant diseases has been considered as an alternative to fungicides (Cook & Baker, 1983; Chet, 1987; Howell, 1990; Harman, 1991; Lewis & Papavizas, 1991) and is becoming an important component of plant disease management (Stasz *et al.*, 1988). One of the fundamental reasons for the growing interest in biocontrol of soilborne diseases is that there is sufficient, though not complete, knowledge of microbial ecology and plant pathology. This renders some possibility of understanding the niche which microorganisms must colonize and the microbial interactions involved. This provides some room for prediction that appropriate diseases for biocontrol can be identified for study (Campbell, 1989).

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Garrette (1965) traced the origin of biocontrol research of soilborne plant pathogens to the 1920s when, in experimental conditions, saprophytic microorganisms were shown to control pathogens if inoculated together with pathogens into pathogen free soils. Thus, Hartley (1921), *cited by* Deacon (1991), showed that a range of microbes could control damping-off of tree seedlings by *Pythium aphanidermatum* (Edson) Fitzp., Millard & Taylor (1927) showed that *Streptomyces griseus* (Krainsky) Waksman & Henrici could control potato scab (*S. scabies* (Thaxter) Waksman & Henrici), and Sanford & Broadfoot (1931) showed that many soil fungi and bacteria could control take-all of wheat (*Gaeumannomyces graminis* var. *tritici* J. Walker).

Likewise, much effort has been made in order to control *Fusarium* spp. by biological means. Ordentlich *et al.* (1991) suggested that biological control could play an important role in long-term, nonpolluting protection of several agricultural crops from *Fusarium*. Ocamb (1994) isolated rhizosphere inhabitants from rhizosphere and non-rhizosphere soils, collected from eastern pine (*P. strobus* L.) that both inhibit and competitively utilize the same carbon sources as root rot-causing *Fusarium* spp., as potential biological control agents. It was also reported that diffusible metabolites of the ectomycorrhiza fungus *Laccaria laccata* (Scop.) Cooke caused distortion of *F. oxysporum* hyphae and inhibited germination of both chlamydospores and microconidia (Sylvia & Sinclair, 1983). Buschenal *et al.* (1995) noted that studies with eastern white pine (*P. strobus*) and red pine (*P. resinosa* Ait.) are underway to develop biological control microorganisms for application to conifer seed since an alternative to soil fumigation with methyl bromide is needed for control of *Fusarium* root rot and damping-off of conifer seedlings. Preliminary results show an ectomycorrhizal fungus, as well as bacteria derived from the rhizosphere, suppress *Fusarium* root rot of eastern white pine.

One of the significant phenomena in biological control of *Fusarium* wilt diseases is the presence of suppressive soils in which microbial activity prevents the appearance of disease in susceptible crops (Alabouvette *et al.*, 1979; Kloepper *et al.*, 1980, *cited by* Sivan & Chet, 1985; Scher & Baker, 1982). Alabouvette *et al.* (1979) showed that the suppressiveness was induced by natural saprophytic fungal communities of *F. oxysporum* and *F. solani*. Soil suppressiveness of *Fusarium* spp. can also occur in the presence of siderophore-producing *Pseudomonas*, which bind Fe^{+3} and make it less available to those microflora unable to produce similar iron transport agents (Kloepper *et al.*, 1980, *cited by*

Sivan & Chet, 1985; Scher & Baker, 1982). Generally microorganisms play a major role in all types of specific suppressiveness (Deacon, 1991).

Much of current research, however, is targeted on the development of inoculant biocontrol agents that can be applied to soil or crops (Deacon, 1991). Two such products have been used routinely on an international scale; they are *Phlebia (Peniophora) gigantea* (Fr:Fr) Donk, introduced commercially 1962 for control of *Heterobasidion annosum* (Fr.) Bref. in pine forests (Rishbeth, 1975), and *Agrobacterium radiobacter* (Beijerinck & van Delden) Conn for control of crown gall (*A. tumefaciens* (Smith & Townsend)) (Kerr, 1980). Biological control of soilborne plant diseases using *Trichoderma* and *Bacillus* species has also become an increasingly popular and promising control alternative in many countries (Broadbent *et al.*, 1971; Elad *et al.*, 1984; Askew & Laing, 1993; Cook *et al.*, 1996; Zhang *et al.*, 1996, *cited by* Kim *et al.*, 1997; Kim *et al.*, 1997).

1.2.4.1 *Trichoderma* as a Biocontrol Agents

Many researchers in the past have established the potential of *Trichoderma* spp. as biocontrol agents of several soilborne plant pathogens. Morrow *et al.* (1938) were among the first to investigate this and showed that *Trichoderma* spp. could be applied to cotton seedlings to control *Rhizoctonia solani* Khün in experimental conditions. Biocontrol researches of soilborne plant pathogenic fungi often report that *Trichoderma* spp. are among the most promising biocontrol agents (Wells *et al.*, 1972; Hadar *et al.*, 1979; Liu & Baker, 1980; Elad *et al.*, 1982b; Elad *et al.*, 1984). Chet & Baker (1981) found an isolate of *T. hamatum* (Bonord.) Bainier in a Colombian soil naturally suppressive to *R. solani*. The antagonist was an effective biocontrol agent when applied to infested soil in the greenhouse. Similarly, various authors found a very promising isolate of *T. harzianum* Rifai capable of controlling *R. solani* and *Sclerotium rolfsii* Sacc. both in the greenhouse and in the field (Hadar *et al.*, 1979; Elad *et al.*, 1980a; Elad *et al.*, 1980b; Elad *et al.* 1982b). Locke *et al.* (1985) reported that the benomyl–tolerant *T. viride* Pers. Biotype T-1-R9 was shown to be a potential biocontrol agent for reducing *Fusarium* wilt in vegetative chrysanthemums. Sivan & Chet (1985) have shown the effectiveness of a newly isolated strain of *T. harzianum* in controlling *Fusarium* wilt of cotton and melons as well as *F. culmorum* (W.G. Sm.) Sacc. in wheat under natural soil conditions. Sivan *et al.* (1987) reported that *T. harzianum* was effective in reducing the incidence of *Fusarium* crown rot of tomato under field conditions in two successive seasons. Marois *et al.* (1981) were the

first to demonstrate successful biological control of *Fusarium* crown rot on tomatoes with *T. harzianum*.

A.   Mechanism of Biocontrol of *Trichoderma*

Suggested mechanisms for biocontrol by *Trichoderma* spp. are antibiosis, lysis, competition, mycoparasitism, and acquired systemic resistance (Papavizas & Lumsden, 1980; Ayers & Adams, 1981; Cook & Baker, 1983; Calderon *et al.*, 1993; Chang *et al.*, 1997).

i)   Antibiosis

Wright (1956a,b) demonstrated that colonization of pea seeds by *T. viride* resulted in the accumulation of significant amounts of the antibiotic gliotoxin in the seeds, and that *Trichoderma* was able to control *Pythium ultimum* Trow when applied as a seed-coat inoculant (Wright, 1956c). Weindling & Emerson (1936) isolated, in crystalline form, an organic metabolite very toxic, even at high dilution, to *Rhizoctonia*. The metabolite was later given the trivial name of gliotoxin (Weindling, 1941). Brian & McGowan (1945) described a second highly fungistatic antibiotic, viridin, produced by *T. viride*. However, Webster & Lomas (1964) did not obtain gliotoxin or viridin from *T. viride*, although this species aggregate is implicated in biocontrol. Dennis & Webster (1971) showed that *Trichoderma* spp. produce antibiotics different from gliotoxin and viridin by nine species aggregates of *Trichoderma*; they showed production of other chloroform-soluble antibiotics such as trichodermin (by *T. viride* and *T. polysporum*) and other peptide antibiotics by *T. hamatum*. They did not exclude the possibility, however, that gliotoxin and viridin are also produced in low concentrations by *Trichoderma* isolates. Papavizas *et al.* (1982) found that several UV-induced mutants of *T. harzianum* produced two unidentified metabolites, one heat-labile and the other heat-stable.

ii)   Mycoparasitism

Many researchers dealing with *Trichoderma* noticed that hyphae of the antagonists parasitized hyphae of other fungi *in vitro* and brought about several morphological changes, coiling, haustoria, disorganization of host cell contents, and penetration of the host (Papavizas, 1985). Durrell (1968) *cited by* Papavizas (1985) using phase contrast and electron microscopy, produced interesting photographs showing haustoria and hyphae of *T. viride* within hyphae of Phycomycetes followed by digestion of their contents.

The relationship between enzymes produced by *Trichoderma* and their importance in host cell degradation has been documented (Cook & Baker, 1983). Chet & Baker (1980, 1981) showed that *Trichoderma* produces cellulase, β-(1-3)-glucanase, and chitinase and degrades the glucans in the walls of *Pythium* spp. and the chitin and glucans in the walls of *R. solani*. There was no evidence that *T. hamatum* (Chet & Baker, 1981) or *T. harzianum* (Hadar *et al.*, 1979) produces any antibiotics. The importance of β-(1-3)-glucanase and chitinase as key enzymes responsible for fungal cell and sclerotial wall lysis and degradation has been stressed by others (Jones *et al.*, 1974; Elad *et al.*, 1982a; Elad *et al.*, 1983). Chérif & Benhamou (1990) presented evidence in their study that a strain of *Trichoderma*, isolated from a sample of peat collected in New Brunswick, is capable of producing several chitinases that likely are involved in chitin breakdown of the pathogenic fungus *F. oxysporum* f. sp. *radicis-lycopersici*.

iii)   Competition

Competition occurs when two or more microorganisms demand more of the same resource than provided by the immediate supply. Competition between the biocontrol agent and the pathogen may lead to disease control (Tronsmo & Hjeljord, 1998). Biological control of *Chondrostereum purpureum* (Pers.) Pouzar, silver leaf pathogen, by *T. viride* on plum trees is the result of competition (Corke & Hunter, 1979); which also seems to be the most potent mechanism employed by *T. harzianum* T-35 in the control of *F. oxysporum* f. sp. *vasinfectum* and *F. oxysporum* f. sp. *melonis* W.C. Snyder & H.N. Hansen in the rhizosphere of cotton and melon (Sivan & Chet, 1989).

Microbes have limited capacity for controlling their microenvironment. In order to survive and compete successfully with other microbes under varying conditions, they have evolved the potential to respond to environmental changes by changing themselves structurally and functionally (Faull, 1988). *Trichoderma* spp. have various responses to these adverse environmental conditions. Although they have relatively slow growth rates, this is balanced by the formation of large numbers of spores, which are easily dispersed to the surrounding areas followed by equally rapid germination (Hawker, 1957). Tolerance to pesticides and other chemical residues can also allow certain fungi to recolonize semi-sterile areas. For example, *Trichoderma* spp. are resistant to doses of soil fumigants and can recolonize soil and wood after such treatment (Warcup, 1951; Evans, 1955; Papavizas *et al.*, 1982).

iv)   Systemic Acquired (Activated) Resistance (SAR)

Recently an exiting and promising means of plant disease control through the use of pathogenic microorganisms or chemical compounds that cause tiny necrotic lesions in the treated plant and by so doing activate the defences of the whole plant against subsequent infections by pathogens of the same or different types. This has been called systemic acquired (activated) resistance (SAR) (Agrios, 1997). Biocontrol agents are also known to trigger SAR as a biochemical weapon to combat plant pathogens.

Some studies have demonstrated that *Trichoderma* spp. can also affect the host plant. Addition of *T. viride* cellulase to grapevine cell cultures induced plant defence reactions such as the hypersensitivity response and phytoalexin production (Calderon *et al.*, 1993). A similar induction of plant defence reactions by *T. longibrachiatum* Rifai in tobacco plants was linked to an increased resistance to *Phytophthora parasitica* Dastur var. *nicotiana* (Breda de Haan) (Chang *et al.*, 1997). This indicates an indirect biocontrol effect of *Trichoderma* through the induction of plant resistance.

1.2.4.2 *Bacillus* as a Biocontrol Agents

Many bacteria including *Bacillus subtilis* (Ehrengerg) Cohn, *Pseudomonas fluorescens* Migula and *P. putida* (Trevisan) Migula have been studied as biocontrol agents of soilborne plant pathogens (Howell & Stipanovic, 1979, 1980; Scher & Baker, 1982; Lamanceau & Alabouvette, 1991; Zhang *et al.*, 1996; de Boer *et al.*, 1999). Seed treatments with *B. subtilis* have since been shown to control various diseases in a variety of crops, including diseases caused by *R. solani* in wheat, brown spot of rice caused by *Drechslera oryzae* van Breda de Haan, and damping-off in tomato and sugar beet (Dunleavy, 1955; Merriman *et al.*, 1974a,b; Nada & Gangopadhyay, 1983, *cited by* Turner & Backman, 1991). Kim *et al.* (1997) recently reported on a unique *Bacillus* sp., designated as Strain L324-92 that suppressed three root rot diseases of wheat, namely take-all caused by *G. graminis* var. *tritici*, Rhizoctonia root rot caused by *R. solani* AG8, and Pythium root rot caused by *Pythium* spp., when applied as seed inoculant. Aldrich & Baker (1970) presented evidence that *B. subtilis* acts as a biocontrol agent. *Bacillus subtilis* applied in a dip or incorporated into the rooting medium controlled the incidence of *Fusarium* stem rot of carnation caused by *F. roseum* f. sp. *dianthi* (Fod). Recently, two *B. subtilis* strains, GB03 and GB07, have been marketed as Kodiak and Epic, respectively by Gustafson Inc., Plano, TX, U.S., for use with several crops as growth promoting

rhizobacteria. Field experiments have shown that cotton yields are increased by seed treatment with these two strains (Minton et al., 1991, Brannen & Backman, 1993, and Kenney & Arthur, 1994, *cited by* Zhang et al. 1996). Similarly, Zhang et al. (1996) have shown that B. subtilis Strains GB03 and GB07 strongly inhibited growth of F. oxysporum f. sp. vasinfectum and other Fusarium spp. in vitro, and reduced the colonization of cotton roots in growth chamber.

Selected strains of B. subtilis, produced by FZB Biotechnic GmbH Berlin, have been tested in model, greenhouse and field experiments in its biocontrol effect on numerous seedling diseases, root rot and wilt diseases inducing soil- and seed-borne fungal pathogens in vegetables, ornamental and agricultural plants cultivated in soil and in hydroponics (Bochow, 1991, Bochow, 1992a,b, and Schmiedeknecht & Bochow, 1993, *cited by* Bochow & Gantcheva, 1995). Results show that specific strains of B. subtilis isolates act as broad-spectrum biocontrol agents with antagonistic and suppressive activities on many pathogenic soil fungi and with plant health promoting actions by enhancing plant growth and yield as well as disease resistance. *Bacillus subtilis,* as a spore forming bacterium, can easily be formulated as a biopreparation and may be applied by way of seed treatment (dipping), soil drench around seedplants or drench introduction into soil substrates or into hydroponic nutrient solutions (Bochow & Gantcheva, 1995).

B. Mechanism of Biocontrol of *Bacillus* spp.

i) Antibiosis
The main mechanism by which B. subtilis strains appear to operate as an antagonist to plant pathogen growth is through antibiosis (Cubeta et al., 1985; Gupta & Utkahede, 1986; Loeffler et al., 1986; McKeen et al., 1986; Seifert et al., 1987; Fravel, 1988; Ferreira et al., 1991). Many Bacillus strains are known to suppress fungal growth in vitro by the production of one or more antifungal antibiotics. Many of the antifungal antibiotics produced in vitro have been identified as peptide antibiotics (Kataz & Demain, 1977).

Leifert et al. (1995) detected three antifungal antibiotics (one with activity against Alternaria brassicicola (Schwein) Wiltshire, one with activity against B. cinerea and one with activity against both fungi) produced by B. subtilis CL27, and one by B. pumilus Meyer & Gottheil CL45. The two antibiotics produced by Strain CL27 with activity

against *A. brassicicola* were identified as peptides. These peptide antibiotics are effective against other gram-negative bacteria and some gram-positive bacteria, moulds and yeast (Brock & Madigan, 1991). Kim *et al.* (1997) found that *Bacillus* sp. Strain L324-92 is one of the several antibiotic-producing bacteria. This strain was shown to possess *in vitro* antibiotic activity against all isolates of *G. graminis* var. *tritici,* as well as species and anastomosis groups of *Rhizoctonia* and all species of *Pythium* tested.

Some of these antibiotic-producing strains were also shown to suppress fungal plant diseases *in vivo* (Baker *et al.*, 1983; Utkhede & Sholberg, 1986; Fravel, 1988; Edward & Seddon 1992; Leifert *et al.*, 1995). The antibiotics produced *in vitro* were generally assumed to be the compounds responsible for biocontrol *in vivo* (Leifert *et al.*, 1995). Edward & Seddon (1992) showed that *B. subtilis* and *B. brevis* Migula produce peptide antibiotics *in vitro* with antifungal activities against fungal plant diseases. These *Bacillus* strains were also found to have *in vivo* activity against fungal plant diseases. In addition to antibiotics, *Bacillus* produces a range of other metabolites including biosurfactants (Edwards & Seddon, 1992), chitinase and other fungal cell wall-degrading enzymes (Priest, 1977; Pelletier & Sygusch, 1990; Frändberg & Schnürer, 1994a,b), volatiles (Friddaman & Rossall, 1993, 1994) and compounds which elicit plant resistance mechanisms (Kehlenbeck *et al.*, 1994).

ii) Competition

The ability of bacteria to survive and proliferate in soil is an important factor in their success as inoculants for promoting biological control, nutrient solubilisation and bioremediation (Young & Burns, 1993). However, many soil inoculants, shown to be beneficial in laboratory experiments fail when used in the field (Lethbridge, 1989). This is probably due to a combination of physical (Rattray *et al.*, 1992), chemical (Acea *et al.*, 1988) and biological (Recorbert *et al.*, 1992) stresses encountered by the introduced species. It may therefore prove more successful to isolate bacteria from the target soil and screen for biologically competitive species, which can then be reintroduced in much larger numbers. Such bacteria may be more likely to survive and express their properties because they are adapted to the recipient soil environment and should compete effectively with the indigenous microorganisms (Young *et al.*, 1993).

Inoculation of spring wheat (*Triticum aestivum* L.) seeds with *B. subtilis* or *B. pumilus* resulted in a rhizosphere population of 10^5 cfu g^{-1} of root tissue of inoculum bacteria one month after treatment (Juhnke *et al.*, 1987). These findings were contrary to the earlier suggestion that *Bacillus* is a relatively poor rhizosphere colonizer (Lockhead, 1940) and therefore demonstrated that *Bacillus* inoculants can effectively colonize the rhizosphere. Various reports have also shown that *Bacillus* can effectively colonize the rhizosphere (Turner *et al.*, 1991; Asaka & Shoda, 1996; Pandey, 1997).

Bacillus species, as a group, have been considered less effective as rhizosphere colonists compared to fluorescent pseudomonads (Kim *et al.*, 1997). However, there is a growing list of reports of rhizosphere colonization and root disease control with *Bacillus* spp. introduced as seed inoculants, including *B. cereus* Frankland & Frankland UW85 for control of damping-off of alfalfa (Handelsman *et al.*, 1990; Halverson *et al.*, 1993), *B. megaterium* de Bary B153-2-2 for control of *Rhizoctonia* root rot of soybean (Liu & Sinclair, 1992, 1993), *B. subtilis* GB03 for control of damping-off of cotton (Mahafee & Beckman, 1993), and *B. mycoides* Flügge for control of wheat take-all (Maplestone & Campbell, 1989). *Bacillus* species have also been reported for the use as 'yield-increasing bacteria' when introduced as a seed inoculant on several crops including wheat and rice (Zhang *et al.*, 1996, *cited by* Kim *et al.*, 1997).

Bacillus spp. as a group offer several advantages over fluorescent pseudomonads and other gram-negative bacteria as seed inoculants for protection against root pathogens, longer shelf life and the broad-spectrum activity of their antibiotics (Kim *et al.*, 1997). One of the best known examples is *B. subtilis* A13, isolated more than 25 years ago in Australia (Broadbent *et al.*, 1971). This strain was selected based on *in vitro* activity to all the nine pathogens tested and was subsequently shown to promote growth of cereals, sweet corn, carrots, and bedding plants when applied as a seed inoculant (Merriman *et al.*, 1975).

iii) Growth stimulant

Bacillus spp. have been used for many years in an attempt to control plant pathogens and increase plant growth (Turner & Backman, 1991; Holl & Chanway, 1992; Mañero *et al.*, 1996; Kim *et al.*, 1997). *Bacillus* spp. Strain L324-92 has been found to show a growth promoting benefit on turf grass when applied to the foliage as a cell suspension (Mathre *et al.*, 1999) and has shown to increase yields of wheat in fields in which one or more of the

three diseases, *G. graminis* var. *tritici*, Rhizoctonia root rot caused by *R. solani* AG8, and Pythium root rot caused by *Pythium* species, were yield limiting factors (Kim *et al.*, 1997). Due to the high growth stimulation response on turf grass, *Bacillus* spp. Strain L324-92 was awarded a license in 1998 for further development and commercialisation for use on turf grass (Mathre *et al.*, 1999). Two *B. subtilis* Strains, GB03 and GB07, have been marketed as Kodiak and Epic respectively by Gustafon Inc. in the U.S. for use with several crops as plant growth promoting rhizobacteria (PGPR). PGPR are believed to improve plant growth by colonizing the root system and pre-empting the establishment of or suppressing deleterious rhizosphere microorganisms on the root (Schroth & Hancock, 1981; Schroth & Hancock, 1982; Suslow & Schroth, 1982). Field trials have shown that yields were increased by seed treatment with these two strains (Zhang *et al.*, 1996). In addition *B. subtilis* MBI 600, registered by the U.S. Environmental Protection Agency for commercial use against soilborne plant pathogens, is also found to improve stand establishment and seedling vigour (Cook *et al.*, 1996).

Propanza *et al.* (1996) isolated two PGPR strains (*B. pumilus* Isolate B.3 and *B. licheniformis* (Weigmann) Chester Isolate B.20) reported as potential plant growth stimulants. The two *Bacillus* strains caused significant increases in growth (182% on aerial length and 163% on aerial surface as compared to the controls) of European alder (*Alnus glutinosa* (L.) Gaertn. Further studies showed that PGPR strains produced auxin-like (IAA-1) compounds at levels of 1.736 and 1.790mg IAA-1L^{-1} culture medium. The filtered bacterial growth culture medium was found to promote plant growth (increase of 64% in aerial surface, 277% in total N content and 32% in aerial length).

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CHAPTER TWO

ISOLATION AND *IN VITRO* SCREENING OF *TRICHODERMA* AND *BACILLUS* ISOLATES FOR ANTAGONISM AGAINST *FUSARIUM OXYSPORUM* F. SP. *CONGLUTINANS*

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Abstract

Bacillus and *Trichoderma* isolates were isolated from the rhizosphere of maize, pine, cabbage and other vegetables to be used as biocontrol agents. They were screened for antagonism *in vitro* in dual culture against the phytopathogenic fungi, *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*). Five *Bacillus* isolates known as JRO1, JRO2, BFO11, B-81 and EX-R inhibited growth of *Foc* by more that 80%. On the other hand, most of the *Trichoderma* isolates (68.3%) showed a very strong degree of antagonism, rating 1 or 2 according to the Bell's rating. *Trichoderma* isolates ET13 and ET23 caused the formation of a brownish yellow colour change and a halo area on the point of contact between the *Trichoderma* isolate and the pathogen, respectively. Results from these studies were not necessarily expected to be related to biocontrol in the field, but they should reflect the potential and genetic variabilities of both the bacterial and fungal isolates as antagonists and of the plant pathogen to resist antagonism.

2.1 Introduction

Microorganisms, as naturally occurring resident antagonists, play an important role in plant disease control and therefore can be managed or exploited to achieve the desired results

(Mathre *et al.*, 1999). Protecting plants from adverse biotic factors, which affect the efficiency and microbiological quality of crops as raw materials, in intensive agricultural production systems is greatly significant. Available plant protection methods have been reviewed in the past decade due to the emergence of sustainable agricultural production systems. Therefore, the importance of using environmentaly-friendly and food-hygienically-safe plant-protection methods, and plant-protecting agents of biological origin, has been greatly emphasized in recent years (Földes *et al.*, 2000).

Many studies in the past have shown the potential of *Trichoderma* and *Bacillus* as biological control agents of several soilborne plant pathogens (Elad *et al.*, 1980; Harman *et al.*, 1980; Hadar *et al.*, 1984; Sivan *et al.*, 1984; Weller, 1988; Turner & Backman, 1991; Kim *et al.*, 1997a,b). Several members of the genus *Bacillus* produce various microbial substances such as antibiotics and secondary metabolites which have been found to show antimicrobial and/or antifungal activity against phytopathogenic and food-borne pathogenic microorganisms (Katz & Demain, 1977; Shoji, 1978). The use of such antimicrobially active species and strains of the genus *Bacillus*, or use of their metabolites, has been suggested as an alternative or supplementary mechanism to chemical plant protection (Swinburne *et al.*, 1975; Pusey & Wilson, 1984; McKeen *et al.*, 1986; Utkhede & Sholberg, 1986; Fravel, 1988; Handelsman *et al.*, 1990; Klich *et al.*, 1994; Potera, 1994; Leifert *et al.*, 1995; Berger *et al.*, 1996; Sharga & Lyon, 1998). Many of these bacilli are soil-inhabiting bacteria or exist as epiphytes and endophytes in the spermophere (Walker *et al.*, 1998; Bacon *et al.*, 2001) and rhizosphere (McKeen *et al.*, 1986; Handelsman *et al.*, 1990). This makes *Bacillus* species ideal candidates for use as biocontrol agents in seed treatment programmes against soilborne pathogens (Walker *et al.*, 1998).

Since *Trichoderma* species are antagonistic to other fungi, they can be used as biocontrol agents (Elad *et al.*, 1980; Elad *et al.*, 1981; Sivan *et al.*, 1984; Sivan & Chet, 1985; Calistru *et al.*, 1997). Some *Trichoderma* spp. used as biocontrol agents are able to produce either antibiotics or extracellular enzymes, or both (Cherif & Benhamou, 1990). They produce a large number of antibiotics (e.g., peptides, cyclic polypeptides), some of which have been characterized (Braeme-Cook & Faull, 1991; Ghisalberti & Sivasithamparam, 1991) as trichodermin, isolated from culture fluid of a strain of *Trichoderma viride* Pers. (Godtfredsen & Vangedal, 1965), and gliotoxin effective against *Pythium* sp. (Whipps & Lumsden, 1991). In addition to antibiotic production, *Trichoderma* spp. are known to be

prolific producers of polysaccharide lyases, proteases and lipases, all of which may be involved in host cell degradation (Benhamou & Chet, 1993). Chitinase, cellulose and β-glucanases are considered important enzymes active against plant pathogens (Chet & Baker, 1981).

Mycoparasitism of plant pathogenic fungi by *Trichoderma* has often being reported in *in vitro* studies. Directed growth of *T. hamatum* (Bonord.) Bainier towards host hyphae and formation of appressoria-like structures after contact, from which penetration took place, has been reported (Chet & Baker, 1981; Chet *et al.*, 1981). Similarly, coiling of *T. harzianum* Rifai around *Rhizoctonia solani* Khün hyphae is an early event preceding hyphal damage of the host (Benhamou & Chet, 1993). It excretes lytic extracellular β-1,3-glucanases and chitinase, and is able to live on hyphal cell walls, living mycelia, and sclerotia of *R. solani* or *Sclerotium rolfsii* Sacc. (Hadar *et al.*, 1979; Elad *et al.*, 1982).

In vitro screening for antibiosis is often used to select potential antagonists, even though *in vitro* antibiosis may not be related to biocontrol in the field (Fravel, 1988). There are several reports of correlation (Austin *et al.*, 1977; Utkhede & Rahe, 1983; Cullen & Andrews, 1984; Zhang *et al.*, 1996) and lack of correlation (Kommedahl & Windels, 1976; Lang & Kommedahl, 1976; Sleesman & Leben, 1976; Papavizas & Lewis, 1982; Suslow & Schroth, 1982; Utkhede & Gaunce, 1983; O'Brien et al., 1984) between assays *in vitro* and biocontrol in the field. Broadbent *et al.* (1971) *cited by* Fravel (1988) carried out an extensive survey of antagonists. They found that approximately 40% of the microorganisms inhibited one or more of the nine pathogens on agar, and about 4% of these were effective biocontrol agents in soil. They noted that, while some microorganisms that inhibited pathogens on agar also did so in soil, those organisms ineffective on agar were also ineffective in soil. Although there is poor correlation with field activity of selected antagonists, the technique has merit when used in conjunction with suitable secondary screens, where it allows economical use of facilities and time (Campbell, 1989).

The main objective of this work was the isolation of antagonistic bacteria and fungi that could control *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*) (Wollenweb.) W.C. Snyder & H.N. Hans *in vitro* and subsequent selection of bioantagonists for secondary *in vivo* greenhouse screening.

2.2 Materials and Methods

2.2.1 Sources of Biocontrol Organisms and Pathogen

2.2.1.1 Isolation of *Trichoderma* Isolates

Sixty isolates of *Trichoderma* spp. were obtained from various sources. Two of these isolates, *T. harzianum* Eco-T[®][1] in formulation and *T. atroviride* P. Karst. SY3A[2] P. Karsten, were provided by Plant Health Products and K.S. Yobo, respectively. Both are known for their potential antagonism against different plant pathogenic fungi. All the other 58 *Trichoderma* isolates were isolated from soils around the root zone of maize, pine, cabbage and squash from Cedara[3], Sunshine Seedlings[4] in Cramond, and commercial vegetable farms in the Tala Valley.

Isolation from soil was carried out on a modified *Trichoderma* selective medium (TSM) using the dilution serial technique. The TSM was prepared as follows: basal medium containing, 0.2g $MgSO_4$; 0.9g K_2HPO_4; 0.15g KCL; 1.0g NH_4NO_3; 3.0g glucose; 0.15g rose bengal; 20g agar and 950ml distilled water was autoclaved at 121°C for 15min. A biocidal medium was prepared in 50ml sterile distilled water containing, 0.25g crystallized chloramphenicol; 0.2g pentachloronitrobenzene (quintozene); 0.2g captab (Elad & Chet, 1983) supplemented with 1.2ml propamocarb (Previcur[®]) (Askew & Laing, 1994) was added to the autoclaved basal medium after it cooled down to 45°C. Seven days later isolated colonies of *Trichoderma* spp. were cultured on to Potato Dextrose Agar (PDA) (MERCK).

2.21.2 Isolation of *Bacillus* Isolates

Bacillus isolates were isolated from soil samples taken from the rhizosphere of maize, cabbage and squash from Cedara[5], commercial vegetable farms in the Tala-Valley, and from roots of greenhouse-grown potted maize and pine plants. Roots of maize and pine were washed off several times with water, cut into small pieces, and crushed using a

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mortar and paste in a 100ml of water. Large root fragments were removed by filtration through one layer of Cheese-cloth. Soil and plant suspension samples were heat treated at 80°C for 10min in a water bath so that endospores could be separated from vegetative cells (Foldes *et al.*, 2000). Using serial dilution, the heat treated soil and plant suspensions were plated onto Casein and Glucose Medium (CAG) containing 10g casein acid hydrolysate; 5g yeast extract; 5g glucose; 4g K_2HPO_4; 17g agar; 1 litre of distilled water; and 10ml of separately sterilized 50% glucose (Shurtleff & Averre III, 1997) and Soil Extract Agar (SEA) containing 1.0g glucose; 0.5 K_2HPO_4; 100ml soil extract; 20g agar; 900ml distilled water. After mixing all the ingredients the pH was adjusted to 6.8-7.0 and the medium autoclaved at 121°C for 15min. Soil extract was prepared by autoclaving 1kg of soil suspended in 1litre of water at 121°C for 30min. Two grams of calcium carbonate was added after being removed from the autoclave and filtered through several disks of filter paper until a clear filtrate was obtained.

Plates were incubated at 30°C for 2d. Colonies that looked like *Bacillus* spp. were identified using the microscopic appearance and Gram-reaction, cultured onto Nutrient Agar (MERCK) and incubated at 30°C.

A total of 96 isolates were prepared for testing. Pre-selected isolates of *Bacillus* sp. JRO1, *Bacillus* sp. JRO2, and *Bacillus* sp. BFO11, *Bacillus* sp. B-81, and *Bacillus* sp. EX-R were obtained from K.S. Yobo[6], M. Morris[7], and B. Kubheka[8], respectively. *Bacillus* isolates were stored in 15% glycerol at −80°C until use (Maniatis *et al.*, 1982).

2.2.2 Pathogen

Fusarium oxysporum f. sp. *conglutinans* was provided by M.D. Laing[9].The pathogen was preserved on silica gel (Windels *et al.*, 1988) and also maintained on Carnation Leaf Agar (CLA) slants at 4°C (Fisher *et al.*, 1982).

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2.2.3 *In Vitro* Screening of *Trichoderma* Isolates in Dual Culture

Tests were performed in Petri dishes (90mm diam.) containing 15ml of PDA. Mycelial disks (5mm×5mm.) were cut from expanding colonies of *Foc* and *Trichoderma* strains grown on PDA, and a single *Trichoderma* disk was placed on the opposite side of a plate 48h after inoculation with *Foc*, 15mm from the Petri dish edge. Since *Foc* grew slower than *Trichoderma* species it was placed on the medium 48h before the *Trichoderma* (Ordentlich *et al.*, 1991). Inoculated plates were incubated in the dark at 25°C for 7d and 14d. Plates inoculated with the pathogen alone were also prepared as controls on PDA plates and incubated for 7d and 14d at 25°C. The experiment was repeated twice and replicated three times.

Degree of antagonism was measured 7d and 14d later on a scale of 1-5 according to the rating system of Bell *et al.* (1982), whereby 1 = *Trichoderma* completely overgrew the pathogen and covered the entire medium surface, 2 = *Trichoderma* overgrew at least two thirds of the medium surface, 3 = *Trichoderma* and *Fusarium* each colonized 50% of the medium surface and neither organism appeared to dominate the other, 4 = *Fusarium* colonized at least two thirds of the medium surface and appeared to withstand encroachment by *Trichoderma*, and 5 = *Fusarium* completely overgrew the entire medium surface. Bell *et al.* (1982) considered an isolate of *Trichoderma* to be antagonistic if the mean score was ≤2, but not if the mean score was ≥3. Askew & Laing (1993) also confirmed that the *in vitro* method of Bell *et al.* (1982) provided good correlation with *in vivo* nursery trials.

2.2.4 Environmental Scanning Electron Microscopic (ESEM) Observation on the Interaction between *Trichoderma* Isolates and *Fusarium oxysporum* f. sp. *conglutinans*

Mycelial plugs (3mm in diam.) were cut from the interaction zone of the *Trichoderma* isolates and *Foc* and fixed overnight in 3% glutaraldehyde in cacodalate buffer (0.1M; pH7). Samples were then dehydrated in a graded series of alcohol ranging from 10 to 100% with 10% interval. Specimens were critical-point-dried with carbon dioxide as a transfusion fluid in a Hitachi HCP-2 and subsequently 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 a Philips XL30 Environmental Scanning Electron Microscopy (ESEM) operating at 15kV.

2.2.5 *In Vitro* Screening of *Bacillus* Isolates in Dual Culture

To determine the effects of *Bacillus* spp. on the growth of *Foc, Bacillus* spp. were cultured on PDA by making a single streak across the center of the Petri dish. After 2d at 25°C, PDA plugs 5mm×5mm, taken from the margins of growing *Foc* colonies were placed on either side of the *Bacillus* spp. streaked at a distance of 2cm. Two replicates were used for each treatment and the experiment was repeated twice. Antagonistic potentials, quantified as percent inhibition of growth, were evaluated after additional 4d at 25°C by measurement of the radii of *Foc* colonies cultured with the *Bacillus* spp. relative to the control plates with *Fusarium* alone. Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = (1 - (\text{Fungal growth} / \text{Control growth})) \times 100$$

2.2.6 Statistical Analysis

Experiments were repeated twice with treatments arranged in a Completely Randomized Design (CRD). All data on the *in vitro* antagonism of *Bacillus* isolates against *Foc* were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 6 Statistical Analysis Software to determine differences between treatment means.

Data on the *in vitro* antagonism of *Trichoderma* isolates against *Foc*, experiments were analyzed using the direct method of Bell *et al.* (1982). Bell *et al.* (1982) considered an isolate of *Trichoderma* to be antagonistic if the mean score was ≤ 2, but not if the mean score was ≥ 3.

2.3 Results

2.3.1 Isolation and Preliminary Characterization of Presumptive Antagonistic Bacteria

A large number of bacterial colonies developed from all sources. The subcultured bacterial isolates were preliminarily characterized as motile, endospore-forming and gram-positive bacteria using microscopic appearance and Gram-stain, which are typical characteristics of the genus *Bacillus*.

2.3.2 Isolation and Preliminary Identification of Presumptive Antagonistic Fungi

Isolated fungal colonies grown on the TSA were subculture onto PDA and identified as members of the genus *Trichoderma* by their fast growing hyaline colonies bearing repeatedly branched conidiophores in tufts with divergent, often irregularly bent, flask-shaped phialides and by their hyaline or more usually green conidia (Domsch *et al.*, 1980).

2.3.3 *In Vitro* Screening of *Trichoderma* Isolates in Dual Culture

The antagonism tests demonstrated that of the sixty *Trichoderma* isolates tested, 68.3% showed a high degree of antagonism (rate = 1 or 2) against *Foc* after 7d of incubation (Table 2.1, 2.2). After 14d of incubation 75% of the *Trichoderma* isolates had a rating of 1 or 2 (Tables 2.1, 2.2).

Within 7d of incubation the available surface of the Petri dish was covered by sporulating hyphae of 10% of the *Trichoderma* isolates tested, which prevented the growth of *Foc*. After continued incubation for 14d, 35% of the *Trichoderma* isolates managed to completely overgrow *Foc*. A distinct colour change was observed between Isolate ET13 and *Foc* when Isolate ET13 overgrew *Foc* (Fig.2.1B). No clear inhibition zone was observed except between Isolate ET23 and *Foc* (Fig.2.1D).

Table 2.1   *In vitro* antagonism of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *conglutinans,* pooled means of three trials

Trichoderma Isolate	Time of Incubation 7 d	14 d	*Trichoderma* Isolate	Time of Incubation 7 d	14 d
ET1	3.0	2.5	ET31	2.0	1.7
ET2	5.0	5.0	ET32	2.0	1.0
ET3	3.0	2.2	ET33	2.0	2.0
ET4	3.0	3.5	ET34	2.0	1.0
ET5	1.0	1.0	ET35	2.3	2.0
ET6	3.0	3.0	ET36	2.0	2.0
ET7	4.0	4.2	ET37	2.0	1.3
ET8	3.0	4.0	ET38	2.0	2.0
ET9	3.0	3.0	ET39	2.0	2.0
ET10	4.0	4.0	ET40	2.2	2.0
ET11	2.0	1.5	ET41	2.0	2.0
ET12	3.0	3.7	ET42	4.0	4.5
ET13	1.0	1.0	ET43	2.0	2.0
ET14	3.0	2.0	ET44	1.0	1.0
ET15	2.0	1.0	ET45	1.0	1.0
ET16	2.0	2.0	ET46	1.5	1.0
ET17	2.0	1.5	TH1	2.0	1.0
ET18	2.0	1.8	TH2	1.7	1.0
ET19	2.0	1.0	TH3	1.5	1.0
ET20	2.0	2.0	TH4	2.0	2.0
ET21	3.0	2.7	TH5	2.0	2.0
ET22	2.0	1.0	TH6	2.0	1.0
ET23	1.0	1.0	TH7	2.0	2.0
ET24	2.0	3.0	TH8	4.0	4.0
ET25	3.0	2.7	TH9	2.0	2.0
ET26	3.0	2.0	TH10	2.0	1.0
ET27	5.0	5.0	SY3	1.0	1.0
ET28	3.0	2.0	T-77B	2.0	1.0
ET29	3.0	2.0	Eco-T®	1.8	1.0
ET30	2.0	2.0	TN	2.0	1.0

Class of antagonism (based on Bell *et al.,* 1982): 1=*Trichoderma* grew and overlap the colony of *Foc* and the whole surface of the media; 2=*Trichoderma* grew and it covered two third of the surface of the media; 3=*Trichoderma* and *Foc* colonized each one half of the surface of the media and did not have dominance; 4= *Focs* grew and it covered two third of the surface of the media; 5= *Foc* grew and overlap the colony of *Trichoderma* and the whole surface of the media.

Table 2.2   Classification of *Trichoderma* isolates antagonism against *Fusarium oxysporum* f. sp. *conglutinans*

Antagonism Class	% Isolates 7 d	14 d
1	10	35
2	58.3	40
3	21.7	10
4	6.7	10
5	3.3	5
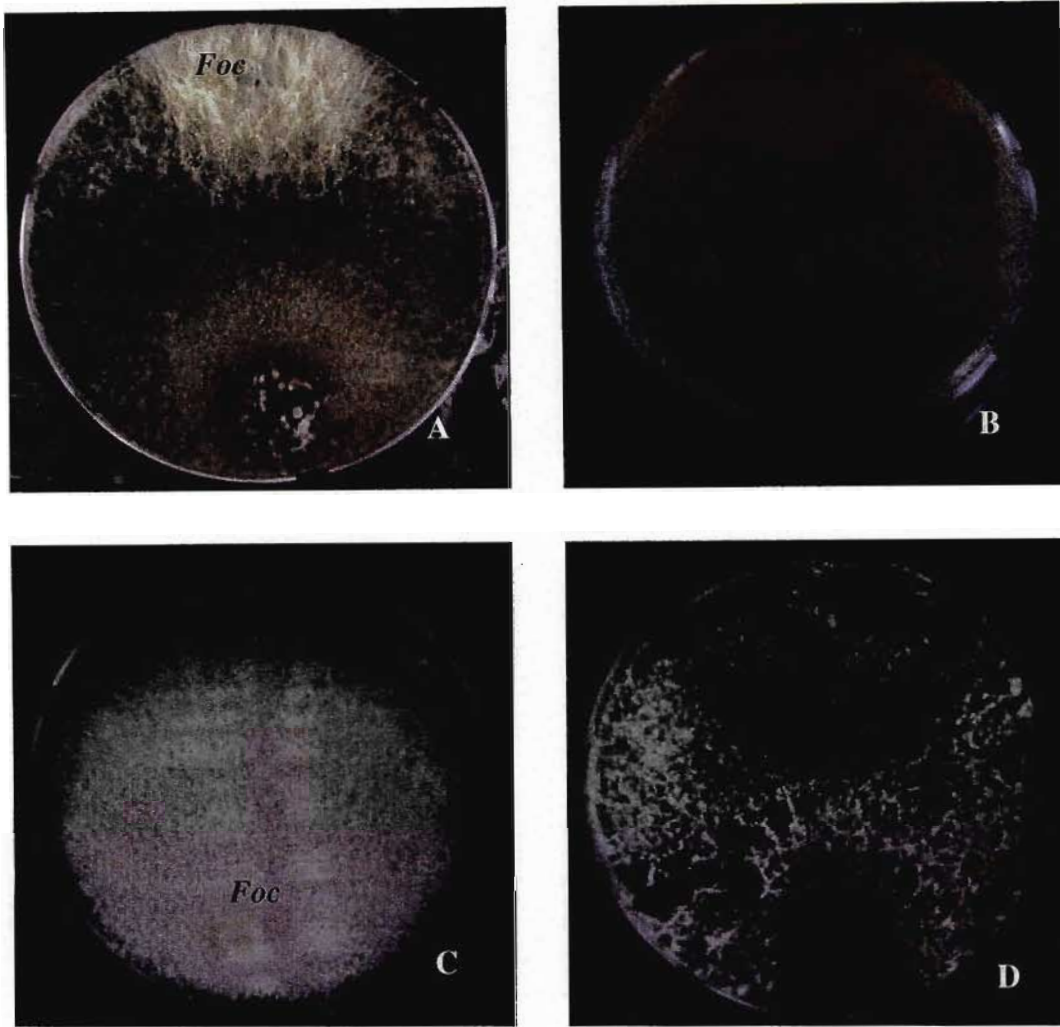
Fig 2.1　　　Plates showing the antagonism of some *Trichoderma* isolates against the phytopathogenic fungus, *F. oxysporum* f. sp. *conglutinans* (*Foc*), incubated at 25°C for 7d. Plate A: showing *Trichoderma* Isolate ET13 overgrowing the pathogen. Plate B: showing a brownish-yellow mycelial discoloration of *Foc* completely overgrown by *Trichoderma* Isolate ET13. Plate C: showing the control whereby *Foc* was inoculated alone. Plate D: showing that *Foc* was aggressively and completely overgrown by a sporulating mycelial mat of *Trichoderma* Isolate ET23; and the formation of a halo region (I) on the point of initial contact.

2.3.4 Environmental Scanning Electron Microscopic (ESEM) Observation on the Interaction between *Trichoderma* Isolates and *Fusarium oxysporum* f. sp. *conglutinans*

ESEM pictures obtained from samples taken from the interaction zone of the three *Trichoderma* isolates and *Foc* show the mycoparasitic nature of the antagonists against the pathogen. *Trichoderma* hyphae tightly attached itself to the hyphae of *Fusarium*, coiled around the hyphae of its victim (Fig. 2.2 A, B, C), caused excessive cell wall degradation and lysis (Fig. 2.2 A, B, C). Pronounced destruction of the mycelium of *Foc* was observed.
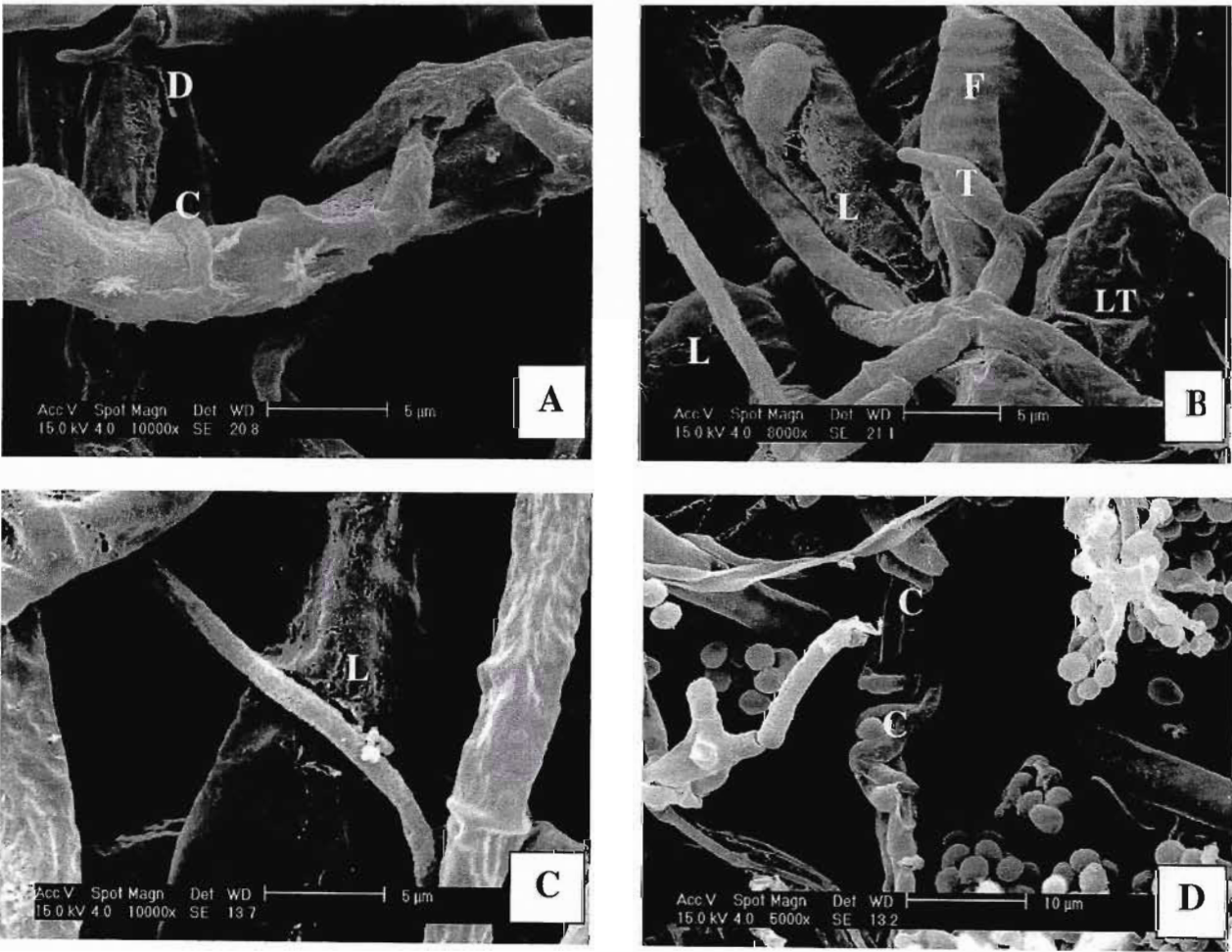
Fig. 2.2 Scanning electron micrograph of *Trichoderma* hyphae interacting with cells of *Foc*. Initial stages of mycoparasitism in which *Trichoderma* hyphae begins to coil (C) around the hyphae of *Foc* (A=*Trichoderma* Isolate ET13, 10000×; B=*T. harzianum* Eco-T®; D=*Trichoderma* Isolate ET23, 5000×). Cell wall degradation (D) lysis due to chemical activity of the *Trichoderma* isolates (A; B; C=*T. harzianum* Eco-T®, 10000×).

2.3.5 *In Vitro* Screening of *Bacillus* Isolates in Dual Culture

Of the 96 *Bacillus* isolates screened for antagonism *in vitro,* only 18 (18.8%) produced a significant antagonism ($P \leq 0.001$) with a clear zone of inhibition (4.75 – 17.25mm); another 18 (18.8%) *Bacillus* isolates showed no significant inhibition ($P \leq 0.001$); 61 (63.5%) isolates showed significant ($P \leq 0.001$) inhibition ($\leq$3mm) relative to the control, but with no clear zone of inhibition was produced between the *Bacillus* isolates and *Foc* tested. *Bacillus* isolates which had insignificant antagonism against the pathogen were eventually overgrown by the pathogen; while most of the *Bacillus* isolates which showed significant antagonism were not completely overgrown.

Five isolates, namely *Bacillus* spp. JRO1, JRO2, EX-R, BFO11 and B-81, showed the highest degree of inhibition ranging from 15.25 to 17.25mm, i.e, >80% inhibition. Inhibition continued as incubation continued. The bacteria grew along the edge of the Petri dish further shrinking the mycelia of the pathogen (Fig 2.3). The results of inhibition tests are presented in Table 2.4.

No colour change was observed during this test as also reported by Montealegre *et al.* (2003). They reported that a dark brown change in mycelial colour was observed closer to the colony end of *R. solani.*

Table 2.3   Growth inhibition of *Fusarium oxysporum* f. sp. *conglutinans* in dual culture against *Bacillus* isolates

% Inhibition	No. of *Bacillus* isolates	% *Bacillus* isolates
0-25	79	82.3
26-50	11	11.5
50-75	1	1
75-100	5	5.2
Total	96	100

Table 2.4 *In vitro* inhibitions of *F. oxysporum* f. sp. *conglutinans* by *Bacillus* isolates, pooled mean of three trials

Bacillus isolates	Inhibition (%)	Bacillus isolates	Inhibition (%)	Bacillus isolates	Inhibition (%)
B1	11^{fghi}	BFO7	5^{bcde}	JRO1	91^{s}
B2	16^{j}	BFO8	12^{ghij}	JRO2	90^{rs}
B3	9^{efgh}	BFO9	13^{hij}	JRO3	9^{efgh}
B4	9^{efgh}	BFO10	7^{cdef}	JRO4	12^{ghij}
B5	7^{cdef}	BFO11	86^{r}	JRO5	9^{efgh}
B6	13^{hij}	CEDB1	11^{fghi}	JRO6	4^{abcd}
B7	12^{ghij}	CEDB2	7^{cdef}	JRO7	5^{bcde}
B8	15^{ij}	CEDB3	9^{efgh}	MM1	8^{defg}
B9	0^{a}	CEDB4	9^{efgh}	MM2	11^{fghi}
B10	7^{cdef}	CEDB5	7^{cdef}	MM3	8^{defg}
B11	11^{fghi}	CEDB6	13^{hij}	MM4	7^{cdef}
BF1	12^{ghij}	CEDB7	9^{efgh}	MM5	7^{cdef}
BF2	9^{efgh}	CEDB8	9^{efgh}	MM6	9^{efgh}
BF3	12^{ghij}	CEDB9	5^{bcde}	MM7	11^{fghi}
BF4	11^{fghi}	CED10	11^{fghi}	MM8	9^{efgh}
BF5	9^{efgh}	CED11	9^{efgh}	MM9	7^{cdef}
BF6	11^{fghi}	CED12	9^{efgh}	MM10	9^{efgh}
BF7	12^{ghij}	CED13	3^{abc}	MM11	4^{abcd}
BF8	7^{cdef}	CED14	12^{ghij}	ACCI-1	50^{p}
BF9	7^{cdef}	CED15	7^{cdef}	ACCI-2	4^{abcd}
BF10	9^{efgh}	CED16	36^{lm}	ACCI-3	2^{ab}
BF11	7^{cdef}	CED17	4^{abcd}	ACCI-4	36^{lm}
BF12	9^{efgh}	CED18	33^{l}	ACCI-5	2^{abc}
BF14	12^{ghij}	CED19	33^{l}	ACCI-6	4^{abcd}
BF16	11^{fghi}	CED20	25^{k}	ACCI-7	4^{abcd}
BF24	9^{efgh}	CED21	7^{cdef}	ACCI-8	3^{abc}
BFO1	11^{fghi}	CED22	42no	ACCI-9	5^{bcde}
BFO2	9^{efgh}	CED32	38^{mn}	ACCI-10	40^{mn}
BFO3	5^{bcde}	CED34	36^{lm}	ACCI-11	50^{p}
BFO4	12^{ghij}	EX-R	90^{rs}	ACCI-12	4^{acd}
BFO5	11^{fghi}	R29	45°	ACCI-13	3^{abc}
BFO6	9^{efgh}	B81	80^{q}	ACCI-14	51^{p}
				CTRL	0^{a}
F test	<0.001	F test	<0.001	F test	<0.001
l.s.d.	4.6	l.s.d.	4.6	l.s.d.	4.6
s.e.d.	2.3	s.e.d.	2.3	s.e.d.	2.3
cv%	14.2	cv%	14.2	cv%	14.2

Means with the same letter are not significantly different at P<0.05.
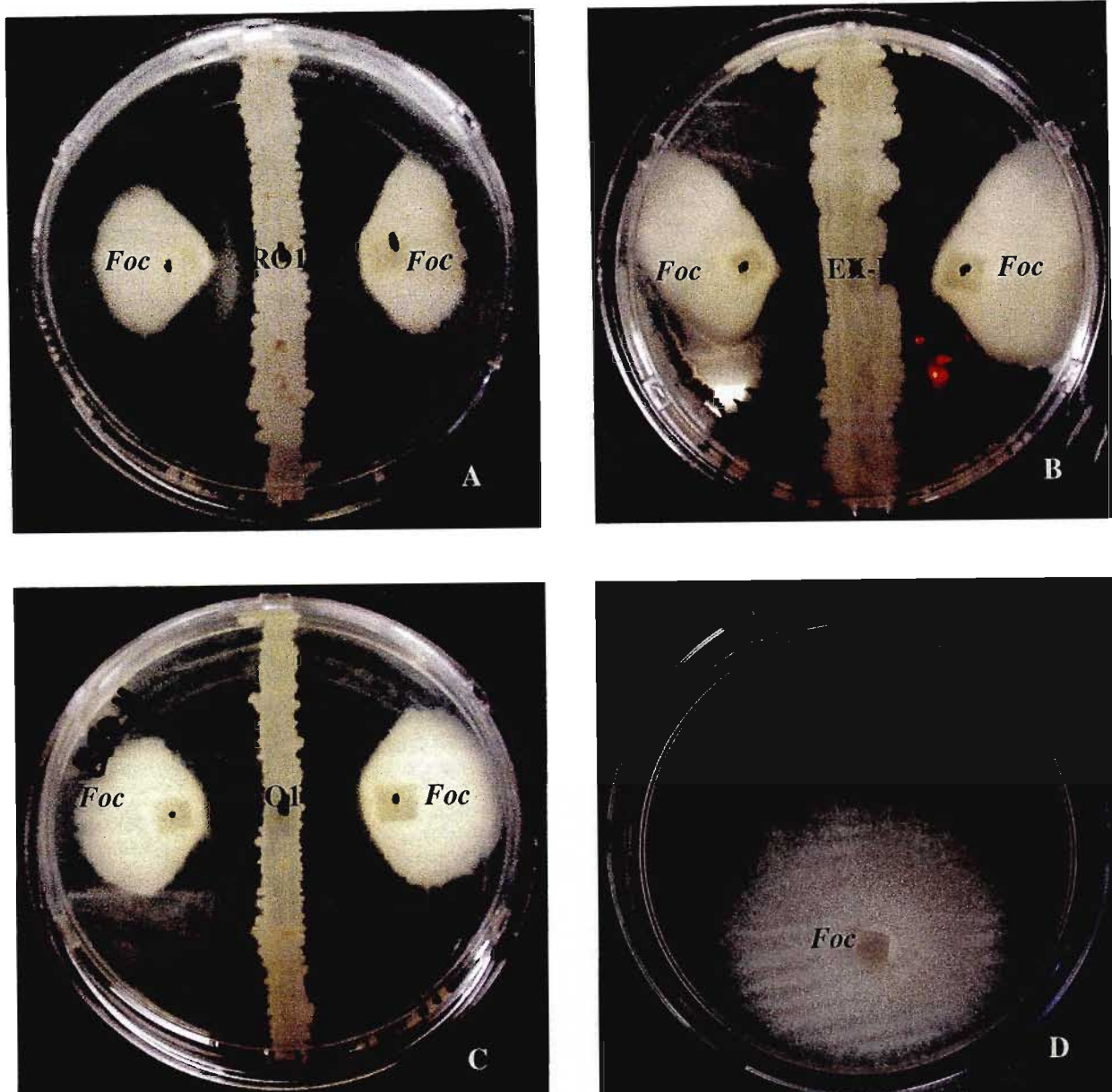
Fig 2.3 Plates showing antibiosis of some *Bacillus* isolates against *Foc* after being incubated at 25°C for 4d. Plate A: showing strong antifungal activity of *Bacillus* Isolate JRO1 inhibiting the growth of *Foc;* the antagonist further shrunk the growth of *Foc* even from the direction of no bacteria. Plate B: showing the antibiotic activity and vigorous growth of *Bacillus* Isolate EX-R. Plate C: showing the antibiotic activity of *Bacillus* Isolate BFO11. Plate D: showing the control where *Foc* was cultured alone.

2.4   Discussion

The development of a proper isolation and *in vitro* screening procedure that provides rapid, repeatable and reliable results is an important initial step in screening efficient antagonists for biocontrol of plant diseases (Anith *et al.*, 2003). The source of the antagonistic bacteria or fungi is also equally important. In this test, the source material for isolation of *Bacillus* and *Trichoderma* was from the rhizosphere of cereal, vegetable and pine plants. Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the front-line defense for roots against attack by pathogens (Podile & Prakash, 1996). The success of all subsequent stages depends on the ability of the initial screening procedure to identify an appropriate candidate (Anith *et al.*, 2003).

Although the dual culture method allows mass screening of isolates and statistical evaluation of data on potential antagonists, poor correlation with field activity of selected antagonists may occur (Papavizas & Lumsden, 1980). However, the technique has merit when used in conjunction with suitable secondary screens, where it allows economical use of facilities and time (Campbell, 1989). The purpose of this study was to isolate *Trichoderma* and *Bacillus* species and screen them *in vitro* against the phytopathogenic *Foc* to find potential antagonists for secondary screening under greenhouse conditions.

Both *Trichoderma* and *Bacillus* are known for their biocontrol potential against soilborne plant pathogens. They have been tested on a wide variety of plant species for their ability to control plant diseases (Cook & Baker, 1983; Sivan & Chet, 1985; Podile & Prakash, 1996; Zhang *et al.*, 1996; Mao *et al.*, 1997).

In the present study, most *Trichoderma* isolates were observed to completely overgrow and inhibit further growth of *F. oxysporum* f. sp. *conglutinans*. Some *Bacillus* isolates also showed very strong antagonism and formation of clear inhibition zone between the *Bacillus* isolates and the pathogen.

The fact that a high percentage (68.3%) of the *Trichoderma* isolates showed strong degree of antagonism (1 or 2) indicates the potential of *Trichoderma* spp. as biocontrol antagonists

against Fusarial diseases. Similar results were also observed by Askew & Laing (1993) when *Trichoderma* isolates were tested against *R. solani in vitro*.

A change to a brownish yellow discoloration in mycelial colour was observed on the common area colonized by both *Trichoderma* Isolate ET13 and *F. oxysporum* f. sp. *conglutinans*, as previously reported by Yobo *et al.* (2004). Thist dark brown colour of mycelia was observed when *T. atroviride* Isolate SY3A was tested for antagonism against *R. solani* on V8 medium. On the other hand, *Trichoderma* Isolate ET23 showed a high degree of antagonism by forming a halo region between the pathogen and the *Trichoderma* sp. itself, quickly overgrew the pathogen and completely covered it with its highly sporulating mycelial mat.

The discoloration of the area around the pathogen by *Trichoderma* Isolate ET13 and the formation of a clear zone by *Trichoderma* Isolate ET23 may be attributed to the production of either antibiotics, inhibitory metabolites (Whipps & Lumsden, 1991; Calistru *et al.*, 1997), or extracellular lytic enzymes such as β-1,3-glucanase and chitinase which enable penetration of host hyphae (Cherif & Benhamou, 1990; Ordentlich *et al.*, 1991) or a combination of these and other antimicrobial substances.

Trichoderma spp. have been known to use mycoparasitism as mechanisms of control of different plant pathogens (Zhang *et al.*, 1999). Microscopic observations using ESEM showed the mycoparasitic and lytic activity of the *Trichoderma* isolates. These modes of action are considered to contribute to disease suppression *in vivo*.

Many *Bacillus* strains are known to suppress fungal growth *in vitro* by the production of one or more antifungal antibiotics (Katz & Demain, 1977). Several members of the genus *Bacillus* produce various antimicrobial substances such as antibiotics and a wide range of antimicrobial substances (Katz & Demain, 1977; Shoji, 1978) that enable the antagonistic bacteria to control fungal soilborne plant pathogens. *Bacillus* spp. also have the advantage of forming a specialized quiescent cell type called endospore, which can remain dormant for extreme periods of time and endure most terrestrial stresses. This makes them appealing candidates for biocontrol because they are tolerant to heat and desiccation, which is an advantage over some root colonizing bacteria. This ability of bacteria to survive and proliferate in soil is an important factor in their success as inoculants for

promoting biological control, nutrient solubilisation and bioremediation (Young & Burns, 1993). The potency of *Bacillus* strains to produce peptide antibiotics and resist adverse environmental conditions is directly related to their ability to sporulate (Ochi & Ohsawa, 1984; Sneath, 1986).

In this study, screening of *Bacillus* focused on endospore-formers; *Bacillus* spp. were isolated by heating the source material at 80°C for 10min. Antagonism of these isolates was tested by means of the direct method of Zhang *et al.* (1996). Thus, it was easy to carry out successful screening because direct interaction was clearly visible.

Although only 5.2% of the *Bacillus* isolates showed a strong degree of inhibition (>80%), good or poor results *in vitro* on agar media do not necessarily mean that the antagonist will be effective or will not be effective *in vivo* (Utkhede & Sholberg, 1986). Some antibiotic producing *Bacillus* strains also suppress fungal plant diseases *in vivo* (Swinburne *et al.*, 1975; Baker *et al.*, 1983; Singh & Deverall, 1984; Pusey & Wilson, 1984; Pusey *et al.*, 1988; Utkhede & Sholberg, 1986; Fravel, 1988). Leifert *et al.* (1995) also noted that *in vitro* antagonism observed could give an indication to what the results will be *in vivo*. For this reason it is a good idea to have antagonists which are well-equipped with different antimicrobial weapons so as to be effective against various pathogens encountered in nature.

Bacillus isolates known as BFO11, JRO1, JRO2, EX-R, and B-81 displayed a strong degree of antagonism against the phytopathogenic fungi, *F. oxysporum* f. sp. *conglutinans,* preventing its hyphal growth by up to 80.3–90.8% owing to the production of antimicrobial compound(s). Though no discoloration of the mycelia was observed, as the incubation continued, the bacilli grew and encircled the fungus and shrunk the mycelial mat (Fig 2.3).

The function of antibiotics is to kill or inhibit the growth of other organisms in nature, thereby providing a competitive advantage to the producing species (Brian, 1957; Priest, 1993). It has been reported 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, plates streaked with *Bacillus* isolates were incubated for 48h at 25°C before being inoculated with the test fungi. Similarly, Foldes *et al.* (2000) indicated that when *Bacillus* isolates were given an advantage, by pre-

incubation, over the test organism with respect to their optimal growth temperature, various metabolites could be produced.

In this test, highly antagonistic bacteria and fungi were obtained that could be used for secondary screening under greenhouse conditions. Results from these studies were expected to reflect the potential and genetic variabilities of both the bacterial and fungal isolates as antagonists and of the plant pathogen to resist antagonism (Bell *et al.*, 1982).

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CHAPTER THREE

SCREENING OF *TRICHODERMA* AND *BACILLUS* ISOLATES AS BIOCONTROL AGENTS AGAINST *FUSARIUM OXYSPORUM* F. SP. *CONGLUTINANS* UNDER GREENHOUSE CONDITIONS

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Abstract

Trichoderma and *Bacillus* isolates, which showed *in vitro* antagonism against *Fusarium oxysporum* f. sp. *conglutinan*s, were screened under greenhouse conditions on cabbage. *Trichoderma* isolates were applied both as a seed treatment and spore suspensions while *Bacillus* isolates were applied by drenching. Some of the isolates significantly (P<0.001) reduced disease incidence and severity, and increased host dry weight when compared to the treatment with the pathogen only. There was also a significant difference between methods of application. Drenching of spore suspensions of the antagonists reduced disease incidence and severity more than seed treatment. Although there was statistically significant reduction in disease incidence and severity due to the introduction of the biocontrol agents, the percent disease decrease was below 50%.

3.1   Introduction

Fusarium spp. specifically attack many agricultural crops causing vascular wilt diseases (Nelson, 1981). These Fusaria have the ability to establish themselves systemically in the xylem vessels of their hosts causing diversified symptoms (MacHardy & Beckman, 1981).

The use of chemical pesticides to control plant diseases in agriculture has raised global concern of its impact on the environment. In addition to environmental concern, the absence of effective chemicals against Fusarial diseases has led to an intensive search for an alternative mechanism of plant protection and disease management strategies (Kok *et al.*, 1996).

Many attempts have been made to control *Fusarium* spp. by biological means. One of the significant phenomena in biological control of *Fusarium* wilt disease is the existence of suppressive soils in which microbial activity prevents the appearance of disease in susceptible crops (Alabouvette *et al.*, 1979; Kloepper *et al.*, 1980; Scher & Baker, 1982).

Many studies have confirmed the potential of *Trichoderma* and *Bacillus* species as valuable biocontrol agents against several soilborne plant pathogens (Sivan & Chet, 1986; Chet, 1990; Zhang *et al.*, 1996; Harman, 2000; Paulitz & Bélanger, 2001). *Trichoderma* has been exceptionally good model with which to study biocontrol because it is ubiquitous, easy to isolate and culture, grows rapidly on many substrates, affects a wide range of plant pathogens, is rarely pathogenic on higher plants, acts as a mycoparasite, competes well for food and site, produces antibiotics, and has an enzyme system capable of attacking a wide range of plant pathogens (Mukerji & Garg, 1988). The ability of bacteria to survive and proliferate in soil is an important factor in their success as introduced biocontrol agents (Young & Burns, 1993). Many bacilli are soil-inhabiting bacteria or exist as epiphytes and endophytes in the spermosphere (Walker *et al.*, 1998; Bacon *et al.*, 2001) and rhizosphere (McKeen *et al.*, 1986; Handelsman *et al.*, 1990). This makes *Bacillus* species ideal candidates for use as biocontrol agents against soilborne pathogens (Walker *et al.*, 1998).

Biocontrol research of soilborne plant pathogenic fungi often report that *Trichoderma* spp. are among the most promising biocontrol agents (Wells *et al.*, 1972; Hadar *et al.*, 1979; Liu & Baker, 1980; Elad *et al.*, 1984). Chet & Baker (1981) found an isolate of *Trichoderma hamatum* (Bonord.) Bainier in a Colombian soil naturally suppressive to *Rhizoctonia solani* Kühn. The antagonist was an effective biocontrol agent when applied to infested soil in the greenhouse. Similarly, Hadar *et al.* (1979), Elad *et al.* (1980), Elad *et al.* (1981b) and Elad *et al.* (1982) found very promising isolates of *T. harzianum* Rifai capable of controlling *R. solani* and *Sclerotium rolfsii* Sacc. both in the greenhouse and in the field. Locke *et al.* (1985) reported that the benomyl–tolerant *T. viride* Pers. Biotype T-1-R9 was

shown to be a potential biocontrol agent for reducing *Fusarium* wilt in vegetative chrysanthemums. Many researchers have shown that *T. harzianum* was an effective biocontrol agent reducing the incidence of crown rot of tomato under field conditions in two successive seasons (Sivan *et al.*, 1987), *Fusarium* wilt of cotton and melons as well as *F. culmorum* (W.G. Sm.) Sacc. on wheat under natural soil conditions (Sivan & Chet, 1986). Marois *et al.* (1981) were the first to demonstrate successful biological control of *Fusarium* crown rot on tomatoes with *T. harzianum*.

Many bacteria, including *Pseudomonas fluorescens* Migula, *P. putida* (Trevisan) Migula and *Bacillus subtilis* (Ehrenberg) Cohn, have been studied as biocontrol agents of soilborne plant pathogens (Howell & Stipanovic, 1979, 1980; Currier *et al.*, 1988; Kloepper, 1991; Brannen & Backman, 1993, 1994). Seed treatments with *B. subtilis* have since been shown to control various diseases in a variety of crops, including diseases caused by *R. solani* in wheat, brown spot of rice, damping-off in tomato and sugar beet, and cotton (Dunleavy, 1955; Merriman *et al.*, 1974a; Merriman *et al.*, 1974b; Nanda & Gangopadhyay, 1983; Zhang *et al.*, 1996). Kim *et al.* (1997) reported on a unique *Bacillus* sp., designated as Strain L324-92 that suppressed three root rot diseases of wheat, namely take-all caused by *Gaeumannomyces graminis* var. *tritici* J. Walker, rhizoctonia root rot caused by *R. solani* AG8, and pythium root rot caused by *Pythium* species, when applied as a seed inoculation. Aldrich & Baker (1970) presented evidence that *B. subtilis* acts as biocontrol agent when it was applied by dipping or incorporated into the rooting medium; it controlled the incidence of *Fusarium* stem rot of carnation caused by *Fusarium roseum* f. sp. *dianthi* (Fod). Recently, two *B. subtilis* strains, GB03 and GB07, have been marketed as Kodiak and Epic, respectively, by Gustafson Inc., Plano, TX, USA, for use with several crops as growth promoting rhizobacteria. Field experiments have shown that cotton yields are increased by seed treatment with these two strains (Minton *et al.*, 1991; Brannen & Backman, 1993; Kenney & Arthur, 1994). Similarly, Zhang *et al.* (1996) showed that *B. subtilis* Strains GB03 and GB07 strongly inhibited growth of *F. oxysporum* f. sp. *vasinfectum* (G.F. Atk.) W.C. Snyder & H.N. Hansen and other *Fusarium* spp. *in vitro*, and reduced the colonization of cotton roots in growth chamber studies.

Selected strains of *B. subtilis*, produced by FZB Biotechnic GmbH Berlin, have been tested in greenhouse and field experiments in their biocontrol effect on numerous seedling disease, root rot and wilt disease inducing soil-and seed-borne fungal pathogens in

vegetables, ornamental and agricultural plants cultivated in soil and hydroponics (Bochow & Gantcheva, 1995). Results show that specific strains of *B. subtilis* isolates act as a broad-spectrum biocontrol agent with antagonistic and suppressive activities on many pathogenic soil fungi together with plant health promoting actions by enhancing plant growth and yield as well as disease resistance. *Bacillus subtilis,* as a spore forming bacterium, can be formulated as a biopreparation relatively easily and can be applied by way of seed treatment (dipping), drenching around plants or introduction into hydroponic nutrient solutions (Bochow & Gantcheva, 1995).

The main objective of this research was to evaluate the ability of the different *Bacillus* and *Trichoderma* isolates to reduce disease incidence and severity of the cabbage yellows caused by *Foc.*

3.2   Materials and Methods

3.2.1   Source of Plant Material

Cabbage, *Brassica oleracea* var. *capitata* L. cv. Glory of Enkhuizen, Reference no. 570101 ABAE, seeds were obtained from Starke Ayres[1] (Pty) Ltd, which is a member of the Pannar® Group.

3.2.2   Biocontrol Microorganisms

Trichoderma *Isolates*

Twenty *Trichoderma* isolates (Table 3.1-3.6) obtained from primary *in vitro* antagonism screening tests against *F. oxysporum* f. sp. *conglutinans* (*Foc*) (Wollenweb.) W.C. Snyder & H.N. Hans (Chapter Two) were used in this secondary greenhouse screening experiment. Of the 20 *Trichoderma* isolates used in this study, *T. harzianum* Eco-T® and *T. atroviride* SY3A were provided by Plant Health Products[2] and K.S. Yobo[3], respectively.

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[1] Starke Ayres[1] (Pty) Ltd, Republic of South Africa.
[2] Plant Health Products (Pty) Ltd, P.O.Box. 207, Nottingham Road, KwaZulu-Natal, Republic of South Africa.
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The rest were isolated from the rhizosphere of cereal and vegetable crops from a maize field in Cedara[4] and commercial vegetable farms in the Tala-Valley, KwaZulu-Natal Province, South Africa.

Bacillus *Isolates*

Eighteen *Bacillus* isolates (Tables 3.6-3.9) previously screened for their potential antagonism in *in vitro* against *Foc* (Chapter Two) were used in this secondary greenhouse screening experiment. Of the eighteen *Bacillus* isolates, EX-R, B81, and BFO11, JRO1 and JRO2 were provided by M. Morris[5], B. Kubheka[6] and K.S. Yobo[7], respectively. The rest were isolated from the rhizosphere of maize and vegetable crops from maize fields in Cedara[4] and commercial vegetable farms in the Tala Valley, KwaZulu-Natal Province, South Africa.

3.2.3 Pathogen

Fusarium oxysporum f. sp. *conglutinans* was provided by M.D. Laing[8]. The pathogen was preserved on silica gel (Perkins, 1962; Windels *et al.*, 1988) and also maintained on Carnation Leaf Agar (CLA) (Fisher *et al.*, 1982) slants at 4°C.

3.2.4 Production of Conidia for Introducing *Fusarium oxysporum* f. sp. *conglutinans* into Soils

Erlenmeyer flasks (250ml) each containing 50ml of liquid yeast extract-glucose (YM) containing yeast extract (MERCK) 5g; peptone (Difco Laboratories) 5g; glucose, 10g; and 1000ml distilled water, were inoculated with mycelial disks from 72h old cultures of *Foc*. Flasks were incubated at 27°C in a water-bath shaker (Gesellschaft für Labortechnik, mbH, D-30938, Burgwedel, Germany) at 120 oscillations min^{-1} for 4d. Conidia were separated by filtration through eight layers of Cheese-cloth. The conidial suspensions were then

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washed three times by centrifugation in a Beckman J2-HS Centrifuge at 9000×g for 30min at 4°C.

Suspensions containing macro-and microconidia of *Foc* were counted using a Neubauer Improved Double Haemocytometer and adjusted to 2×10^7 conidia ml^{-1}. Four millilitres of this suspension were added to each seedling in a Speedling® 24 tray. The pathogen was inoculated 4d after the cabbage seeds were sown to encourage the germination of conidia due to the release of plant exudates from the roots of the cabbage seedlings.

3.2.5   Production of Conidia for Introducing *Trichoderma* Isolates into Soils

A conidial suspension of *Trichoderma* isolates was prepared from conidia collected from cultures grown on V8 agar medium, containing 200ml V8 tomato juice; 3g $CaCO_3$ (uniLAB®); 15g agar (MERCK) and 800ml of distilled water and autoclaved for 15min at 121°C. V8 agar plates were inoculated with agar blocks from half strength PDA plates kept at 4°C and incubated for 7d at 25°C. Then 5ml sterile distilled water was pipetted onto plates and conidia were collected by scraping the plate with a cotton wool-tipped sterile Pasteur pipette.

***Trichoderma* isolates were applied in two ways:**

a.   *Seed Treatment*

The conidial suspensions of the 20 *Trichoderma* isolates were counted using Neubauer Improved Double Haemocytometer and adjusted to 5×10^9 conidia ml^{-1}. Cabbage seeds were soaked in conidial suspensions from each isolate supplemented with 2% (g v^{-1}) carboxylmethycellulose (CMC), which served as an adhesive, then immediately dried by ventilation under a laminar flow.

The density of conidia of the *Trichoderma* isolates on the seed coat surface was determined by a plate dilution technique from treated and untreated seeds, using a modified *Trichoderma*-selective medium (Elad *et al.*, 1981a; Askew & Laing, 1994).

b.   *Seedling Drench*

For drenching, the conidial suspension of the *Trichoderma* isolates was counted using a Neubauer Improved Double Haemocytometer and adjusted to 10^7 conidia ml^{-1}. Then each cabbage seedling in the Speedling® 24 tray was inoculated with 4ml of *Trichoderma* conidial suspension. In this experiment, cabbage seedlings were inoculated both with the biocontrol agents and the pathogen at the same time 4d after sowing to encourage germination and growth of conidia of both the *Trichoderma* and *Fusarium* due to the release of root exudates from the already established seedling.

3.2.6   Preparation of Antagonistic *Bacillus* isolates for Introducing into Soils

Bacillus isolates were grown in 250ml Erlenmeyer flasks each containing a 100ml nutrient broth (MERCK) for 4d at 30°C in a water bath (Gesellschaft für Labortechnik, mbH, D-30938, Burgwedel, Germany) shaker at 150 oscillations min^{-1}. Flasks were inoculated with *Bacillus* isolates previously grown in Tryptone Soy Agar (TSA) (MERCK) for 48h. After 4d *Bacillus* isolates were harvested by centrifugation in a Beckman J2-HS Centrifuge at 9000×g for 15min. The broth was decanted and bacterial pellets were resuspended in sterile distilled water. Bacterial cells were then counted using a plate dilution technique in TSA and adjusted to a concentration of 10^7 c.f.u. ml^{-1} of water.

Bacillus isolates were applied by drenching 4ml of the bacterial suspension into the soils of the 4d old cabbage seedlings grown in Speedling® 24 tray. Seedlings were inoculated at 4d in order to stimulate both the pathogen and bacterial spores to germinate owing to the release of root exudates.

3.2.7   Sterilization of Speedling® 24 Trays

Since the Speedling® 24 trays were previously used by other researchers, sterilization of the trays was necessary to eliminate the effect of other microbial agents. Trays were sterilized by dipping in Plaz-Dip® and immediately air-dried. Plaz-Dip® is a 12% copper hydroxide suspension in PVA paint.

3.2.8 Plant Growing Medium

Cabbage seedlings were grown in a composted pine bark medium. It was used as a sole plant-growing medium for the entire study. Since composted pine bark is nutritionally poor and has a poor water holding capacity, irrigation was provided three times a day for 5min supplemented with NPK soluble fertilizer [3:1:3(38)].

3.2.9 Controls

There were two control treatments:
1. neither antagonist nor pathogen
2. pathogen only

3.2.10 Disease Rating

Disease incidence and disease severity were recorded based on visual assessment of symptoms arising from infection by *Foc* according to a five-point key (Carver *et al.*, 1996):

0 = healthy plants

1 = initial signs of wilting (yellowing)

2 = up to 25% of the leaves with symptoms

3 = up to 50% of the leaves with symptoms

4 = up to 75% of the leaves with symptoms

5 = plants dead

The disease incidence and severity were calculated as follows (Zhang *et al.*, 1996):

Disease incidence = $100\% \times (n1 + n2 + n3 + n4 + n5)$

Disease severity = $100 \times (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)/5n$

3.2.11 Statistical Analysis

Experiments were repeated twice with treatments arranged in a Randomized Complete Block Design (RCBD). All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 6 Statistical Analysis Software to determine differences

between treatment means. Percentage of disease incidence and disease severity were calculated on a per plot basis and transformed using angular transformation, while percentage of germination was transformed using square root transformation prior to analysis. All least significant differences were determined at $P < 0.05$.

The usual interpretation of the analysis of variance is valid only when certain mathematical assumptions concerning the data are met. These assumptions are (i) additive effects, (ii) independence of errors, (iii) homogeneity of variance and (iv) normal distribution. Failure to meet one or more of these assumptions affects both the level of significance and sensitivity of the F test in the analysis of variance. Experiments designed to evaluate the incidence of disease, count data such as number of infested plants per plot or percent plants infested with a disease, violate some of the assumptions of variance. Thus, any drastic departure from one or more of the assumptions must be corrected before the analysis of variance is applied (Gomez & Gomez, 1984).

Therefore, the remedial measure believed to be appropriate in this research is 'data transformation'. This also applies to Chapters 4, 5 and 6. The following rules were used in choosing the proper transformation scale for percentage data derived from count data (Gomez & Gomez, 1984).

Rule 1. For percentage data lying within the range of 30 to 70%, no transformation needed.

Rule 2. For percentage data lying within the range of either 0 to 30% or 70 to 100%, but not both, the square-root transformation should be used.

Rule 3. For percentage data that do not follow the ranges specified in either Rule 1 or Rule 2, the arc sine (angular) transformation should be used.

3.3 Results

3.3.1 *Trichoderma* Isolates Applied as Seed Treatments

Twenty isolates of *Trichoderma* applied as seed treatments to cabbage seeds were tested for their potential to reduce the percentage of disease incidence and disease severity of the cabbage yellows fungus, *Foc*. Of all the isolates tested, 61% of the *Trichoderma* isolates very significantly (P<0.001) reduced percent disease incidence by 13-35%; and 65% of the isolates very significantly (P<0.001) reduced percent disease severity by 15-38% when averaged over the three trials. Of all the isolates tested, only two isolates reduced disease incidence and severity by more than 32% and one isolate reduced diseases incidence and severity by more than 25%. Results on seed treatment of cabbage seeds with *Trichoderma* isolates are presented in Tables 3.1-3.3.

The effect of *Trichoderma* isolates was also highly significant (P<0.001) on dry matter content of cabbage seedlings relative to the control infested with the pathogen only. Treatments with Isolates ET13, ET23 and *T. harzianum* Eco-T® significantly increased the dry weight of plots inoculated with *Foc*. As expected, the pathogen caused a significant reduction (P<0.001) in the dry weight of cabbage between the pathogen inoculated and non-inoculated treatments.

Some *Trichoderma* isolates applied as seedcoating were shown to significantly (P<0.001) affect the germination of cabbage seeds in the first and second experiment (Table 3.1). However, in the third experiment, no significant difference was observed on the percentage germination of cabbage seeds coated with spores of *Trichoderma* isolates (Table 3.2, 3.3). *Foc* was assumed to have no effect on the germination of cabbage seeds since inoculation was carried out 4d after sowing, when cabbage seeds had already germinated.

Cabbage seedlings infested with spore suspensions of *Foc* only showed disease incidence and disease severity of 87-91% and 78-88%, respectively. No disease was observed on seedlings inoculated with neither the pathogen nor the antagonists.

Table 3.1   Biocontrol effects of *Trichoderma* isolates applied as a seed treatments against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 1

Trichoderma Isolates	Germination (%)	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	86(9)^b	67(55)^b	61(51)^{bc}	2.58^{cd}
ET13	81(9)^b	69(56)^{bc}	54(48)^b	2.67^d
ET15	81(9)^b	82(65)^{bcdef}	74(60)^{cdef}	1.26^a
ET19	74(9)^b	81(64)^{bcdef}	68(55)^{bcde}	1.47^a
ET22	85(9)^b	84(67)^{def}	79(63)^{ef}	1.11^a
ET23	71(8)^a	71(58)^{bcd}	64(53)^{bcd}	2.48^{bcd}
ET32	85(9)^b	74(60)^{bcde}	70(57)^{bcdef}	1.63^{ab}
ET34	79(9)^b	77(62)^{bcdef}	75(60)^{cdef}	1.43^a
ET44	79(9)^b	73(60)^{bcde}	68(56)^{bcde}	1.23^a
ET45	71(8)^a	75(60)^{bcde}	71(58)^{cdef}	1.19^a
ET46	87(9)^b	77(62)^{bcdef}	70(57)^{bcdef}	1.63^{ab}
ET5	81(9)^b	82(66)^{cdef}	68(55)^{bcde}	1.29^a
SY3A	92(10)^c	77(62)^{bcdef}	72(58)^{cdef}	1.35^a
ET47	74(9)^b	76(61)^{bcdef}	73(59)^{cdef}	1.73^{abc}
ET48	82(9)^b	86(69)^{ef}	74(60)^{cdef}	1.26^a
TH1	78(9)^b	76(61)^{bcdef}	67(55)^{bcde}	1.16^a
TH2	78(9)^b	86(68)^{def}	77(61)^{def}	1.26^a
TH3	76(9)^b	75(60)^{bcde}	73(59)^{cdef}	1.88^{abcd}
TH6	86(9)^b	77(62)^{bcdef}	71(57)^{bcdef}	1.86^{abcd}
TH10	83(9)^b	75(60)^{bcde}	70(57)^{bcdef}	1.08^a
Foc only	94(10)^c	89(71)^f	83(66)^f	1.55^a
Water only	87(9)^b	0(6)^a	0(6)^a	3.92^e
F test	(<0.007)	(<0.001)	(<0.001)	<0.001
l.s.d	(0.6)	(10.7)	(9.4)	0.915
s.e.d	(0.6)	(5.3)	(4.7)	0.453
cv%	(4.3)	(10.9)	(10.3)	32.9

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using square-root transformation for germination (%) and angular transformation for disease incidence (%) and disease severity (%).

Table 3.2   Biocontrol effects of *Trichoderma* isolates applied as a seed treatment against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 2

Trichoderma Isolates	Germination (%)	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	89(9)^a	60(51)^{bc}	50(45)^c	2.83^b
ET13	82(9)^a	54(47)^b	40(39)^b	2.60^b
ET15	86(9)^a	79(63)^{fgh}	63(53)^{efg}	1.34^a
ET19	76(9)^a	83(66)^{hi}	70(57)^{gh}	1.05^a
ET22	89(9)^a	78(62)^{fgh}	67(55)^{fg}	1.02^a
ET23	78(9)^a	63(52)^{bc}	53(47)^{cd}	2.58^b
ET32	88(9)^a	73(59)^{ef}	63(53)^{efg}	1.14^a
ET34	85(9)^a	79(63)^{fgh}	66(54)^{fg}	1.13^a
ET44	83(9)^a	64(53)^{cd}	56(49)^{cde}	1.14^a
ET45	75(9)^a	82(65)^{ghi}	71(57)^{gh}	1.10^a
ET46	90(10)^b	72(58)^{def}	63(53)^{efg}	1.36^a
ET5	88(9)^a	68(56)^{cde}	54(47)^{cd}	1.40^a
SY3A	94(10)^b	69(56)^{cde}	60(51)^{def}	1.49^a
ET47	83(9)^a	74(60)^{efg}	66(54)^{fg}	1.30^a
ET48	79(9)^a	81(65)^{ghi}	70(57)^{gh}	1.17^a
TH1	82(9)^a	76(61)^{efgh}	64(53)^{efg}	1.47^a
TH2	82(9)^a	88(70)^i	75(60)^{hi}	1.11^a
TH3	81(9)^a	77(61)^{efgh}	65(54)^{fg}	1.40^a
TH6	90(10)^b	78(62)^{fgh}	69(56)^{gh}	1.23^a
TH10	86(9)^a	83(66)^{hi}	75(60)^{hi}	1.14^a
Foc only	89(9)^a	87(69)^i	78(62)^i	1.01^a
Water only	88(9)^a	0(6)^a	0(6)^a	3.53^c
F test	(<0.001)	(<0.001)	(<0.00.)	<0.001
l.s.d	(0.3)	(5.0)	(4.6)	0.512
s.e.d	(0.1)	(2.5)	(2.3)	0.254
cv%	(1.8)	(5.3)	(5.5)	20.4

- Means with the same letter in the same experiment are not significantly different at P<0.05.

- Values in parenthesis represent transformed means using square-root transformation for germination (%) and angular transformation for disease incidence (%) and disease severity (%).

Table 3.3 Biocontrol effects of *Trichoderma* isolates applied as a seed treatment against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 3

Trichoderma Isolates	Germination (%)	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	89(9)^a	42(40)^b	33(35)^b	2.87^c
ET13	85(9)^a	44(42)^{bc}	32(34)^b	2.75^{bc}
ET15	88(9)^a	63(53)^{bcde}	45(42)^{bcd}	1.47^a
ET19	83(9)^a	80(64)^{ef}	65(54)^{defgh}	1.07^a
ET22	92(10)^b	67(56)^{cde}	56(49)^{cdefg}	1.29^a
ET23	88(9)^a	56(48)^{bcd}	46(43)^{bcde}	2.83^c
ET32	86(9)^a	70(57)^{de}	57(49)^{cdefg}	1.02^a
ET34	92(10)^b	73(59)^{de}	57(49)^{cdefg}	1.17^a
ET44	90(9)^a	61(52)^{bcde}	52(46)^{bcdef}	1.21^a
ET45	85(9)^a	83(66)^{ef}	74(60)^{ghi}	1.15^a
ET46	90(10)^b	65(54)^{bcde}	54(48)^{cdefg}	1.82^a
ET5	89(9)^a	55(48)^{bcd}	42(40)^{bc}	1.70^a
SY3A	90(9)^a	65(54)^{bcde}	52(46)^{bcdef}	1.72^a
ET47	94(10)^b	74(59)^{de}	59(50)^{cdefg}	1.19^a
ET48	78(9)^a	80(64)^{ef}	70(57)^{fgh}	1.13^a
TH1	85(9)^a	69(58)^{de}	58(50)^{cdefg}	1.84^{ab}
TH2	86(9)^a	82(65)^{ef}	69(56)^{fgh}	1.04^a
TH3	89(9)^a	73(59)^{de}	62(52)^{cdefg}	1.20^a
TH6	93(10)^b	77(62)^{def}	67(55)^{efgh}	1.21^a
TH10	85(9)^a	97(84)^g	87(70)^i	1.09^a
Foc only	97(10)^b	91(76)^{fg}	80(65)^{hi}	0.96^a
Water only	94(10)^b	0(6)^a	0(6)^a	2.87^c
F test	(0.135)^{NS}	(<0.001)	(<0.001)	<0.001
l.s.d	(0.6)	(14.3)	(12.3)	0.911
s.e.d	(0.3)	(7.1)	(6.1)	0.452
cv%	(3.6)	(15.6)	(15.6)	34.7

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-NS = Not Significant

-Values in parenthesis represent transformed means using square-root transformation for germination (%) and angular transformation for disease incidence (%) and disease severity (%).

3.3.2 *Trichoderma* Isolates Applied as Drenches

Significant differences (P<0.001) in the reduction of disease incidence and severity were observed when *Trichoderma* isolates were applied as spore suspensions. Of the 20 *Trichoderma* isolates tested, 73% and 70% of the isolates significantly reduced disease incidence and severity, respectively; disease incidence and severity were reduced by 13-47% and 12-49%, respectively when averaged over the three trials. The breakdown of results is shown in Table 3.4. Results on the biocontrol effect of *Trichoderma* isolates applied as spore suspensions are presented in Tables 3.5-3.7.

Owing to the effect of the treatments a very highly significant (P<0.001) difference of dry weights of cabbage plots was observed (Table 3.5-3.7). Thirty percent of the *Trichoderma* isolates significantly increased dry weight compared to the treatment with the pathogen only. Of the 30% *Trichoderma* isolates, 13% caused a 61-96% increase in dry weight and 17% caused a 102-137% increase in dry weight compared to the effect of the pathogen only. Except in Trial 1, none of the *Trichoderma* isolates applied as a spore suspension exceeded the dry weight of the non-infested control. On the other hand, germination had no effect on plot dry weight because seeds had already germinated when treatments were applied.

Cabbage seedlings inoculated with spore suspensions of *Foc* showed a high percent of disease incidence and disease severity of 84% and 66%, respectively. Due to the effect of disease incidence and severity dry weight was significantly reduced. No disease was observed on seedlings inoculated with water only.

Table 3.4   Percentage of *Trichoderma* isolates (applied by drenching), which caused reduction in percentage of disease incidence and severity

Trichoderma Isolates (%)	Reduction in Disease Incidence (%)	*Trichoderma* Isolates (%)	Reduction in Disease Severity (%)
15	13-20	20	12-25
25	26-36	30	29-39
20	40-48	40	41-48

Table 3.5   Biocontrol effects of *Trichoderma* isolates applied as a spore suspension against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 1

Trichoderma Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	41(40)^{bcd}	25(30)^{bcde}	2.77^{efg}
ET13	36(37)^{bc}	15(23)^{b}	3.18^{g}
ET15	41(40)^{bcd}	17(24)^{bc}	3.01^{fg}
ET19	80(63)^{jkl}	50(45)^{hij}	1.71^{abcde}
ET22	74(60)^{ijkl}	48(44)^{hij}	1.03^{a}
ET23	35(36)^{b}	16(23)^{b}	2.87^{fg}
ET32	56(49)^{efgh}	31(33)^{def}	2.76^{efg}
ET34	78(62)^{jkl}	52(46)^{hij}	0.97^{a}
ET44	49(45)^{cdef}	26(31)^{bcde}	2.51^{efg}
ET45	81(64)^{kl}	58(49)^{jk}	1.17^{abc}
ET46	43(41)^{bcde}	24(29)^{bcd}	2.34^{defg}
ET5	54(47)^{defg}	28(32)^{cdef}	2.31^{cdefg}
SY3A	39(39)^{bcd}	23(28)^{bcd}	2.62^{efg}
ET47	66(55)^{ghij}	34(35)^{defg}	1.96^{abcdef}
ET48	76(60)^{ijkl}	56(48)^{ijk}	1.29^{abcd}
TH1	78(62)^{jkl}	49(44)^{hij}	1.15^{ab}
TH2	70(57)^{hijk}	42(40)^{fghi}	2.07^{abcdefg}
TH3	63(53)^{fghi}	38(38)^{efgh}	2.19^{bcdefg}
TH6	76(61)^{ijkl}	47(43)^{ghij}	1.11^{ab}
TH10	77(61)^{ijkl}	49(45)^{hij}	1.24^{abcd}
Foc only	85(67)^{l}	67(55)^{k}	1.34^{abcd}
Water only	0(6)^{a}	0(6)^{a}	2.83^{efg}
F test	(<0.001)	(<0.001)	<0.001
l.s.d	(8.4)	(8.7)	1.140
s.e.d	(4.2)	(4.3)	0.565
cv%	(10.2)	(14.6)	34.3

-Means with the same letter in the same experiment are not significantly different at $P<0.05$.

- NS = Not Significant

- Values in parenthesis represent transformed means using angular transformation.

Table 3.6   Biocontrol effects of *Trichoderma* isolates applied as a spore suspension against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 2

Trichoderma Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	38(38)^{b}	25(29)^{bc}	2.86^{g}
ET13	38(38)^{b}	23(29)^{bc}	2.56^{fg}
ET15	59(50)^{cd}	36(35)^{cd}	1.71^{abcdef}
ET19	79(63)^{gh}	52(46)^{fghi}	1.72^{abcdef}
ET22	80(64)^{gh}	57(49)^{hij}	0.88^{a}
ET23	37(38)^{b}	22(27)^{b}	2.37^{defg}
ET32	59(50)^{cd}	38(38)^{de}	2.48^{efg}
ET34	78(62)^{gh}	58(49)^{hij}	0.93^{a}
ET44	54(47)^{c}	33(35)^{cd}	2.25^{cdefg}
ET45	80(64)^{gh}	57(49)^{hij}	1.17^{ab}
ET46	58(50)^{cd}	41(40)^{def}	1.53^{abcd}
ET5	67(55)^{def}	41(40)^{def}	1.6^{abcde}
SY3A	52(46)^{c}	34(36)^{d}	1.88^{bcdef}
ET47	72(58)^{efg}	43(41)^{defg}	1.46^{abc}
ET48	79(63)^{gh}	59(50)^{ij}	1.22^{ab}
TH1	77(61)^{fgh}	50(45)^{fghi}	1.14^{ab}
TH2	74(59)^{efg}	54(47)^{ghi}	1.56^{abcd}
TH3	66(54)^{de}	46(43)^{efgh}	1.95^{bcdef}
TH6	79(62)^{gh}	57(49)^{hij}	0.96^{a}
TH10	80(63)^{gh}	55(48)^{hij}	1.13^{ab}
Foc only	83(66)^{h}	66(54)^{j}	1.37^{abc}
Water only	0(6)^{a}	0(6)^{a}	3.83^{h}
F test	(<0.001)	(<0.001)	<0.001
l.s.d	(6.4)	(6.4)	0.892
s.e.d	(3.2)	(3.2)	0.442
cv%	(7.4)	(9.7)	30.9

-Means with the same letter in the same experiment are not significantly different at P<0.05.

- Values in parenthesis represent transformed means using angular transformation.

Table 3.7   Biocontrol effects of *Trichoderma* isolates applied as a spore suspension against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 3

Trichoderma Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	42(40)^{b}	27(31)^{bc}	2.89^{fgh}
ET13	38(38)^{b}	22(28)^{b}	3.09^{gh}
ET15	51(46)^{cd}	30(33)^{bcd}	1.82^{abcd}
ET19	83(66)^{ij}	55(48)^{h}	1.32^{ab}
ET22	79(63)^{hi}	59(50)^{h}	1.98^{bcde}
ET23	37(38)^{b}	22(28)^{b}	2.95^{fgh}
ET32	59(50)^{de}	38(38)^{def}	2.75^{efg}
ET34	84(67)^{ij}	59(50)^{h}	1.9^{bcd}
ET44	52(46)^{cd}	33(35)^{cde}	2.19^{cdef}
ET45	86(68)^{j}	61(52)^{hi}	1.46^{abc}
ET46	54(47)^{d}	36(37)^{def}	1.69^{abcd}
ET5	63(53)^{ef}	39(39)^{ef}	2.44^{defg}
SY3A	45(42)^{bc}	33(35)^{cde}	1.36^{ab}
ET47	74(59)^{gh}	42(41)^{f}	1.86^{bcd}
ET48	83(66)^{ij}	62(52)^{hi}	1.06^{a}
TH1	83(66)^{ij}	53(47)^{gh}	1.78^{abcd}
TH2	75(60)^{h}	54(47)^{gh}	1.45^{abc}
TH3	68(55)^{fg}	45(42)^{fg}	2.24^{cdef}
TH6	81(65)^{ij}	58(50)^{h}	1.64^{abc}
TH10	83(66)^{ij}	57(49)^{h}	1.91^{bcd}
Foc only	91(73)^{k}	70(57)^{i}	1.36^{ab}
Water only	0(6)^{a}	0(6)^{a}	3.56^{h}
F test	(<0.001)	(<0.001)	<0.001
l.s.d	(4.9)	(5.7)	0.796
s.e.d	(2.5)	(2.8)	0.394
cv%	(5.6)	(8.6)	23.4

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

3.3.3 *Bacillus* Isolates Applied as Drenches

Some *Bacillus* isolates inoculated onto young emerging cabbage seedlings as a spore suspension resulted in a significant (P<0.001) decrease in disease incidence and severity, and an increase in dry weight as compared to the treatment with the pathogen only. Results on the biocontrol effect of *Bacillus* isolates applied as spore suspensions are presented in Tables 3.8-3.10.

Disease incidence inflicted by the vascular fungus, *Foc*, was significantly reduced by 10-49%. Of the total number of *Bacillus* isolates tested, 70% and 80% caused a statistically significant decrease in disease incidence and severity, respectively. The breakdown is shown in Table 3.11.

Differences in seedling dry weight between the treatments was significant (P<0.001). A total of 60% of the *Bacillus* isolates significantly increased seedling dry weight beyond the treatment with the pathogen only. Thirty five percent of the isolates increased dry weight by up to 51-99% and 25% of the isolates increased dry weight by up to 130-173%. Although it was not statistically significant (P<0.05) *Bacillus* isolates, BFO11 and JRO2, showed 11 and 16% increase in seedling dry weight over the treatment with water only, respectively. Dry weight was not affected by the number of germinated seedlings since there was no statistically significant difference in the number of germinated seedlings, though the number of germinated seedlings was not actually the same for each treatment. Treatments had no effect on the number of germinated seedlings since they were applied after the seeds had already germinated.

Cabbage seedlings inoculated with spore suspensions of *Foc* showed a high percentage of disease incidence and disease severity of 68-77% and 58-68%, respectively. Due to the effect of disease incidence and severity, plot dry weight was significantly reduced compared to the treatment inoculated with water only. No disease was observed on seedlings inoculated with water only.

Table 3.8 Biocontrol effects of *Bacillus* isolates applied as a spore suspension against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 1

Bacillus Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
ACCI1	79(64)[j]	59(50)[gh]	0.54[a]
ACCI4	55(48)[fghi]	45(42)efgh	2.65[fgh]
ACCI10	55(48)[fghi]	42(40)[defg]	1.96[cdefg]
ACCI11	68(56)[hij]	51(46)[fgh]	2.04[defg]
ACCI14	57(50)[ghij]	45(42)[efgh]	1.28[abcd]
CED16	50(45)[defghi]	30(33)[cdef]	1.56[bcde]
CED18	79(63)[j]	67(55)[h]	0.78[ab]
CED19	52(46)[efghi]	28(32)[cde]	1.38[abcd]
CED20	45(42)[defgh]	33(34)[cdef]	2.9[ghi]
CED22	53(47)[efghi]	29(32)[cde]	2.45[efgh]
CED32	53(48)[fghi]	45(42)[efgh]	1.8[cdef]
CED34	42(40)[defg]	29(32)[cde]	2.64[fgh]
JRO1	30(33)[bcde]	24(29)[cde]	2.92[ghi]
JRO2	40(39)[cdefg]	34(35)[cdef]	3.45[hi]
BFO11	19(25)[bc]	16(23)[bc]	3.33[hi]
EX-R	30(31)[bcd]	24(28)[cd]	2.57[fgh]
B81	11(19)[ab]	5(13)[ab]	3.76[i]
R29	33(34)[bcdef]	21(27)[cd]	2.81[ghi]
Foc only	71(58)[ij]	58(50)[gh]	1.2[abc]
Water only	0.0(6)[a]	0.0(6)a	3.24[hi]
F test	(<0.001)	(<0.001)	<0.001
l.s.d	(14.6)	(13.4)	1.002
s.e.d	(7.2)	(6.6)	0.495
cv%	(21.0)	(23.4)	26.8

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

Table 3.9 Biocontrol effects of *Bacillus* isolates applied as a spore suspension against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 2

Bacillus Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
ACCI1	69(56)^{efg}	56(48)^{gh}	1.20^{ab}
ACCI4	69(56)^{efg}	56(49)^{gh}	1.27^{ab}
ACCI10	68(56)^{efg}	51(45)^{fg}	1.22^{ab}
ACCI11	70(57)^{efg}	55(48)^{gh}	1.55^{abc}
ACCI14	69(56)^{efg}	56(48)^{gh}	1.24^{ab}
CED16	62(52)^{ef}	48(44)^{efg}	1.50^{abc}
CED18	72(59)^{fg}	58(50)^{gh}	1.03^{a}
CED19	79(63)^{g}	65(54)^{hi}	1.51^{abc}
CED20	71(58)^{efg}	58(49)^{gh}	1.05^{ab}
CED22	69(56)^{efg}	55(48)^{gh}	1.56^{abc}
CED32	69(56)^{efg}	56(49)^{gh}	1.59^{abc}
CED34	70(57)^{efg}	57(49)^{gh}	1.78^{bcde}
JRO1	37(37)^{bc}	26(31)^{bc}	2.33^{def}
JRO2	43(41)^{c}	32(34)^{cd}	2.36^{ef}
BFO11	27(31)^{b}	19(26)^{b}	2.72^{f}
EX-R	37(38)^{bc}	28(32)^{bcd}	2.69^{f}
B81	47(43)^{cd}	38(38)^{de}	1.32^{ab}
R29	59(50)^{de}	42(41)^{ef}	1.61^{abcd}
Foc only	77(62)^{g}	69(56)^{i}	1.08^{ab}
Water only	0.0(6)^{a}	0.0(6)^{a}	2.09^{cdef}
F test	(<0.001)	(<0.001)	<0.001
l.s.d	(7.4)	(6.7)	0.730
s.e.d	(3.7)	(3.3)	0.360
cv%	(9.1)	(9.7)	27.0

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

Table 3.10 Biocontrol effects of *Bacillus* isolates applied as a spore suspension against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 3

Bacillus Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
ACCI1	69(56)^k	55(48)^{kl}	0.84^a
ACCI4	47(43)^{de}	37(38)^{efgh}	1.97^{gh}
ACCI10	52(46)^{efg}	37(38)^{efgh}	1.42^{bcde}
ACCI11	55(48)^{fgh}	40(39)^{fgh}	1.57^{cdefg}
ACCI14	59(50)^{ghi}	48(44)^{ijk}	1.20^{abc}
CED16	52(46)^{efg}	36(37)^{efg}	1.48^{cdef}
CED18	67(55)^{jk}	57(49)^l	1.32^{bcd}
CED19	64(53)^{ijk}	45(42)^{hij}	1.43^{bcde}
CED20	52(46)^{efg}	41(40)^{ghi}	1.88^{fgh}
CED22	61(51)^{hij}	41(40)^{ghi}	1.83^{efgh}
CED32	61(51)^{hij}	52(46)^{jkl}	1.65^{defg}
CED34	50(45)^{ef}	37(38)^{efgh}	2.21^{hi}
JRO1	33(35)^c	25(30)^{cd}	2.60^{ijk}
JRO2	41(40)^d	32(35)^{ef}	2.92^{kl}
BFO11	22(28)^b	17(24)^b	3.05^l
EX-R	31(34)^c	25(30)^{cd}	2.64^{jkl}
B81	28(32)^{bc}	21(27)^{bc}	2.60^{ijk}
R29	46(43)^{de}	32(34)^{de}	2.18^{hi}
Foc only	68(56)^k	59(50)^l	1.05^{ab}
Water only	0.0(6)^a	0.0(6)^a	2.47^{ij}
F test	(<0.001)	(<0.001)	<0.001
l.s.d	(4.7)	(4.3)	0.428
s.e.d	(2.3)	(2.1)	0.211
cv%	(6.6)	(7.1)	13.5

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

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Table 3.11 Percentage of *Bacillus* isolates (applied by drenching) which caused a reduction in percentage of disease incidence and severity

Bacillus Isolates (%)	Reduction in Disease Incidence (%)	*Bacillus* Isolates (%)	Reduction in Disease Severity (%)
45	10-26	55	10-29
25	30-49	25	30-44

3.4 Discussion

Disease incidence and severity, and yield increases have been used to evaluate the effect of biocontrol treatments on crops (Kommedahl & Windels, 1981). In this study percentage of disease incidence and severity calculated (as shown in the Section 3.2.10) and plot dry weight (as yield) were taken to evaluate biocontrol treatments on cabbage.

Some *Trichoderma* and *Bacillus* isolates used in this experiment were found to be effective in reducing the incidence and severity of cabbage yellows on seedlings. Significant differences were observed between the performances of the individual bacterial or fungal antagonists when compared among each other (Table 3.1-3.9). Some fungal isolates such as *T. harzianum* Eco-T, and *Trichoderma* Isolates ET23 and ET13, and some *Bacillus* isolates, B-81, BFO11, EX-R, and JRO1, showed relatively higher and statistically significant reduction in disease incidence and severity.

Although disease incidence and severity was significantly reduced owing to the effect of the biocontrol treatments compared to the treatment with the pathogen only, the level of disease observed was still high. This may be explained in association with some of the factors that influence the effectiveness of a biocontrol agent. These are:

(i)   Mechanism(s) of action

(ii)   Optimum rate(s) and concentration of antagonist applied to target areas

(iii)   The inoculum density of a pathogen

(iv)   Carrier or preparation substrate

(v)   Method(s) of application

(vi)   Nature of pathogen to be controlled (Baker, 1981; Hebbar *et al.*, 1992)

(vii)   Timing of application

Inoculum density of a pathogen contributes to its potential to infect a plant and also to the severity of disease. Inoculum density of fungal pathogens required to produce disease varies widely among different pathogens and depends on the type of the infecting unit (Cook & Baker, 1983). Hebbar *et al.* (1992) reported that for seedling bioassays of populations of *F. moniliforme* J. Sheldon greater than 10^4 c.f.u. g^{-1} soil, the suppression of fungal infection by antagonistic bacteria was ineffective, and below 10^3 c.f.u. g^{-1} of soil, infection by *F. moniliforme* was not clearly manifested. Similarly, Sivan & Chet (1986) reported that when a lower concentration of *Trichoderma* preparation was used, no significant difference in disease reduction was obtained from the untreated control. In a similar experiment conducted in soil infested with a smaller amount of the pathogen it was shown that disease incidence was significantly reduced.

The concentrations of the antagonists used for seed treatment and drenching with *Trichoderma* and drenching with *Bacillus* isolates may not have been high enough to fully colonize plant roots and overcome the pathogen's activity. Correct concentrations of inoculum of the antagonists are essential for effective biocontrol of plant pathogens (Adams, 1990).

Wilt pathogens which penetrate into the vascular system of their hosts, usually succeed in escaping from antagonistic microflora. This reduces the stages in the life cycle in which the pathogen is exposed to antagonists (Baker, 1981). Therefore, the main effort in the biocontrol of *F. oxysporum* must take place before infection. Most of the seedlings infected with the vascular pathogen were dead at end of the experiments.

It is generally assumed that root colonization by introduced antagonists is essential for biocontrol of root pathogens and that increasing the population of an introduced antagonist on the root should enhance disease control (Suslow, 1982). The extent of root colonization is one of the factors that influence the effectiveness of biocontrol agents. Variable root colonization by introduced antagonist, including colonization from plant-to-plant, and root-to-root, on a given plant, is probably the main reason for inconsistent control by biological control agents (Weller, 1988).

The antagonistic activity of the biocontrol agents used may depend on the production of metabolites or substances toxic to other microorganisms or plant pathogens (Omoifo & Ikotum, 1987; Weller, 1988). If the mode of action of the biocontrol antagonists used in this study were antibiosis or the production of toxic antimicrobial substances, then it may be possible that the pathogen had infected the plant before the antagonist was able manifest itself and produce those antimicrobial compounds. The medium in which the plants were grown might have an impact on fast population build up of the antagonist, probably due to low nutrient status. This may have reduced bacterial activities such as growth and multiplication, root colonization and possibly production of antibiotics and other vital metabolites necessary for biological control.

A relatively higher percent of disease incidence and severity was reduced when *Trichoderma* spp. were applied as spore suspensions rather than seed treatments. This may be attributed to the fact that cabbage seeds are small in size that the amount of inoculum carried on the surface of the seeds was not enough to colonize the root area and protect the cabbage roots from infection. Moreover, the antagonist's spores are mainly concentrated around the seed whereas the vascular pathogen, *F. oxysporum*, normally grows towards an infection court such as wounds or cracks on root tips of the host plant through the differentiating tissues and then become established in the xylem. When the microconidia of *Foc* were drenched onto the pine bark medium, where the cabbage seedlings were grown, they normally spread all over the medium beyond the reach of the antagonists which mainly concentrate around the seed.

Ideally, a seed-applied biocontrol agent should spread from its site of application to give long-term protection of a root system. The seed-applied biocontrol agent probably could not spread at the rate of root extension, which it seldom can by active growth, whereby the young root regions were exposed to infection by the vascular pathogen. Seed treatment may give effective control of pre-emergence disease but less effective control of post-emergence disease initiated at or near the soil surface, away from the treatment site (Deacon, 1991). In contrast, drenched spore suspensions of the antagonist has the chance to spread all over the medium which allows the antagonist to protect young fast growing roots.

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CHAPTER FOUR

COMPARISONS OF SINGLE VERSUS MIXED BIOCONTROL AGENTS AGAINST *FUSARIUM OXYSPORUM* F. SP. *CONGLUTINANS* ON CABBAGE

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Abstract

Intra-and inter-species interaction were evaluated to test the compatibility between *Bacillus* Isolates EXR, B81, BFO11 and JRO1, and *T. harzianum* Eco-T® and *Trichoderma* Isolates ET23 and ET13. Further observation was also conducted using Environmental Scanning Electron microscopy (ESEM) of the interaction between *Trichoderma* isolates. Greenhouse experiments using mixtures of *Bacillus* isolates or *Trichoderma* isolates were carried out to see if mixtures of the biocontrol agents would give a better result than when applied singly. All *Trichoderma* isolates suffered severe inhibition by all *Bacillus* isolates. Interaction between *Trichoderma* isolates generally resulted in equal colonization of the agar plate by all isolates. However, formation of thick mycelial mats and colour changes during the interaction between Isolates ET23 and ET13 and between Isolates Eco-T® and ET13 could be signs of incompatibility. Further microscopic observation revealed conflicting images showing extensive mycelial damage in some areas and inter-weaving of mycelia without visible signs of damage in other areas. *Bacillus* Isolates B81 and BFO11 inhibited growth of Isolate JRO1 while Isolate EXR was inhibited by all other *Bacillus* isolates. In a greenhouse experiment, the use of mixtures of *Bacillus* or *Trichoderma* isolates was not significantly different from the application of the biocontrol agents singly.

4.1   Introduction

Fusarium wilts are widespread, destructive plant diseases, which cause severe losses on most vegetables and flowers, several field crops, plantation crops, and a few shade trees (Agrios, 1997). Vascular wilt of cabbage (cabbage yellows), caused by *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*) (Wollenweb.) W.C. Snyder & H.N. Hansen, is a difficult disease to control because inoculum persists in the soil in the form of chlamydospores for a long period of time (Smith & Snyder, 1975), making cultural practices ineffective. Though resistant varieties are available, pathogenic forms of *Fusarium* may still arise as recombinants or mutations from within the diverse and malleable population of *F. oxysporum* (Beckman, 1987) as in the case in Southern California. A new race appeared in Southern California, where cabbage is rarely, if ever, attacked by the yellows fungus, and the disease appeared in a field where no previous occurrences of Fusarium yellows of crucifers had been reported (Ramirez-Villupadua *et al.*, 1985). When monogenic resistance, controlled by a single gene pair, is relied upon, a step mutation in the pathogen will bring out a race towards which the resistance gene pair is not effective (Walker, 1957). Increasing public pressure over the real and imagined risks associated with crop protection by the use of chemical pesticides on endangered species, farm-workers safety and environmental pollution (Jacobsen & Backman, 1993); the high cost of discovering new chemicals; and the development of resistance by pathogens (Delp, 1977; Lewis, 1977; Campbell, 1989) all suggest the need for safe and environmentally friendly control mechanisms.

Much interest has recently focused on developing alternative strategies to chemical control (Bèlanger *et al.*, 1995; Dik & Elad, 1999). However, the efficacy of biological control is occasionally inadequate and variability in control efficacy may be high due to the influence of biotic and abiotic conditions on microbial biocontrol agents (Guetsky *et al.*, 2002). Guetsky *et al.* (2002) indicated that since different mechanisms of control employed by biocontrol organisms may be dissimilarly influenced by those conditions, it is possible that if multiple mechanisms are involved, under a certain set of conditions one mechanism may compensate for the other. It is also likely that most cases of naturally occurring biological control results from mixtures of antagonists, rather than from high populations of a single antagonist (Raupach & Kloepper, 1998). For example, mixtures of antagonists are

considered to account for protection with disease-suppressive soils (Lemanceau & Alabouvette, 1991; Schippers, 1992). Consequently, application of a mixture of introduced biocontrol agents would more closely resemble the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of control (Dandurand & Knudsen, 1993; Duffy *et al.*, 1996), and allow the combination of various mechanisms without the need for genetic engineering (Janisiewicz, 1988).

However, there are also reports of combinations of biological control agents that do not result in improved suppression of disease compared with the application of separate antagonists (Hubbard *et al.*, 1983; Sneh *et al.*, 1984; Miller & May, 1991; Dandurand & Knudsen, 1993). There has been little investigation of the extent to which biocontrol bacteria that inhibit plant pathogens may also inhibit biocontrol fungi or mixtures of fungi or bacteria may inhibit or out-compete each other. Fravel (1988) discussed the possibility of deleterious effects of antibiotics and antibiotic-like compounds, produced by biocontrol agents, on beneficial microorganisms. In the spermosphere or rhizosphere, where microbes are in closer proximity in a relatively nutrient-rich environment, interactions may be more likely. Production of the antibiotic phenazine-1-carboxylic acid by the biocontrol agent *Pseudomonas fluorescens* Migula Strain 2-79RN$_{10}$ had this effect on *Trichoderma harzianum* Rifai Isolate ThID1 in sterile soil, although the effect was diminished in nonsterile field soil (Bin *et al.*, 1991). Hubbard *et al.* (1983) stated that seed-colonizing pseudomonads were largely responsible for the failure of *T. hamatum* (Bonord) Bainier as a seed protectant in New York soil. Kloepper (1983) also reported that mixtures of two or more rhizobacteria resulted in significant reductions in tuber· infestation by *Erwinia carotovora* (Jones) Bergey *et al.*; however, control by mixed strains was not greater than that obtained by any single strain. It is not known whether the biocontrol agents were truly compatible, or if biocontrol was a result of one dominant strain.

The overall goal of this investigation was to determine the compatibility of *Bacillus* and/or *Trichoderma* isolates, which showed a certain level of control in previous tests against the vascular pathogen, *Foc*, and if multiple combinations of *Bacillus* and/or *Trichoderma* isolates could improve efficacy and enhance control consistency.

4.2 Materials and Methods

4.2.1 Source of Plant Material

Cabbage seeds, *Brassica oleracea* var. *capitata* L. cv. Glory of Enkhuizen, Reference no. 570101 ABAE, were obtained from Starke Ayres[®1] (Pty) Ltd, which is a member of the Pannar[®] Group.

4.2.2 Microorganisms

Biocontrol Agents

Three *Trichoderma* isolates, *T. harzianum* Eco-T[®2] and *Trichoderma* Isolates ET23 and ET13 and four *Bacillus* isolates, BFO11, JRO1[3], B81[4] and EX-R[5], which showed good performance in previous experiments against *Foc* on cabbage (Chapters Two and Three) were used.

Pathogen

Fusarium oxysporum f. sp. *conglutinans* was provided by M.D. Laing[6]. The pathogen was preserved on silica gel at 4°C (Perkins, 1962; Windels *et al.*, 1988) and also maintained on Carnation Leaf Agar (CLA) (Fisher *et al.*, 1982) slants at 4°C.

4.2.3 Compatibility Test of Biocontrol Agents *In Vitro*

Three different compatibility tests were conducted. These were:

 i) Compatibility between *Trichoderma* isolates

 ii) Compatibility between *Bacillus* isolates, and

 iii) Compatibility between *Trichoderma* and *Bacillus* isolates

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i) Compatibility between *Trichoderma* Isolates

Three *Trichoderma* isolates, *T. harzianum* Eco-T®, ET13 and ET23, were tested for compatibility. Tests were performed on Potato Dextrose Agar (PDA) (MERCK) plates (90mm diam.). Mycelial disks (approximately 5mm×5mm) were cut from expanding colonies of the *Trichoderma* isolates grown on PDA, and a single *Trichoderma* disk was placed on the opposite side of a plate 15mm from the Petri dish edge.

Degree of compatibility or antagonism between the *Trichoderma* isolates was measured 5d and 10d later on a scale of 1-5, similar to the rating system of Bell *et al.* (1982), whereby 1 = *Trichoderma* Isolate A completely overgrew *Trichoderma* Isolate B and covered the entire medium surface, 2 = *Trichoderma* Isolate A overgrew at least two thirds of the medium surface, 3 = each *Trichoderma* isolate colonized 50% of the medium surface and neither organism appeared to dominate the other, 4 = *Trichoderma* Isolate B colonized at least two thirds of the medium surface and appeared to withstand encroachment by *Trichoderma* Isolate A, and 5 = *Trichoderma* Isolate B completely overgrew the entire medium surface.

ii) Compatibility between *Bacillus* Isolates

The test bacteria were first grown in 250ml Erlenmeyer flasks containing 100ml of Tryptone Soy Broth (TSB) (MERCK) inoculated with 1ml of each *Bacillus* isolate with a concentration of 10^6 c.f.u ml^{-1} and incubated at 30°C in a water-bath shaker (Gesellschaft für Labortechnik, mbH, D-30938, Burgwedel, Germany) at 120 oscillations min^{-1} for 24h. *Bacillus* isolates were tested for compatibility between each other on Tryptone Soya Agar (TSA) (MERCK) plates by making many streaks of one *Bacillus* isolate 20mm starting from the edge of the plate; and a single streak of each of the other three *Bacillus* isolates was made at right angle to the test bacteria with the tip of each streak nearly touching the edge of the test bacteria (Mkhize *et al.*, 1997). After 4d at 30°C the compatibility between the four *Bacillus* isolates was compared.

iii) Compatibility between *Trichoderma* and *Bacillus* Isolates

To determine the effects of *Bacillus* isolates on the growth of *Trichoderma* isolates, *Bacillus* isolates were cultured on PDA by making a single streak across the center of the Petri dish. After 2d at 25°C, PDA plugs 5mm×5mm, taken from the margins of growing *Trichoderma* isolates colonies were placed on either side of the *Bacillus* isolates streak at a

distance of 15mm from the edge of the plate. Two replicates were used for each treatment and the experiment was repeated twice. Antagonistic potentials, quantified as percent inhibition of growth, were evaluated after additional 4d at 25°C by measurement of the radii of *Trichoderma* isolatescolonies cultured with the *Bacillus* isolate relative to the control plates with *Trichoderma* alone. Percent inhibition was calculated as follows:

$$\% \text{ inhibition} = (1- (\text{Fungal growth} / \text{Control growth})) \times 100$$

4.2.4 Environmental Scanning Electron Microscopic (ESEM) Observation on the Compatibility between *Trichoderma* Isolates

Mycelial plugs (3mm×3mm) were cut from the interaction zone of the *Trichoderma* isolates 5d post-inoculation and fixed overnight in 3% glutaraldehyde in cacodylate buffer (0.1M; pH7.0). Samples were then dehydrated in a graded series of alcohol ranging from [10, 20, 50, 70, 80%] and twice in 100% (v/v). Specimens were dried in a critical point drier, Hitachi HCP-2 critical point drier-CPD, with carbon dioxide as a transfusion fluid in and subsequently mounted on copper stubs using double-sided carbon tape. All stubs were then coated with gold-palladium in a Polaron E500 Sputter Coater and kept in a desiccator until viewed under a Philips XL30 Environmental Scanning Electron Microscopy (ESEM) operating at 15kV.

4.2.5 Preparation of the Pathogen's Inoculum

Erlenmeyer flasks (250ml) each containing 50ml of liquid yeast extract-glucose (YM) containing yeast extract (MERCK) 5g; peptone (MERCK) 5g; glucose, 10g, and 1000ml of distilled water were inoculated with mycelial disks from 72h old cultures of *Foc*. Flasks were incubated at 27°C in a water-bath shaker (Gesellschaft für Labortechnik (GFC), mbH, D-30938, Burgwedel, Germany) at 120 oscillations min^{-1} for 4d. Conidia were separated by filtration through eight layers of cheese-cloth. The conidial suspensions were then washed three times by centrifugation in a Beckman J2-HS Centrifuge at 9000×g for 30min at 4°C.

Suspensions containing microconidia of *Foc* were counted using a Neubauer Improved Double Haemocytometer and adjusted to 2×10^7 microconidia ml^{-1}. Three millilitres of this suspension was added to each seedling in a Speedling® 24 tray. The pathogen was inoculated 4d after the cabbage seeds were sown to encourage germination of conidia due to the release of plant exudates from the roots of the cabbage seedlings.

4.2.6   Preparation of Inocula of the Biocontrol Agents

Trichoderma *Isolates*

A conidial suspension of the *Trichoderma* isolates was prepared from conidia collected from cultures grown on V8 agar medium, containing 200ml V8 tomato juice; 3g CaCO$_3$ (uniLAB®); 15g agar (MERCK) and 800ml of distilled water and autoclaved for 15min at 121°C. V8 agar plates were inoculated with agar blocks from half strength PDA plates kept at 4°C and incubated for 7d at 25°C. Then 5ml sterile distilled water was pipetted into plates and conidia were collected by scraping the plate with cotton-tipped sterile Pasteur pipette. The conidial suspension of the *Trichoderma* isolates was counted using a Neubauer Improved Double Haemocytometer and adjusted to 10^7 conidia ml^{-1}.

Bacillus *Isolates*

Bacillus isolates were grown in a 250ml Erlenmeyer flasks each containing a 100ml Nutrient Broth (NB) (MERCK) for 4d at 30°C in a water-bath shaker (Gesellschaft für Labortechnik, mbH, D-30938, Burgwedel, Germany) at 150 oscillations min^{-1}. Flasks were inoculated with *Bacillus* isolates previously grown in TSA for 48h. After 4d *Bacillus* isolates were harvested by centrifugation in a Beckman J2-HS centrifuge at 9000×g for 15min. The broth was decanted and bacterial pellets were resuspended in sterile distilled water. Bacterial cells were then counted using the plate dilution technique in a TSA medium and adjusted to a concentration of 10^7 c.f.u. ml^{-1} of water.

4.2.7   Application Method of Biocontrol Inoculum

Seedling drenching was the sole method of application of the biocontrol inoculum. This was chosen based on the results of previous experiments (Chapter Three). Furthermore,

drenching of young cabbage seedlings in the nursery before being transplanted to the field is a commonly used method by farmers on the field (B. Neumann, 2004, pers. comm.[7]).

4.2.8 Biocontrol Experiments

4.2.8.1 Greenhouse Experiments using Mixtures of *Trichoderma* or *Bacillus* Isolates

Each cabbage seedling in the Speedling® 24 tray was inoculated with 4ml of 10^7 conidia ml^{-1} of single or a mixture of *Trichoderma* isolates or 4ml of 10^7 c.f.u. ml^{-1} of suspensions of *Bacillus* isolates as biocontrol inoculum. Similarly, 3ml of 2×10^7 conidia ml^{-1} of *Foc* was applied to each cabbage seedling in each treatment except in the control where neither pathogen nor biocontrol agent was applied.

In this experiment, cabbage seedlings were inoculated both with the biocontrol agents and the pathogen at the same time 4d after sowing to encourage germination and growth of the fungal and bacterial biocontrol agents and the pathogen due to the release of root exudates from the already established seedling. The experiment was repeated twice with treatments randomised in a complete block design (RCBD) and replicated three times.

4.2.9 Sterilization of Speedling® 24 Trays

Since the Speedling® 24 trays were previously being used by other researchers, sterilization of the trays was necessary to eliminate the effect of other microbes. They were sterilized by dipping the trays in Plaz-Dip® and immediately air-dried. Plaz-Dip® is a 12% copper hydroxide suspension in PVA paint.

4.2.10 Plant Growing Medium

Cabbage seedlings were grown in a composted pine bark medium. It was used as a sole plant-growing medium for the entire study. Composted pine bark is nutritionally poor as well as having poor water holding capacity due to it coarse textured nature; therefore, irrigation, supplemented with NPK soluble fertilizer [3:1:3(38)], was supplied three times a day for 5min.

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4.2.11 Controls

There were two control treatments:

1. neither antagonist nor pathogen
2. pathogen only

4.2.12 Disease Rating

Disease incidence and disease severity were recorded based on visual assessment of symptoms arising from infection of *Foc* according to a five-point key (Carver *et al.*, 1996):

0 = healthy plants

1 = initial signs of wilting (yellowing)

2 = up to 25% of the leaves with symptoms

3 = up to 50% of the leaves with symptoms

4 = up to 75% of the leaves with symptoms

5 = plants dead

Disease incidence and severity were calculated as follows (Zhang *et al.*, 1996):

Disease incidence = $100\% \times (n1 + n2 + n3 + n4 + n5)$

Disease severity = $100 \times (0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4 + 5n_5)/5n$

4.2.13 Statistical Analysis

Greenhouse experiments were repeated twice with treatments arranged in a RCBD while all *in vitro* experiments were arranged in a Completely Randomized Design (CRD). All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 6 Statistical Analysis Software to determine differences between treatment means. Percentage of disease incidence and disease severity were calculated on a per plot basis and transformed using angular transformation prior to analysis. All least significant differences were determined at $P<0.05$.

Data on *in vitro* compatibility tests between *Trichoderma* isolates was analyzed using the direct method of Bell *et al.* (1982). They considered an isolate of *Trichoderma* to be antagonistic if the mean score was ≤2, but not if the mean score was ≥3.

4.3   Results

4.3.1   Compatibility between *Trichoderma* and *Bacillus* Isolates

In this experiment all three *Trichoderma* isolates were highly significantly inhibited by all *Bacillus* isolates ($P<0.001$). None of the *Trichoderma* isolates was able to withstand the antibiotic or other biochemical activity of the *Bacillus* isolates, resulting in a significant reduction in growth relative to the control, where *Trichoderma* was cultured alone (Tables 4.1-4.3; Fig.4.4). *Trichoderma harzianum* Eco-T® was relatively less sensitive to the biochemical activity of the *Bacillus* isolates, particularly to *Bacillus* Isolate B81, than *Trichoderma* Isolates ET23 and ET13.

Table 4.1   Compatibility tests between *Trichoderma harzianum* Eco-T® and four *Bacillus* isolates *in vitro*

Bacillus isolates	Growth of Eco-T® (mm)		
	Trial 1	Trial 2	Trial 3
None	60.00^c	61.00^d	61.00^c
×B81	38.67^b	40.67^c	42.00^b
×BFO11	23.00^a	23.67^a	24.00^a
×EXR	24.33^a	25.00^{ab}	24.67^a
×JRO1	23.67^a	25.67^b	24.67^a
F test	<0.001	<0.001	(<0.001)
l.s.d.	3.254	2.933	3.787
s.e.d.	1.461	1.317	1.700
cv%	5.3	4.6	5.9

-Means with the same letter in the same column and within the same experiment are not significantly different at $P<0.05$.
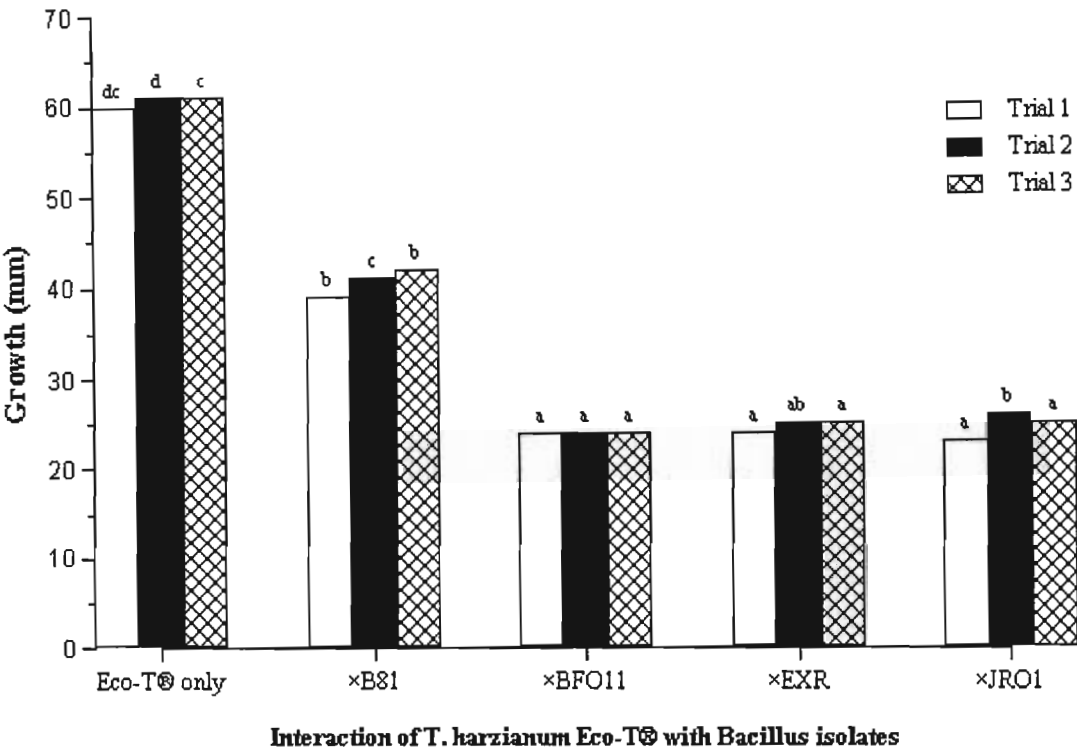
NB = Means with the same letter in the same trial are not significantly different at $P<0.05$.

Fig. 4.1   *In vitro* dual culture interaction test on the compatibility between *Trichoderma harzianum* Eco-T® and four *Bacillus* isolates

Table 4.2 Compatibility tests between *Trichoderma* Isolate ET23 and four *Bacillus* isolates *in vitro*

Bacillus isolates	Growth of ET23 (mm)		
	Trial 1	Trial 2	Trial 3
None	68.67^c	69.00^d	68.33^c
×B81	30.33^b	29.67^c	28.67^b
×BFO11	22.67^a	22.00^a	23.00^a
×EXR	25.33^{ab}	25.67^b	24.67^a
×JRO1	22.67^a	23.33^{ab}	24.33^a
F test	<0.001	<0.001	(<0.001)
l.s.d.	5.438	3.355	3.387
s.e.d.	2.440	1.506	1.520
cv%	8.8	5.4	5.6

-Means with the same letter in the same column and within the same experiment are not significantly different at P<0.05.
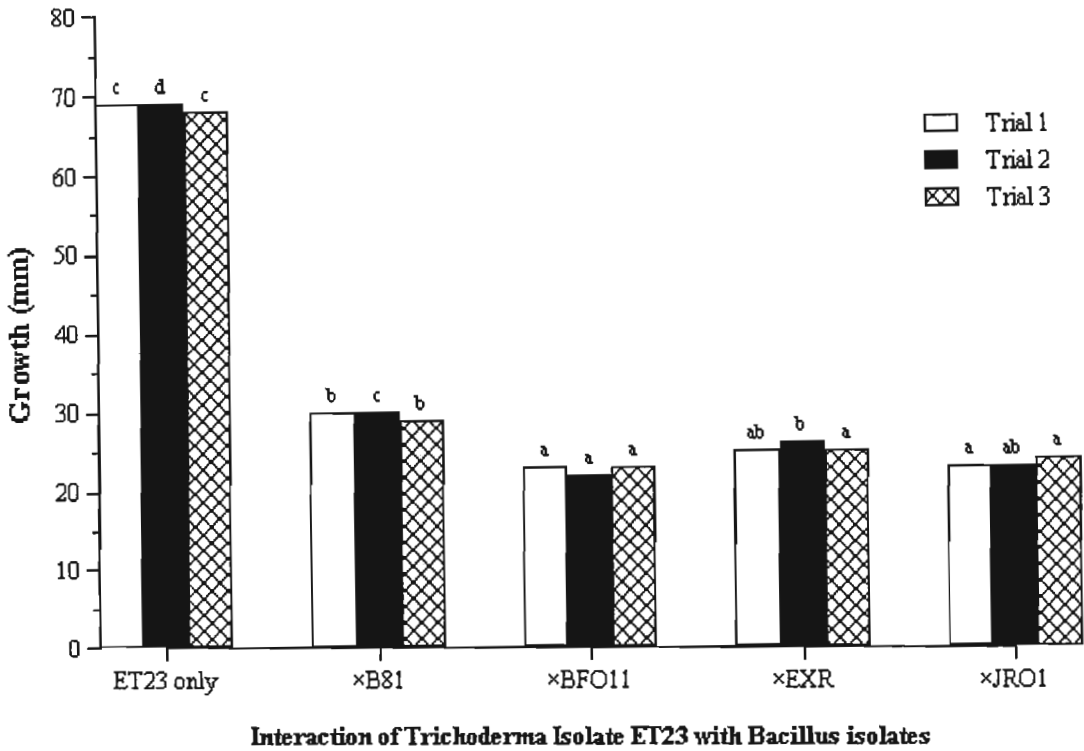
NB = Means with the same letter in the same trial are not significantly different at P<0.05.

Fig. 4.2 *In vitro* dual culture interaction test on the compatibility between *Trichoderma* Isolate ET23 and four *Bacillus* isolates

Table 4.3   Compatibility tests between *Trichoderma* Isolate ET13 and four *Bacillus* isolates *in vitro*

Bacillus isolates	Growth of ET13 (mm)		
	Trial 1	Trial 2	Trial 3
None	77.67^c	77.33^c	77.00^c
×B81	22.33^a	23.00^a	23.33^a
×BFO11	24.67^{ab}	25.00^a	25.33^a
×EXR	28.00^b	28.67^b	29.67^b
×JRO1	23.67^a	24.33^a	24.67^a
F test	<0.001	<0.001	(<0.001)
l.s.d.	3.387	2.301	3.387
s.e.d.	1.520	1.033	1.520
cv%	5.3	3.5	5.2

-Means with the same letter in the same column and within the same experiment are not significantly different at $P<0.05$.
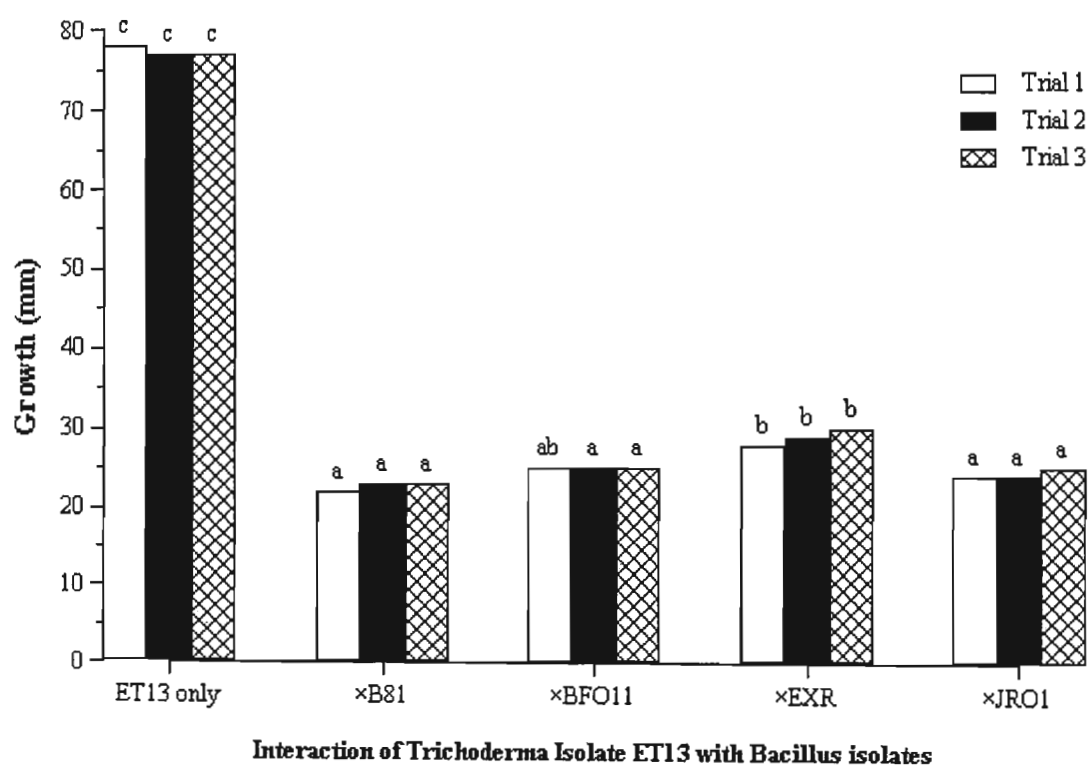
NB = Means with the same letter in the same trial are not significantly different at $P<0.05$

Fig. 4.3   *In vitro* dual culture interaction test on the compatibility between *Trichoderma* Isolate ET13 and four *Bacillus* isolates
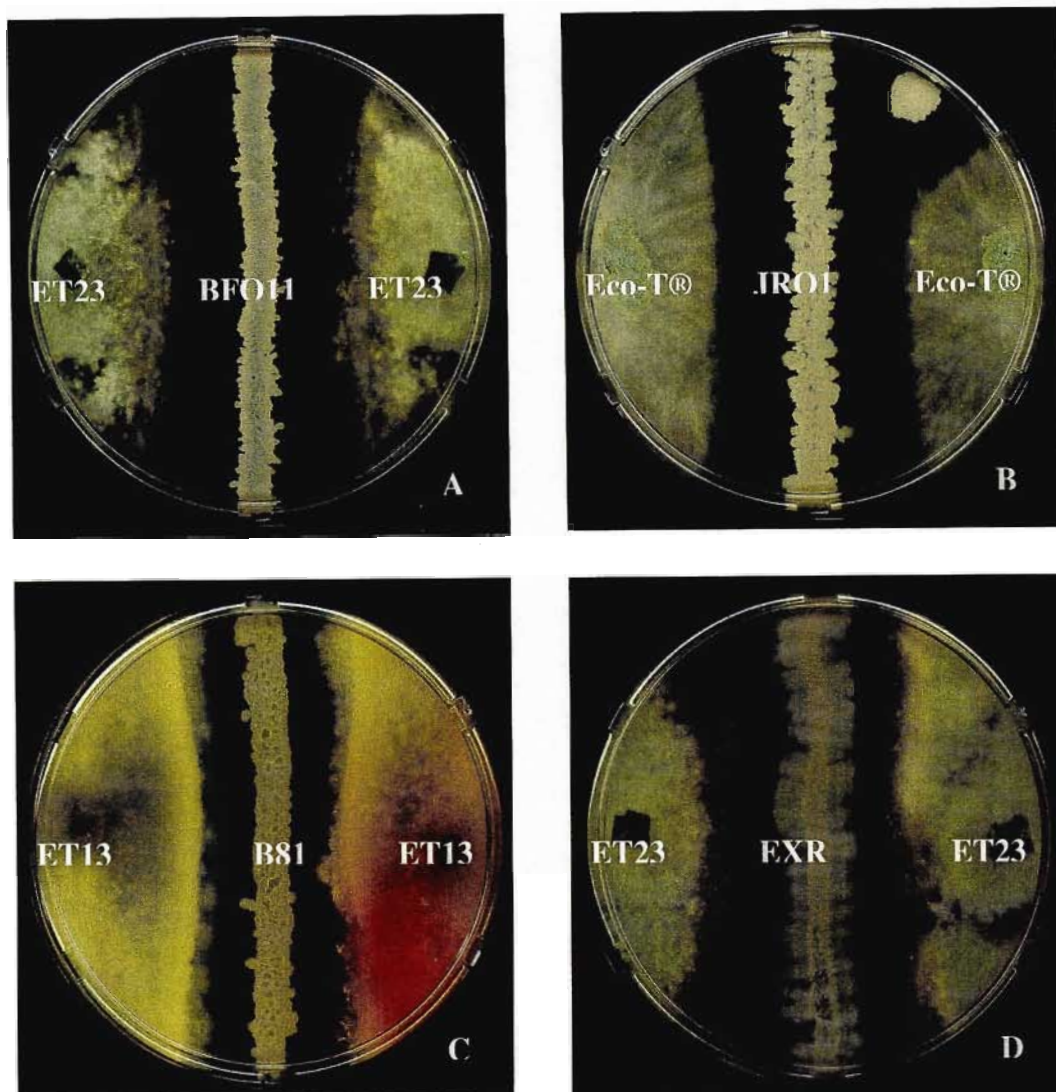
Fig. 4.4   Plates showing the compatibility between *Trichoderma* and *Bacillus* isolates, incubated at 25°C for 4-5d. Plate A, D: showing *Trichoderma* Isolate ET23 inhibited by *Bacillus* Isolates BFO11 and EXR, respectively. Plate B: *T. harzianum* Eco-T® being inhibited by *Bacillus* Isolate JRO1. Plate C: *Trichoderma* Isolate ET13 inhibited by *Bacillus* Isolate B81 and changes in colour due to antagonism of the two organisms.

4.3.2 Compatibility between *Trichoderma* Isolates

The three *Trichoderma* isolates showed some degree of competition and signs of antagonism among them. In the interaction between *Trichoderma* Isolate ET23 and *T. harzianum* Eco-T®, the plate was almost equally colonized by each isolate in most cases (Fig. 4.5A). A yellowish colour change was common in the interaction between *Trichoderma* Isolate ET13 and the other two *Trichoderma* isolates. *Trichoderma* Isolate ET23 in all cases formed a thick mycelial mat at the interaction point with *Trichoderma* Isolate ET13 (Fig. 4.5B). Although both colonized the agar almost equally, *Trichoderma* Isolate ET23 and *T. harzianum* Eco-T® usually grew on top of *Trichoderma* Isolate ET13, especially after 10d (Fig. 4.5D).

Microscopic observation from samples taken from the interaction zone of the three *Trichoderma* isolates using ESEM revealed conflicting images. Pictures from the interaction between *Trichoderma* Isolates ET23 and ET13 shows that there was mycelial damage (Fig. 4.6A) in some areas, while in other areas there was excessive inter-weaving of mycelia without any visible damage. Similar images were also found in the case of *T. harzianum* Eco-T® and *Trichoderma* Isolate ET13 (Fig. 4.6C). No penetration of mycela or coiling was observed. No visible mycelial damage was observed as a result of the interaction between *T. harzianum* Eco-T® and *Trichoderma* Isolate ET23. Thickening of mycelia at the interaction point was observed (Fig. 4.5D). Pictures on microscopic observation are presented in Fig. 4.6.

Table 4.4   Compatibility between *Trichoderma* isolates *in vitro,* Trial 1

Interactions	Mean Rating	
	5d	**10d**
Eco-T*ET23	2.9	2.9
Eco-T*ET13	3.0	2.7
ET23*ET13	2.9	2.7

Class of antagonism (based on Bell *et al.,* 1982): 1=*Trichoderma* Isolate A grew and overlap the colony of *Trichoderma* Isolate B and the whole surface of the media; 2=*Trichoderma* Isolate A grew and it covered two third of the surface of the media; 3=*Trichoderma* Isolate A and *Trichoderma* Isolate B colonized each one half of the surface of the media and did not have dominance; 4= *Trichoderma* Isolate B grew and it covered two third of the surface of the media; 5= *Trichoderma* Isolate B grew and overlap the colony of *Trichoderma* Isolate A and the whole surface of the medium.

Table 4.5   Compatibility between *Trichoderma* isolates *in vitro,* Trial 2

Interactions	Mean Rating	
	5d	**10d**
Eco-T*ET23	2.6	2.9
Eco-T*ET13	3.1	2.7
ET23*ET13	2.9	2.7

Class of antagonism (based on Bell *et al.,* 1982): 1=*Trichoderma* Isolate A grew and overlap the colony of *Trichoderma* Isolate B and the whole surface of the media; 2=*Trichoderma* Isolate A grew and it covered two third of the surface of the media; 3=*Trichoderma* Isolate A and *Trichoderma* Isolate B colonized each one half of the surface of the media and did not have dominance; 4= *Trichoderma* Isolate B grew and it covered two third of the surface of the media; 5= *Trichoderma* Isolate B grew and overlap the colony of *Trichoderma* Isolate A and the whole surface of the medium.
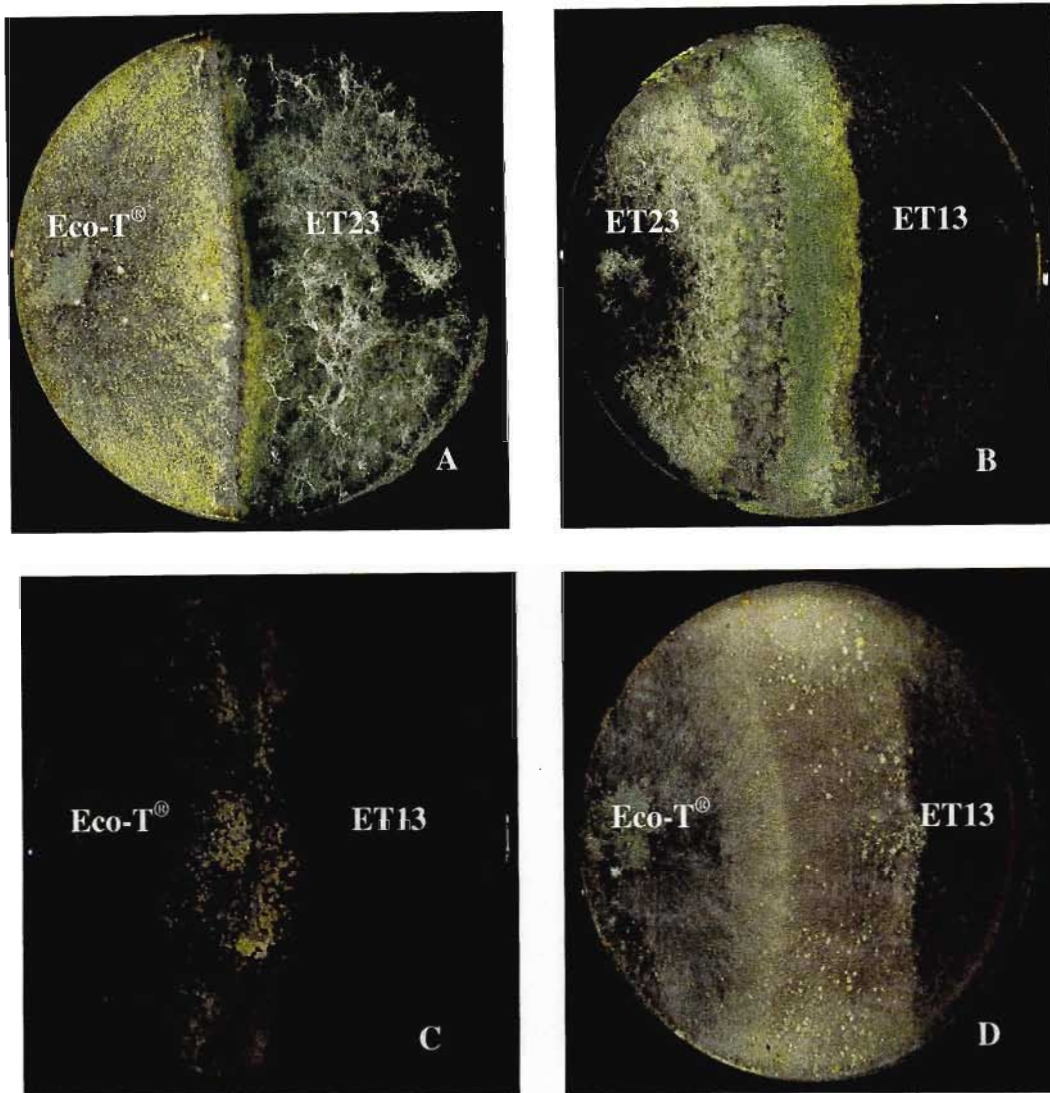
Fig. 4.5 Plates showing the compatibility between three *Trichoderma* isolates (Eco-T®, ET23 and ET13), incubated at 25°C for 5d. Plate A: showing both *Trichoderma* isolates growing at equal rate without overgrowing one another. Plate B: showing an equal colonization of the plate by both isolates with the formation of thick mycelial mat at the point of contact. Plate C: showing *Trichoderma* isolate ET13 colonizing a slightly bigger area than *T. harzianum* Eco-T®, and production of a high number of spores at the point of contact. Plate D: showing both isolates growing equally towards one another with *T. harzianum* Eco-T® growing on top of Isolate ET13 after 10d.
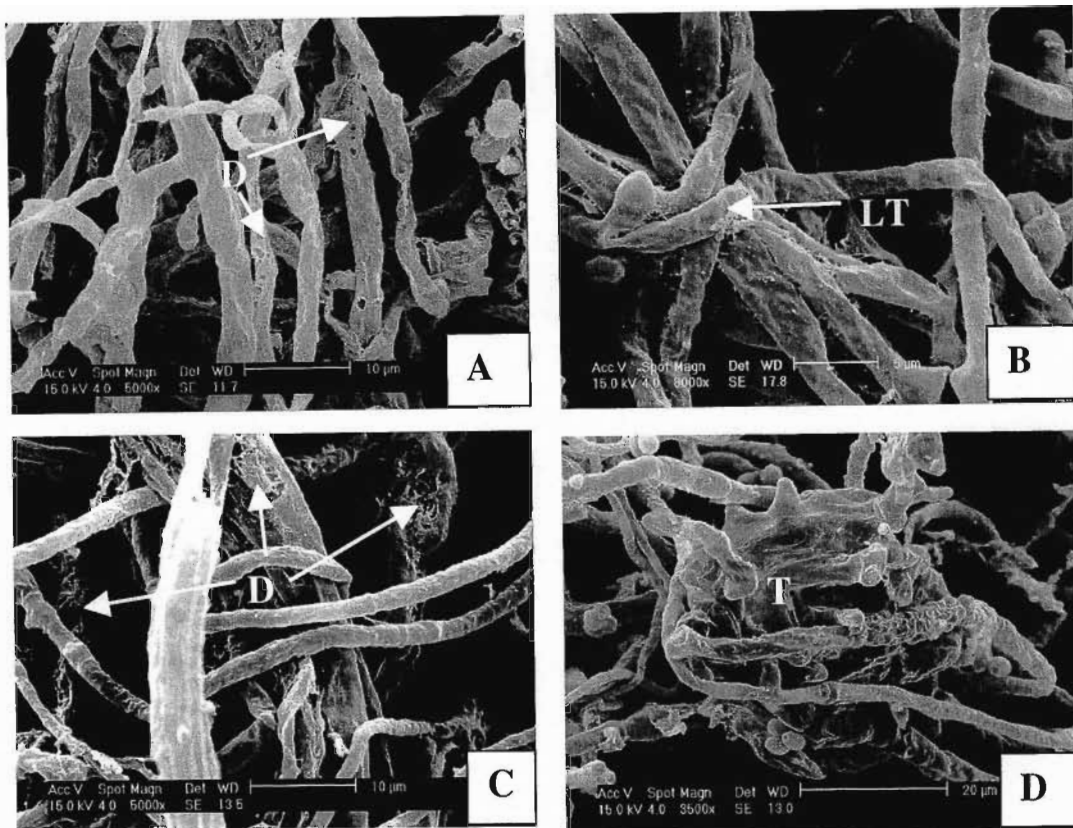
Fig. 4.6 Scanning electron micrograph showing the compatibility between three *Trichoderma* isolates, ET23, ET13 and *T. harzianum* Eco-T®. Degradation (lysis) of mycelium due to biochemical activity during interaction between ET13, and ET23 and Eco-T® (A; C, respectively) and Loss of turgor (LT) during interaction between ET13 and Eco-T® (B). Thickening (T) of mycelium during interaction between ET23 and Eco-T® (D).

4.3.3 Compatibility between *Bacillus* Isolates

In this experiment some of the four *Bacillus* isolates appeared to be inhibitive to others. The growth of *Bacillus* Isolate EXR was inhibited by all three isolates (Fig. 4.7A). The area close to the streaks of the other isolates was cleared while EXR were growing in between the streaks forming in and out in a zigzag manner. The growth of *Bacillus* Isolate JRO1 was also slightly inhibited by *Bacillus* Isolates B81 and BFO11 (Fig. 4.7D). However, the inhibition zone was smaller than what was observed on EXR. *Bacillus* Isolates B81 and BFO11 were not inhibited by any of the other isolates (Fig. 4.7B; C). In

this experiment no numbers were involved since the inhibition zone observed was too small to measure.
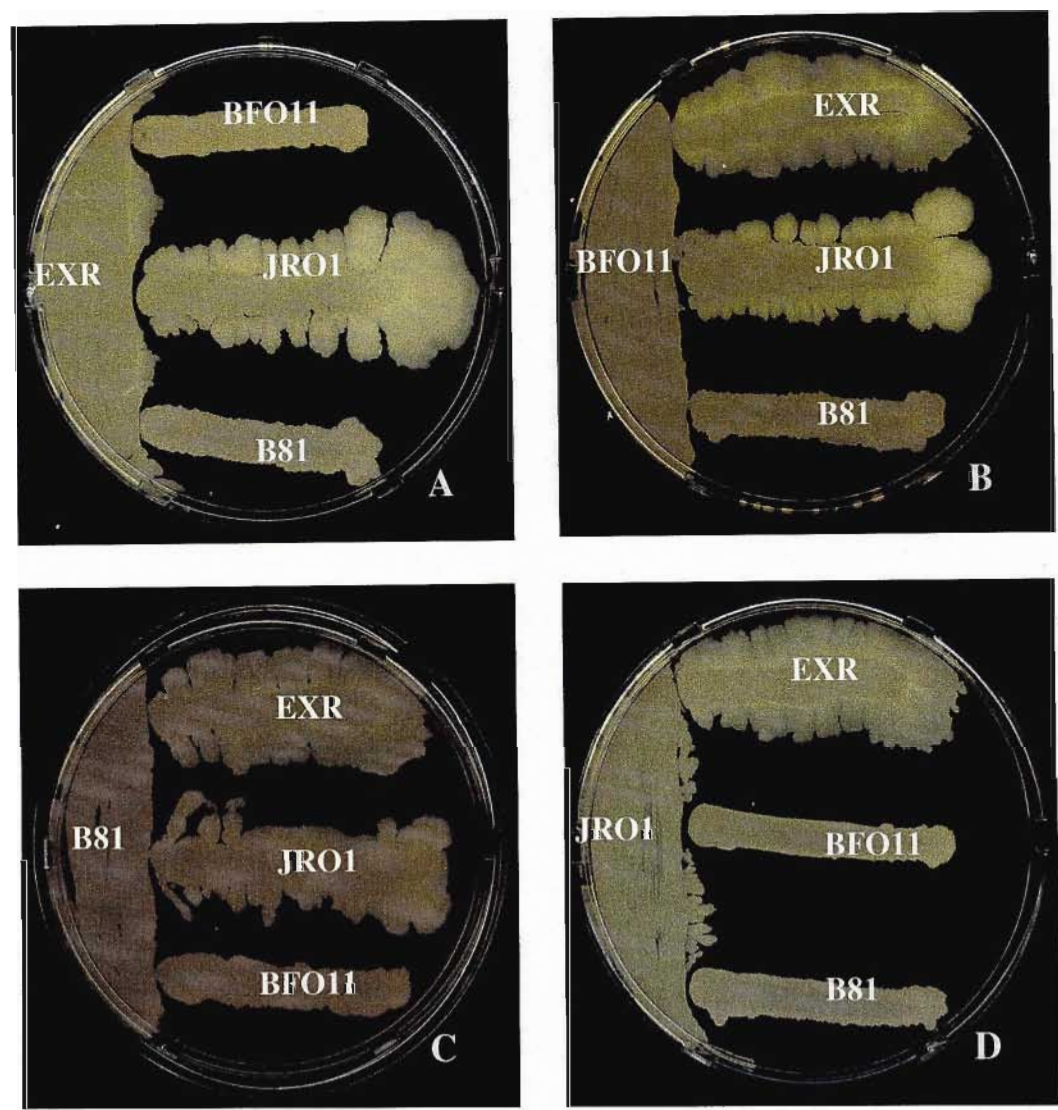
Fig. 4.7 Plates showing the compatibility between four *Bacillus* isolates (EXR, BFO11, B81 and JRO1), incubated at 30°C for 5d. Plate A: all three *Bacillus* isolates inhibited growth of EXR. Plate B: No inhibition of growth of BFO11 by any of the other isolates. Plate C: B81 slightly inhibited growth of JRO1 and EXR. Plate D: growth of JRO1 was inhibited by B81 and slightly inhibited by BFO11.

4.3.4 Greenhouse Experiments using Mixtures of *Trichoderma* or *Bacillus* Isolates

The level of disease control obtained due to biocontrol treatments varied among the three trials of the experiment; and to some extent the trend for the treatment effects on disease was not consistent (Tables 4.6a-4.8b). Pooled mean of the three trials showed that there

was no significantly higher reduction in disease due to mixtures of the biocontrol agents (Tables 4.9a,b; Fig. 4.8a,b). However, the treatment effects for disease control were significantly different than the control treated with the pathogen alone.

Application of mixtures of two or all of the three *Trichoderma* isolates resulted either in non-significant differences or significantly worse disease reduction levels as compared to individual applications. In all three trials, mixtures of *T. harzianum* Eco-T® with the other two *Trichoderma* isolates resulted in non-significant differences in suppression of disease than when applied singly (Tables 4.6b-4.9b). *Trichoderma* Isolate ET23 caused significant reduction of disease in Trial 2, but a non-significant difference in disease suppression in Trial 1 and 3 when applied in combination with one or both of the other two *Trichoderma* isolates than when it was applied singly (Tables 4.6b; 4.7b). On the other hand, a significantly higher level of disease symptoms was observed in Trial 1 when *Trichoderma* Isolate ET13 was applied in a mixture with one or both of the other two *Trichoderma* isolates than when it was applied individually (Table 4.6b).

Application of mixtures of *Bacillus* isolates also showed a similar trend in that there was a lack of consistency among the three trials. Combinations of *Bacillus* Isolate B81 with one, two or three of the other *Bacillus* isolates caused no significant decrease in the level of disease except in Trial 1 when it was mixed with *Bacillus* Isolate EXR (Table 4.6a). However, a significantly higher level of disease was observed in Trial 2 when *Bacillus* Isolate B81 was applied in a mixture with *Bacillus* Isolate JRO1 (Table 4.7a). The magnitude of disease protection was non-significant when *Bacillus* Isolate BFO11 was applied in a mixture with one, two or three of the other three *Bacillus* isolates in Trials 1 and 2 (Tables 4.6a; 4.7a). However, there was a significantly higher level of disease control when applied in combination with one, two or three of the other *Bacillus* isolates in Trial 1 (Table 4.6a). Non-significant and significantly lower disease reductions were induced by both *Bacillus* Isolate EXR and JRO1 applied in combination with one, two or three of the other *Bacillus* isolates in Trials 2 and 3, respectively (Table 4.7a; 4.8a). Significantly higher and significantly lower levels of disease were also observed in Trial 1 when *Bacillus* Isolate EXR was applied in a mixture with the other three *Bacillus* isolates and *Bacillus* Isolate B81, respectively (Table 4.6a). Significant reductions in the extent of disease were induced when *Bacillus* Isolate JRO1 was applied in combination with *Bacillus* Isolates BFO11, EXR, and EXR and BFO11.

Means of disease reduction were 36.3% for mixtures of *Trichoderma* isolates and 40.6% for single isolates; 33.8% for mixtures of *Bacillus* isolates and 39.2% for single strains. Overall mean disease reductions for both *Trichoderma* and *Bacillus* were 36.5% for mixtures and 38.5% for single strains.

Table 4.6a   Biocontrol effects of single or mixtures of *Bacillus* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 1

Single and Mixtures of *Bacillus* Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
B81	31(34)^{ghi}	24(29)^{g}	3.08^{jk}
BFO11	21(27)^{bc}	12(20)^{c}	2.56^{fg}
EXR	30(33)^{fgh}	19(26)^{ef}	2.78^{ghij}
JRO1	36(37)^{jk}	23(28)^{fg}	2.64^{fg}
B81×JRO1	38(38)^{k}	19(26)^{ef}	2.99^{hijk}
BFO11×B81	30(33)^{fgh}	18(25)^{de}	3.00^{hijk}
BFO11×JRO1	26(30)^{de}	19(26)^{ef}	2.22^{cd}
EXR×B81	25(30)^{de}	12(23)^{d}	2.54^{efg}
EXR×BFO11	29(32)^{efg}	20(26)^{ef}	2.18^{c}
EXR×JRO1	30(33)^{fgh}	20(27)^{efg}	2.09^{b}
BFO11×JRO1×B81	32(35)^{hij}	20(27)^{efg}	2.24^{cde}
EXR×BFO11×B81	30(33)^{fgh}	21(27)^{efg}	2.35^{cdef}
EXR×BFO11×JRO1	29(33)^{fgh}	22(28)^{fg}	1.85^{ab}
EXR×JRO1×B81	32(35)^{hij}	23(29)^{g}	3.03^{ijk}
EXR×BFO11×JRO1×B81	35(36)^{ijk}	23(29)^{g}	2.52^{defg}
Foc only	60(51)^{l}	43(41)^{h}	1.64^{a}
Water only	0(6)^{a}	0(6)^{a}	3.29^{k}
F test	(<0.001)	(<0.001)	(<0.001)
l.s.d.	(2.9)	(2.5)	0.308
s.e.d.	(1.5)	(1.2)	0.153
cv%	(5.7)	(6.0)	7.2

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

Table 4.6b Biocontrol effects of single or mixtures of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 1

Single and Mixtures of *Trichoderma* Isolates	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	27(31)^{ef}	18(25)^{de}	2.67^{g}
ET13	19(25)^{b}	10(19)^{bc}	2.62^{fg}
ET23	20(27)^{bc}	9(17)^{b}	3.07^{jk}
Eco-T®×ET13	25(30)^{de}	19(26)^{ef}	2.70^{gh}
Eco-T®×ET23	23(28)^{cd}	12(20)^{c}	2.74^{ghi}
ET23×ET13	25(30)^{de}	18(25)^{de}	2.58^{fg}
Eco-T®×ET23×ET13	25(30)^{de}	20(26)^{ef}	3.00^{hijk}
Foc only	60(51)^{l}	43(41)^{h}	1.64^{a}
Water only	0(6)^{a}	0(6)^{a}	3.29^{k}
F test	(<0.001)	(<0.001)	(<0.001)
l.s.d.	(2.9)	(2.5)	0.308
s.e.d.	(1.5)	(1.2)	0.153
cv%	(5.7)	(6.0)	7.2

-Means with the same letter in the same experiment are not significantly different at $P<0.05$.

-Values in parenthesis represent transformed means using angular transformation.

Table 4.7a Biocontrol effects of single and mixtures of *Bacillus* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 2

Biocontrol Agent	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
B81	30(33)^{b}	21(27)^{bc}	2.82^{cde}
BFO11	22(27)^{b}	13(20)^{bc}	2.60^{cde}
EXR	25(30)^{b}	14(21)^{bc}	2.50^{cde}
JRO1	32(35)^{b}	20(26)^{bc}	2.73^{cde}
B81×JRO1	36(36)^{b}	16(22)^{bc}	3.03^{de}
BFO11×B81	26(30)^{b}	14(22)^{bc}	3.13^{de}
BFO11×JRO1	22(28)^{b}	16(24)^{bc}	2.21^{abcde}
EXR×B81	22(28)^{b}	12(20)^{bc}	2.86^{cde}
EXR×BFO11	32(34)^{b}	23(28)^{bc}	1.84^{abc}
EXR×JRO1	30(33)^{b}	20(26)^{bc}	2.09^{abcd}
BFO11×JRO1×B81	30(33)^{b}	17(24)^{bc}	2.67^{cde}
EXR×BFO11×B81	28(32)^{b}	20(26)^{bc}	2.38^{bcde}
EXR×BFO11×JRO1	24(29)^{b}	17(24)^{bc}	1.40^{ab}
EXR×JRO1×B81	34(35)^{b}	22(28)^{bc}	2.69^{cde}
EXR×BFO11×JRO1×B81	34(35)^{b}	23(29)^{c}	2.94^{de}
Foc only	61(51)^{c}	48(44)^{d}	1.19^{a}
Water only	0(6)^{a}	0(6)^{a}	3.25^{e}
F test	(<0.001)	(<0.002)	0.045
l.s.d.	(11.9)	(11.1)	1.075
s.e.d.	(5.9)	(5.5)	0.534
cv%	(23.7)	(28.6)	25.6

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

Table 4.7b Biocontrol effects of single and mixtures of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 2

Biocontrol Agent	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	28(32)^b	17(24)^{bc}	2.48^{cde}
ET13	20(25)^b	11(17)^{ab}	2.88^{cde}
ET23	21(27)^b	10(17)^{ab}	2.89^{cde}
Eco-T®×ET13	23(28)^b	17(24)^{bc}	2.61^{cde}
Eco-T®×ET23	24(29)^b	13(20)^{bc}	2.99^{de}
ET23×ET13	26(30)^b	15(22)^{bc}	2.59^{cde}
Eco-T®×ET23×ET13	20(26)^b	17(23)^{bc}	2.56^{cde}
Foc only	61(51)^c	48(44)^d	1.19^a
Water only	0(6)^a	0(6)^a	3.25^e
F test	(<0.001)	(<0.001)	0.045
l.s.d.	(11.9)	(11.1)	1.075
s.e.d.	(5.9)	(5.5)	0.534
cv%	(23.7)	(28.6)	25.6

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

Table 4.8a Biocontrol effects of single and mixtures of *Bacillus* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 3

Biocontrol Agent	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
B81	34(36)^{ghi}	26(31)^g	3.13^{lm}
BFO11	30(33)^{defg}	20(27)^{ef}	2.61^{efghijk}
EXR	21(27)^b	13(27)^{ef}	2.77^{ghijkl}
JRO1	38(38)^i	26(31)^g	2.60^{defghijk}
B81×JRO1	37(37)^{hi}	18(25)^{de}	2.99^{ijklm}
BFO11×B81	34(36)^{ghi}	20(27)^{efg}	2.98^{hijklm}
BFO11×JRO1	32(35)^{fghi}	23(28)^{efg}	2.16^{bcd}
EXR×B81	28(32)^{def}	19(26)^{def}	2.54^{cdefgh}
EXR×BFO11	33(35)^{fghi}	24(29)^{fg}	2.15^{bc}
EXR×JRO1	33(35)^{fghi}	23(29)^{fg}	2.06^b
BFO11×JRO1×B81	35(36)^{ghi}	21(28)^{efg}	2.23^{bcde}
EXR×BFO11×B81	31(34)^{efgh}	22(28)^{efg}	2.35^{bcdefg}
EXR×BFO11×JRO1	26(31)^{bcdf}	19(26)^{def}	2.26^{bcdef}
EXR×JRO1×B81	34(35)^{fghi}	23(29)^{fg}	3.03^{klm}
EXR×BFO11×JRO1×B81	32(35)^{fghi}	24(29)^{fg}	2.48^{bcdefg}
Foc only	72(58)^j	50(45)^h	1.28^a
Water only	0(6)^a	0(6)^a	3.33^m
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(3.8)	(3.2)	0.443
s.e.d.	(1.9)	(1.6)	0.220
cv%	(7.0)	(7.4)	10.4

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation

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Table 4.8b   Biocontrol effects of single and mixtures of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), Trial 3

Biocontrol Agent	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	$26(31)^{cde}$	$15(23)^{cd}$	$2.55^{cdefghi}$
ET13	$30(33)^{defg}$	$21(27)^{ef}$	$2.69^{fghijkl}$
ET23	$22(28)^{bc}$	$11(19)^{b}$	3.10^{lm}
Eco-T®×ET13	$29(33)^{defg}$	$21(27)^{ef}$	2.74^{ghijkl}
Eco-T®×ET23	$25(30)^{bcd}$	$13(21)^{bc}$	2.79^{ghijkl}
ET23×ET13	$31(34)^{efgh}$	$20(27)^{ef}$	$2.58^{cdefghij}$
Eco-T®×ET23×ET13	$25(30)^{bcd}$	$18(25)^{de}$	3.01^{jklm}
Foc only	$72(58)^{j}$	$50(45)^{h}$	1.28^{a}
Water only	$0(6)^{a}$	$0(6)^{a}$	3.33^{m}
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(3.8)	(3.2)	0.443
s.e.d.	(1.9)	(1.6)	0.220
cv%	(7.0)	(7.4)	10.4

-Means with the same letter in the same experiment are not significantly different at $P<0.05$.
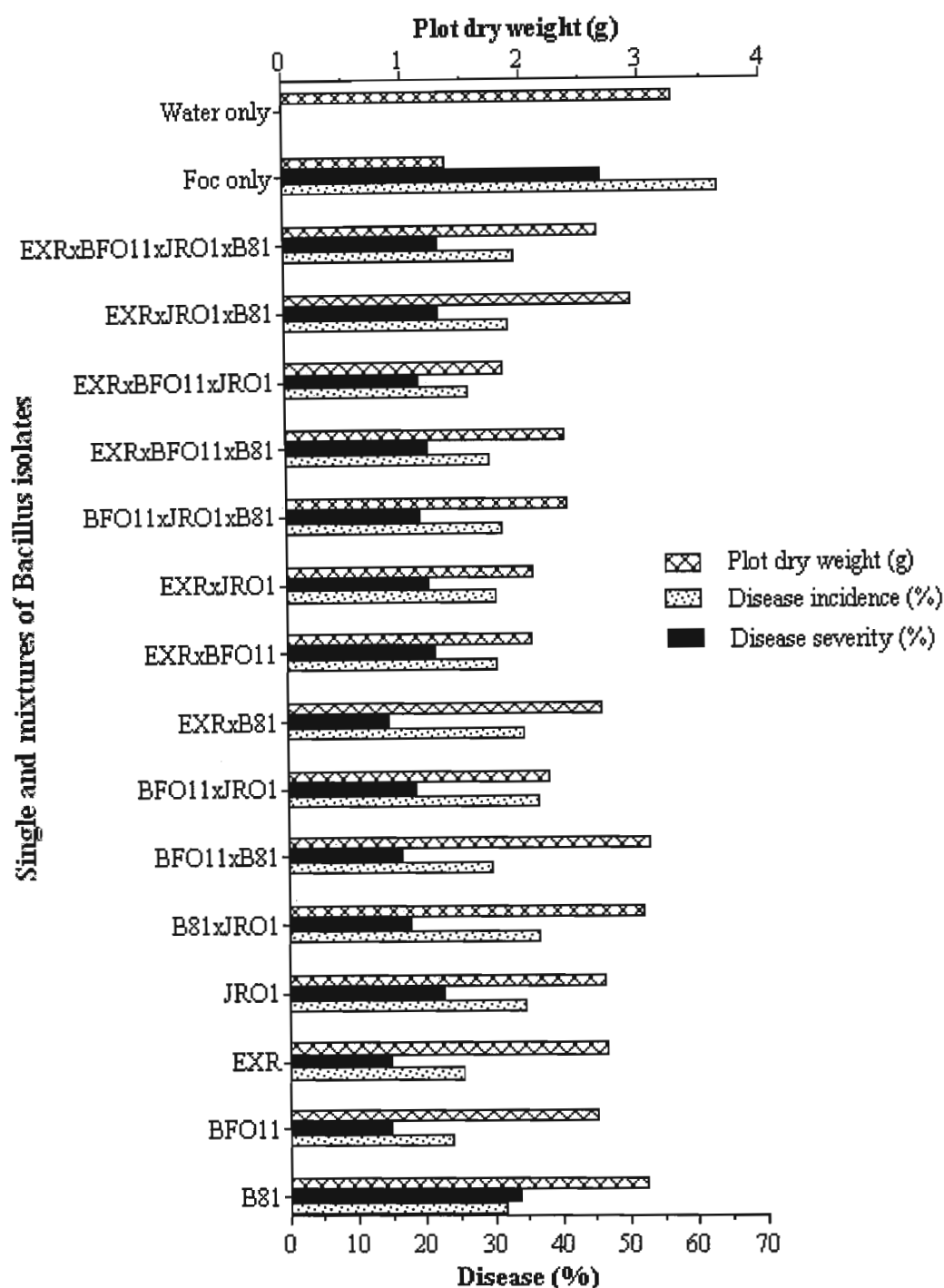
-Values in parenthesis represent transformed means using angular transformation.

Table 4.9a   Biocontrol effects of single and mixtures of *Bacillus* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), pooled mean of three trials

Biocontrol Agent	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
B81	32(34)^{fgh}	24(29)^{i}	3.01^{jkl}
BFO11	24(30)^{cd}	15(23)^{cde}	2.59^{fgh}
EXR	26(30)^{cd}	15(23)^{cde}	2.68^{hi}
JRO1	35(36)^{hi}	23(29)^{i}	2.66^{ghi}
B81×JRO1	37(37)^{i}	18(25)^{efg}	3.00^{jkl}
BFO11×B81	30(33)^{efg}	17(25)^{efg}	3.04^{kl}
BFO11×JRO1	27(31)^{cde}	19(26)^{fgh}	2.20^{cde}
EXR×B81	25(30)^{cd}	15(23)^{cde}	2.65^{fghi}
EXR×BFO11	31(34)^{fgh}	22(28)^{hi}	2.06^{bc}
EXR×JRO1	31(34)^{fgh}	21(27)^{ghi}	2.08^{bcd}
BFO11×JRO1×B81	32(35)^{ghi}	20(26)^{fgh}	2.38^{efg}
EXR×BFO11×B81	30(33)^{efg}	21(27)^{ghi}	2.36^{def}
EXR×BFO11×JRO1	27(31)^{cde}	20(26)^{fgh}	1.84^{b}
EXR×JRO1×B81	33(35)^{ghi}	23(29)^{i}	2.92^{ijk}
EXR×BFO11×JRO1×B81	34(36)^{hi}	23(29)^{i}	2.65^{fghi}
Foc only	64(53)^{j}	47(43)^{j}	1.37^{a}
Water only	0(6)^{a}	0(6)^{a}	3.29^{l}
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(2.9)	(2.8)	0.298
s.e.d.	(1.4)	(1.4)	0.148
cv%	(5.5)	(6.8)	7.0

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

*Graphs for disease incidence and severity (%) were drawn using untransformed data.

*x = indicates combination of the *Bacillus* isoates

Fig. 4.8a   Biocontrol effects of single and mixtures of *Bacillus* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), pooled mean of three trials

Table 6.9b Biocontrol effects of single and mixtures of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), pooled mean of three trials

Biocontrol Agent	Disease Incidence (%)	Disease Severity (%)	Plot Dry Weight (g)
Eco-T®	$27(27)^{b}$	$17(24)^{def}$	2.57^{fgh}
ET13	$23(29)^{bc}$	$14(22)^{cd}$	2.73^{hij}
ET23	$21(27)^{b}$	$10(18)^{b}$	3.02^{jkl}
Eco-T®×ET13	$26(31)^{cde}$	$19(26)^{fgh}$	2.68^{hi}
Eco-T®×ET23	$24(29)^{bc}$	$12(21)^{c}$	2.84^{hijk}
ET23×ET13	$27(32)^{def}$	$18(25)^{efg}$	2.58^{fgh}
Eco-T®×ET23×ET13	$23(29)^{bc}$	$18(25)^{efg}$	2.86^{hijk}
Foc only	$64(53)^{j}$	$47(43)^{j}$	1.37^{a}
Water only	$0(6)^{a}$	$0(6)^{a}$	3.29^{l}
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(2.9)	(2.8)	0.298
s.e.d.	(1.4)	(1.4)	0.148
cv%	(5.5)	(6.8)	7.0

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

Plot dry weight (g)

Single and mixtures of Trichoderma isolates

Disease (%)

Legend:
- Plot dry weight (g)
- Disease incidence (%)
- Disease severity (%)

*Graphs for disease incidence and severity (%) were drawn using untransformed data while the letters showing significance were given based on the transformed data.

*x = indicates combination of the *Trichoderma* isolates

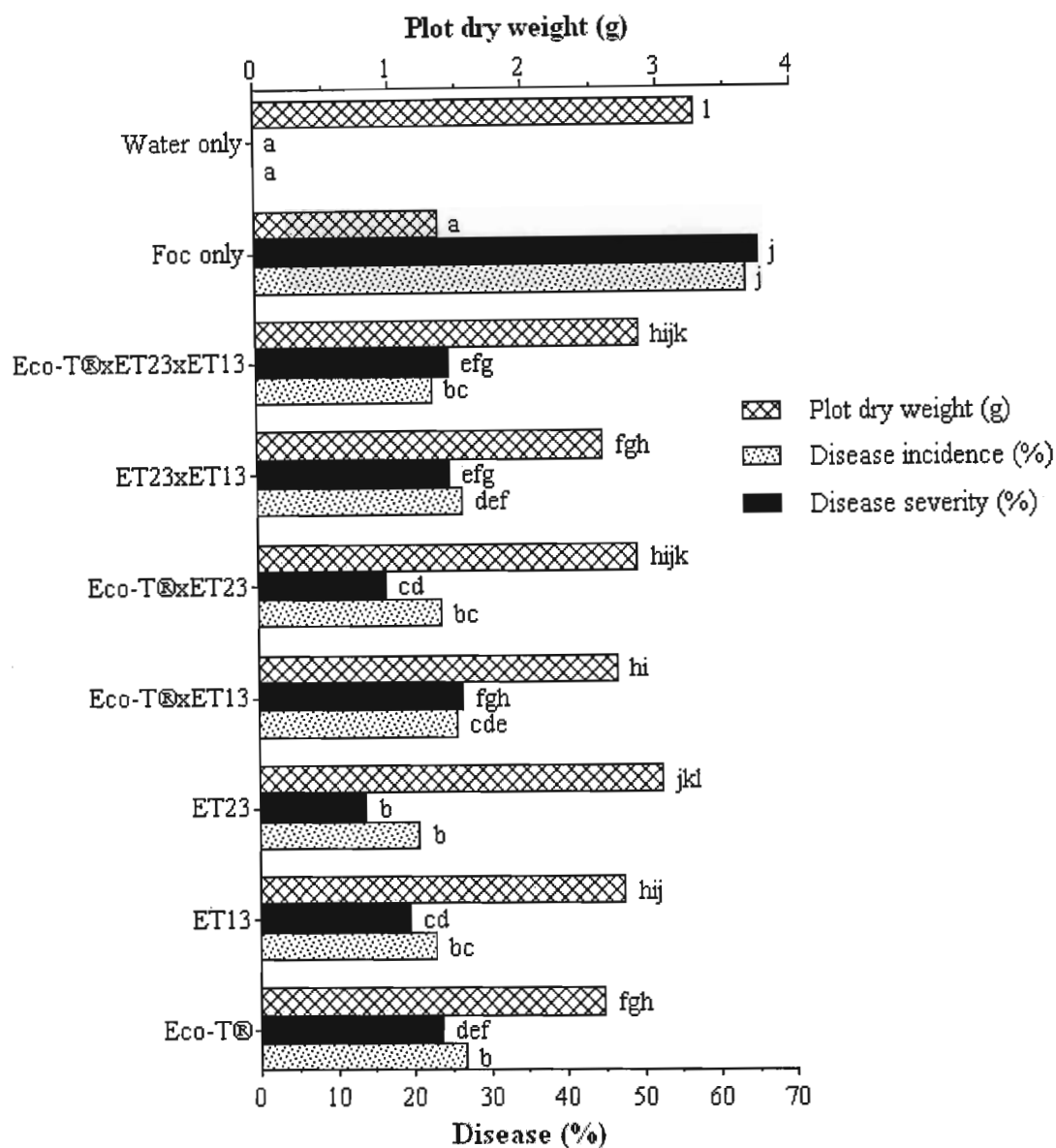**Fig. 4.8b**   Biocontrol effects of single and mixtures of *Trichoderma* isolates against *Fusarium oxysporum* f. sp. *conglutinans* (*Foc*), pooled mean of three trials

4.4   Discussion

Mixed bioinocula have been pioneered in the control of soilborne diseases (Sivasithamparam & Parker, 1978) and many researchers have reported the role of application of mixtures of biocontrol agents to overcome the variable and inconsistent performance of biocontrol agents, particularly under field conditions, where they are highly influenced by biotic and abiotic conditions (Duffy *et al.*, 1996; Raupach & Kloepper, 1998; Fukui *et al.*, 1999; Guetsky *et al.*, 2001; Guetsky *et al.*, 2002).

However, selecting the best compatible mixtures of biocontrol agents is a prime prerequisite because biocontrol agents may also inhibit each other. Under normal natural conditions microorganisms generally compete for nutrients and space and the competition becomes aggressive when resources are limited. Some microorganisms may suppress the growth of other microorganisms by releasing antimicrobial substances even when resources are not limited just because the other microorganism is naturally sensitive to those biochemical substances. The use of filtrate extracts of bacterial antagonists grown alone, for instance, inhibits the growth of many fungal pathogens, at least in bioassay tests.

4.4.1   Compatibility between *Trichoderma* and *Bacillus* Isolates *In Vitro*

Results from *in vitro* test presented here demonstrate that the use of combinations of *Trichoderma* and *Bacillus* may not be possible because *Trichoderma* isolates suffered antagonism from the biochemical, possibly antibiotics, effect of *Bacillus* isolates (Tables 4.1-4.3). Although relatively little investigation has been conducted on the extent to which biocontrol bacteria that inhibit plant pathogens may also inhibit biocontrol fungi, there are reports that show that biocontrol bacteria produce deleterious antibiotics and antibiotic-like compounds that inhibit biocontrol fungi and other beneficial microorganisms (Hubbard *et al.*, 1983; Fravel, 1988; Bin *et al.*, 1991). Bin *et al.* (1991) and Hubbard *et al.* (1983) reported that co-inoculation of the pigmented antibiotic phenazine-1-carboxylic acid producing *P. fluorescens* Strains 2-79 with *T. harzianum* and pea seed colonizing *Pseudomonas* spp. with *T. hamatum* in New York soils detrimentally altered the effectiveness of the biocontrol ability of the *Trichoderma* species.

4.4.2 Compatibility between *Trichoderma* Isolates

One of the useful functions of taxonomy is its predictive value and if one organism is known to have certain properties, then a taxonomically related organism will often have similar properties (Cook *et al.*, 1996). However, different species in the genus *Trichoderma*, or even different strains of the same species, may have different mechanisms of control against plant pathogens. This difference may cause incompatibility.

The results from the *in vitro* test show that there was some degree of defensiveness between the isolates although they grew at almost equal rate. For example, in the test between *T. harzianum* Eco-T$^®$ and *Trichoderma* Isolate ET23 both organisms used half of the plate equally. On the other hand, the test between *Trichoderma* Isolates ET13 and ET23 shows that ET23 was relatively more vigorous, growing on top of ET13 and formed a thick mycelial mat at the point of contact with ET13. This, together with the formation of a yellowish colour on the area of contact, may indicate that there was a certain degree of incompatibility although both organisms used the plate more or less equally. The formation of yellowish colour was also observed in the interaction between *Trichoderma* Isolate ET13 and *T. harzianum* Eco-T$^®$ though it was relatively more yellow compared to what was observed when *Trichoderma* Isolate ET13 was tested against *Foc* in the dual culture test (Chapter Two). Therefore, the change in colour could have resulted from the release of biological substances by *Trichoderma* Isolate ET13.

Electron microscopic observations from samples taken from the point of contact of *T. harzianum* Eco-T$^®$ and *Trichoderma* Isolate E23, and *Trichoderma* Isolate E13 using ESEM also shows that there was lysis of mycelia that could have resulted either from the release of biochemical substances from either or both sides or due to the death of old mycelia. However, there was also inter-weaving of mycelia without visible signs of lysis.

4.4.3 Compatibility between *Bacillus* Isolates

The slight antagonism observed between some of the *Bacillus* isolates is an indication to the fact that, although they are species of the same genus, they may exhibit a certain degree of defensiveness against some members of their own genus or species as the non

pathogenic *F. oxysporum* does to pathogens which belong to the species *F. oxysporum* (Komada, 1990).

A certain degree of antagonism may be acceptable in biocontrol experiments with mixed bioinocula as long as the combination provides better control compared to the results obtained when applied singly. Moreover, some biochemical substances released by biocontrol agents, which cause serious damages to other organisms, may not be manifested in soil as it does on agar plates. However, the incompatibility observed *in vitro* is still important as it highlights what may happen when grossly incompatible organisms are applied together into root zone or other plant parts.

4.4.4 Greenhouse Experiments using Mixtures of *Trichoderma* or *Bacillus* Isolates

A greenhouse study using single and mixed bioinocula was conducted using biocontrol organisms obtained from preliminary greenhouse screening tests against the same pathogen used in this test. They caused a certain level of disease suppression. However, the level of control obtained was not high enough. Therefore, to improve the level of disease reduction, mixed bioinocula were investigated in the hope of finding better control with the best possible combinations of compatible isolates. However, mixtures of *Trichoderma* or *Bacillus* isolates generally resulted either in non-significant differences in disease reduction or higher levels of disease than when applied singly.

Different *Trichoderma* and *Bacillus* species have different mechanisms of control against plant pathogens and may have different ecological requirements. All *Trichoderma* and *Bacillus* species do not manifest all the mechanisms of control employed against plant pathogens by all species in their genera. Therefore, when compatible strains of *Trichoderma* or *Bacillus* species with different mechanisms of control are applied together, significantly better result would be obtained. Guetsky *et al.* (2002) proved their hypothesis that control efficacy achieved by biocontrol agents exhibiting several distinct mechanisms of control will result in an additive effect or synergism, but not antagonistic, effects. They further noted that it is possible that if multiple mechanisms are involved, under a certain set of conditions one mechanism may compensate for the other.

Therefore, the lack of improvement in disease control with the use of mixtures of *Trichoderma* or *Bacillus* isolates was probably because there was no additive effect due to mixing of biocontrol agents.

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CHAPTER FIVE

BIOCONTROL OF *FUSARIUM* SPP. AND *RHIZOCTONIA SOLANI* USING *TRICHODERMA* AND *BACILLUS* ISOLATES ON MAIZE

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Abstract

Several *Fusarium* isolates were isolated from diseased kernels and cobs of maize and subsequently tested for pathogenicity under greenhouse conditions. However, none of them caused any visible disease such as seed rot, root rot, seedling blight or stalk rot. Therefore, biocontrol experiments against *Fusarium* could not be conducted on maize. On the other hand, three *Trichoderma* and four *Bacillus* isolates previously selected for their activity against *F. oxysporum* f. sp. *conglutinans* on cabbage were tested against Rhizoctonia damping-off caused by *R. solani* on maize. Application of *T. harzianum* Eco-T® and *Trichoderma* Isolate ET23 significantly ($P<0.001$) increased seedling emergence. However, none of the *Bacillus* isolates reduced percentage of preemergence damping-off. Damping-off by *R. solani* on maize was strictly preemergence.

5.1   Introduction

Soilborne plant pathogens cause seed rot, damping off, root rot, wilt, and fruit rot, which results in an annual loss of $4-5b in the United State alone (Jewell, 1987 *cited by* Lewis *et al.*, 1998).

The fungus, *Fusarium verticillioides* (Sacc.) Nirenberg (=*F. moniliforme* J. Sheldon (teleomorph *Gibberella fujikuroi* ((Sawada) Wollenw.) not only poses a threat to crop

productivity but also to the health of humans and animals eating products contaminated with the fungus (Gelderblom *et al.*, 2001). *Fusarium verticillioides* can cause severe financial losses during the preharvest life of maize plants (*Zea mays* L.) and postharvest in the processing and storage of products. The fungus has been associated with root, stalk, ear, and kernel rots, as well as seedling blights (Kommedahl & Windels, 1981). On the other hand, *Rhizoctonia solani* Kühn (teleomorph = *Thanatephorus cucumeris* (Frank) Donk.) is a ubiquitous soilborne pathogen best known as the major cause of seedling damping-off diseases (Fiddaman & Rossall, 1995). The pathogen causes a range of diseases and has a wide host range. In maize, the pathogen has been reported to cause crown and adventitious root rot, where the roots of the germinating seeds and young plants brown and decay with sunken brown lesions on the root and crown thus reducing seedling stand (Anonymous, 2004). Maize culture has also been reported to support *R. solani* growth saprophytically, thus increasing the inoculum level in the field, causing severe root and hypocotyl rot in the following crop (Sumner *et al.*, 1986).

No currently available fungicide, resistant cultivar, or cultural practices provide for the effective management of *F. verticillioides* in maize (Munkvold & Desjardins, 1997). Similarly, measures recognized for the control of *R. solani*, such as rotation of cultures, use of resistant varieties and treatment of seeds and/or soil with fungicides, are usually unsuitable or ineffective, mainly due to the genetic variability present in the pathogen to the host range, capacity to survive in the soil and in the seeds, and its physiologic flexibility to infect different hosts (Leach & Garber, 1970). In addition to the cost of development of new fungicides (Delp, 1977; Chet, 1990) and the continuous emergence of new pathogenic strains with resistance to these fungicides (Shatla & Sinclair, 1963), the increasing use of potentially hazardous fungicides in agriculture has been a growing world-wide public concern. These factors have pushed for the development of alternative non-chemical measures to control these and other soilborne plant pathogens.

The use of environmentally friendly microbial antagonists to control *F. verticillioides* and *R. solani* has been widely investigated as an alternative control method (Elad *et al.*, 1980; Chet & Baker, 1981; Elad *et al.*, 1982; Sumner *et al.*, 1992; Fiddaman & Rossall, 1995; Calistru *et al.*, 1997; Kim *et al.*, 1997; Mao *et al.*, 1997; Mao *et al.*, 1998; Yates *et al.*, 1999; Bacon *et al.*, 2001; Montealegre *et al.*, 2003). The potential for use of *Trichoderma* species as biocontrol agents was suggested more than 60 years ago by Weindling (1932),

who was the first to demonstrate the parasitic activity of members of this fungal genus to pathogens such as *R. solani*. Similarly, biological control of soilborne diseases by bacterial antagonists has been widely reported (Weller, 1988; Hornby, 1990). *Bacillus* spp. have been tested on a wide variety of plant species for their ability to control diseases (Weller, 1988). There are several reports in which *Trichoderma* and *Bacillus* species controlled *R. solani* on different crops (Elad *et al.*, 1980; Chet & Baker, 1981; Elad *et al.*, 1982; Sumner *et al.*, 1992; Fiddaman & Rossall, 1995; Kim *et al.*, 1997; Montealegre *et al.*, 2003). A few investigations have reported *Trichoderma* and *Bacillus* species as control organisms for phytopathogenic *Fusarium* spp. in maize (Calistru *et al.*, 1997; Mao *et al.*, 1997; Mao *et al.*, 1998; Yates *et al.*, 1999; Bacon *et al.*, 2001).

Emphasis of this research was centered on evaluation of the effectiveness of the bacterial and fungal biocontrol agents to reduce disease incidence and severity due to *Fusarium* spp. and *R. solani*.

5.2   Materials and Methods

5.2.1   Source of Plant Material

Maize seed, *Zea mays* L., of the cultivar PAN 6479, seed lot 5PL CODE I/208409/GII, was obtained from Pannar Seed Company[1] (Pty).

5.2.2   Microorganisms

Pathogens

Twenty isolates of *Fusarium* were isolated from infected maize kernels and cobs provided by H. Gevers[2], Pannar Seed Company[1] and B. Flett[3], from maize fields at Ukulinga, Greytown and Potchefstroom, respectively. Infected maize kernels were first surface sterilized in 3.5% commercial bleach for 10-15min and rinsed three times in sterile distilled water. Each grain was cut half, and placed cut side down onto a Peptone-

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[1] Pannar Seed (Pty) P.O.Box 19, Greytown 3250, Republic of South Africa.
[2] Prof. H. Gevers, Quality Seeds (Pty) Ltd., Pietermaritzburg, Republic of South Africa.
[3] Dr Brad Flett, ARC, Potchefstroom, Republic of South Africa.

Pentachloronitrobenzene Agar (PCNB) medium (Nash & Snyder, 1962). Plates were then incubated at 25°C for 7-10d. *Fusarium* isolates were subcultured onto Carnation Leaf Agar (CLA) (Fisher *et al.*, 1982) before being stored frozen on 15% glycerol at -80°C and at 4°C on silica gel (Perkins, 1962).

Rhizoctonia solani culture was isolated by C. Clark[4] and maintained on a half-strength V8 agar slants.

Biocontrol Agents

Three *Trichoderma* isolates, *T. harzianum* Eco-T[®5] and *Trichoderma* Isolates ET23 and ET13 and four *Bacillus* isolates, BFO11[5], JRO1[5], B81[6] and EX-R[7], which were previously screened against *F. oxysporum* f. sp. *conglutinans* on cabbage (Chapters Two and Three) were used.

5.2.3 Preparation of Pathogen Inocula

Fusarium isolates stored on silica gel were cultured on Oat Meal Agar (OMA) and incubated at 25°C for 10-14d. The OMA was prepared by blending 60g of ground rolled oats suspended in 600ml of distilled water and 12g of agar dissolved by heat in 400ml of distilled water. The agar was then half-filled into 250ml bottles and autoclaved at 121°C for 90min. Inoculum consisting of both conidia and mycelium was obtained by flooding the agar plate with 15ml of sterile distilled water. Inoculum with a concentration of 10^7 c.f.u. ml^{-1} was used to cause satisfactory infection (Bacon *et al.*, 1994).

Inoculum of *R. solani* was prepared by culturing V8 Agar medium, containing 200ml V8 tomato juice; 3g CaCO$_3$ (uniLAB[®]); 15g agar (MERCK) and 800ml of distilled water and autoclaved for 15min at 121°C. Agar block approximately 5mm×5mm was used per hole or per seed in the 24 Speedling[®] tray. The V8 agar block fully colonized by *R. solani* was placed at the center of a planting hole half-filled with composted pine bark and covered

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[5] Plant Health Products (Pty) Ltd, P.O.Box 207, Nottingham Road, Republic of South Africa.
[6] B. Kubheka, Discipline of Plant Pathology, School of Applied Environmental Sciences, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, Pietermaritzburg, Republic of South Africa.
[7] Dr M. Morris, Plant Health Products (Pty) Ltd, P.O.Box 207, Nottingham Road, KwaZulu-Nata, Republic of South Africa.

with a thin film of composted pine bark. Maize seeds were then sown on top and covered with composted pine bark to fill the hole.

5.2.4 Greenhouse Pathogenicity Test of *Fusarium* Isolates

The relative ability of the different isolates of *Fusarium* obtained from the three different places was compared in a polycarbonate greenhouse tunnel with temperatures ranging from 26 to 28°C for most of the time and supplied with irrigation water maintained at 20°C and supplemented with NPK soluble fertilizer [3:1:3(38)] three times a day for 5min. Experiments were arranged in a completely randomized block design with each treatment replicated three times and each experiment repeated once. Two different inoculation techniques were used:

a. *Drenching*

After maize seeds planted in Speedling® 24 trays just started to break the soil surface, each seedling was drenched with 4ml of the inoculum suspension at a concentration of 10^7 c.f.u. ml^{-1}. After inoculation, plants were monitored for 6wk for any symptoms of the disease such as seed rot leading to poor germination, root rot, the initial water-soaked appearance of entire leaves and stems and stalk rot with the appearance of white to pink mycelia within tissue (Voorhees, 1934; Shurtleff, 1980).

b. *Root Wounding*

Maize seedlings were allowed to grow in Speedling® 24 for 3wk. The young maize seedlings were lifted from trays and the roots were wounded by cutting the root tips with a pair scissor. Wounded roots were then inoculated by dipping in inoculum suspensions of *Fusarium* isolates with concentrations of 10^7 c.f.u. ml^{-1}. After 4wk, plants were checked for any symptom of infection of *Fusarium*.

5.2.5 Preparation of Inocula of the Biocontrol Agents

Trichoderma *Isolates*

A conidial suspension of the *Trichoderma* isolates was prepared from conidia collected from cultures grown on V8 agar medium. V8 agar plates were inoculated with agar blocks from Potato Dextrose Agar (PDA) (MERCK) plates kept at 4°C and incubated for 7d at

25°C. Then 5ml sterile distilled water was pipetted into plates and conidia were collected by scraping the plate with cotton wool-tipped curved sterile Pasteur pipette. The conidial suspensions of the *Trichoderma* isolates were then counted using a Neubauer Improved Double Haemocytometer and adjusted to 5×10^9 conidia ml^{-1}.

Bacillus *Isolates*

Four *Bacillus* isolates were grown in 250ml Erlenmeyer flasks each containing a 100ml Nutrient Broth (NB) (MERCK) for 4d at 30°C in a water bath shaker (Gesellschaft für Labortechnik, mbH, D-30938, Burgwedel, Germany) at 150 oscillations min^{-1}. Flasks were inoculated with the *Bacillus* isolates previously grown in Tryptone Soy Agar (TSA) (MERCK) for 48h. After 4d the *Bacillus* isolates were harvested by centrifugation in a Beckman J2-HS Centrifuge at 9000×g for 15min. The broth was decanted and bacterial pellets were resuspended in sterile distilled water. Bacterial cells were then counted using a plate dilution technique in a TSA medium and adjusted to a concentration of 10^7 c.f.u. ml^{-1} of water.

5.2.6 Application of Biocontrol Agents

Biocontrol agents were applied only as a seed treatment since it is only economical to use seed treatments on low value field crops such as maize. In addition, the exudates that comes out of the seed during germination help the spores of the biocontrol agents to germinate and establish quickly in the rhizosphere.

Maize seeds were soaked in a conidial suspension of 10^9 conidia ml^{-1} supplemented with 2% (w v^{-1}) carboxylmethycellulose (CMC), which served as an adhesive, and immediately dried on a Laminar Flow bench overnight. The density of *Trichoderma* isolates' conidia on the seed surface was determined by a plate dilution technique from treated and untreated seeds, using a modified *Trichoderma*-selective medium (Askew & Laing, 1993).

5.2.7 Sterilization of Speedling® 24 Trays

Since the Speedling® 24 trays were previously being used by other researchers, sterilization of the trays was necessary to eliminate the effect of other microbes. They were

sterilized by dipping the trays in Plaz-Dip® and immediately air-dried. Plaz-Dip® is a 12% copper hydroxide suspension in PVA paint.

5.2.8 Plant Growing Medium

Composted pine bark medium was used as a sole plant-growing medium through out the entire study. However, since composted pine bark is nutritionally poor and has poor water holding capacity it was supplemented with NPK soluble fertilizer [3:1:3(38] at a rate of three times a day for 5min.

5.2.9 Controls

In the pathogenicity test of *Fusarium* isolates there was only one control treatment, which was treated only with water.

In the biocontrol experiments there were two control treatments:
1. neither antagonist nor pathogen
2. pathogen only

5.2.10 Disease Rating

In the biocontrol experiment the effect of *R. solani* and the biocontrol agent was determined by measuring percentage seedling emergence and survival. Therefore, percent emergence and survival were calculated as follows:

% Emergence or Survival = 100 × (number of seedlings germinated or survived)/24
Where,

24 = number of seeds sown

5.2.11 Statistical Analysis

Biocontrol experiments were repeated twice with treatments randomized and replicated four times in a block design. All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 6 Statistical Analysis Software to determine

differences between treatment means. Percentage of emergence and survival was calculated on a per plot basis and transformed using angular transformation prior to analysis. All least significant differences were determined at P<0.05.

5.3   Results

5.3.1   Pathogenicity Test of *Fusarium* Isolates

Of all the *Fusarium* isolates tested for pathogenicity none caused any visible symptoms of seed rot, root rot, seedling blight or stalk rot. Since the isolates were unable to cause visible disease symptoms to maize plants, the biocontrol experiments were not able to be carried out. However, in a pilot experiment (data not shown) where seedlings of 4wk old injected at the stem area with 1ml suspensions of the isolates with an average concentration of 10^7 c.f.u. ml^{-1} caused extensive symptom of seedling blight and stalk rot for most of the isolates though the disease severity was decreasing with some of the plants almost completely recovered.

5.3.2   Greenhouse Biocontrol Test of *Rhizoctonia solani*

The damping-off pathogen, *R. solani*, caused significant level of disease, which was only a preemergence damping-off, ranging from 61 to 75% (Table 4.1-4.3; Fig. 4.1-4.3). There was no significant difference between percent emergence and final stand since there was no postemergence damping-off due to the pathogen.

Percentage of damping-off was significantly reduced owing to the effect of biocontrol treatments. Two *Trichoderma* isolates, *T. harzianum* Eco-T® and *Trichoderma* Isolate ET23, were very significantly (P<0.001) suppressive to *R. solani*, reducing percentage of damping-off by 35-41% and 34-39%, respectively, over the pathogen-treated control. Though there were some *Bacillus* isolates which caused statistically significant reductions in percentage of damping-off, these were not high nor consistent (Table 4.1-4.3; Fig. 4.1-4.3).

Biocontrol treatment also increased plot dry weight significantly (P<0.001) compared to the treatment with pathogen only. *Trichoderma* Isolate ET23 and *T. harzianum* Eco-T® significantly increased plot weight ranging from 36 to 43% and 36 to 41%, respectively, compared to the treatment treated with *R. solani* only. Some *Bacillus* isolates also showed a significant increase in plot dry weight relative to the pathogen-treated control despite the fact that there was a lack of consistency and the level of increase in plot dry weight was lower (Tables 4.1-4.3).

Table 5.1   Effects of treatment of maize seeds with *Trichoderma* and *Bacillus* isolates on *Rhizoctonia solani* in greenhouse, Trial 1

Treatments	Emergence (%)	Plot Dry Weight (g)
Bacillus Isolate B81	30(33)^{ab}	4.98^a
Bacillus Isolate BFO11	38(38)^{ab}	6.16^{ab}
Bacillus Isolate EXR	44(41)^b	7.23^b
Bacillus Isolate JRO1	32(34)^{ab}	5.24^a
Trichoderma Isolate Eco-T®	62(52)^c	10.14^c
Trichoderma Isolate ET13	28(32)^a	4.48^a
Trichoderma Isolate ET23	65(53)^c	10.49^c
Rhizoctonia solani only	26(31)^a	4.30^a
Water only	87(71)^d	14.09^d
F test	(<0.001)	<0.001
l.s.d	(8.4)	1.904
s.e.d	(4.1)	0.923
cv%	(13.5)	17.5

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation
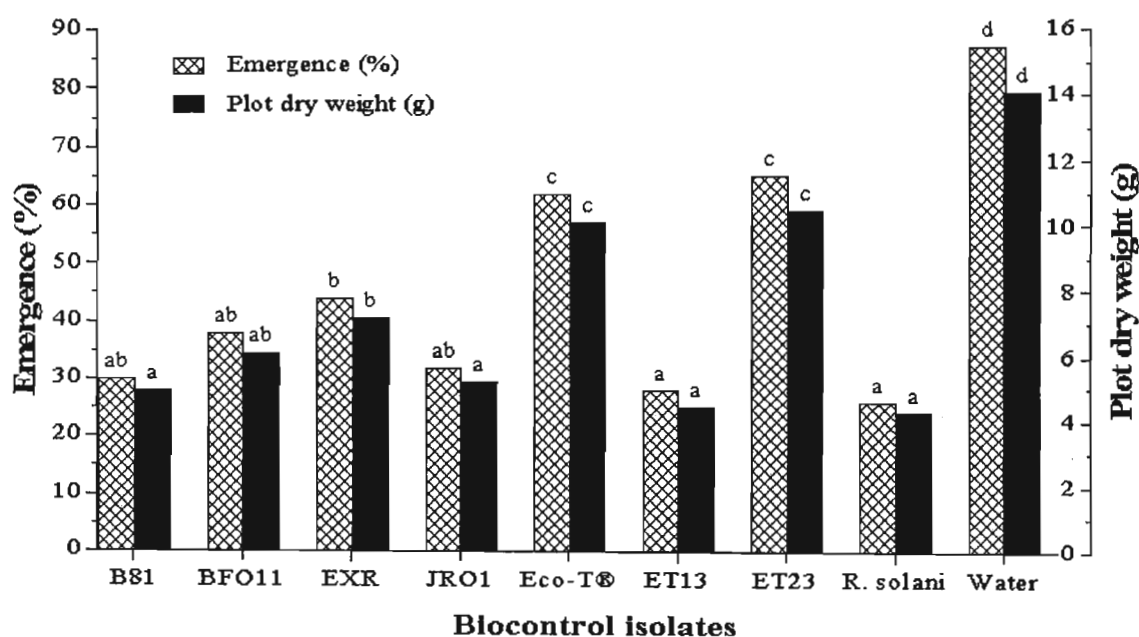
*Graph for emergence (%) was drawn using untransformed data while the letters showing significance were given based on the transformed data.

Fig. 5.1 Effects of treatment of maize seeds with *Trichoderma* and *Bacillus* isolates on *Rhizoctonia solani* in greenhouse, Trial 1

Table 5.2   Effects of treatment of maize seeds with *Trichoderma* and *Bacillus* isolates on *Rhizoctonia solani* in greenhouse, Trial 2

Treatments	Emergence (%)	Plot Dry Weight (g)
Bacillus Isolate B81	23(28)^{ab}	5.86^a
Bacillus Isolate BFO11	26(31)^{ab}	6.25^a
Bacillus Isolate EXR	31(34)^b	6.72^a
Bacillus Isolate JRO1	28(32)^{ab}	6.45^a
Trichoderma Isolate Eco-T®	60(51)^c	11.18^b
Trichoderma Isolate ET13	25(30)^{ab}	6.70^a
Trichoderma Isolate ET23	56(49)^c	10.51^b
Rhizoctonia solani only	19(25)^a	4.44^a
Water only	94(78)^d	16.57^c
F test	(<0.001)	<0.001
l.s.d	(8.7)	3.384
s.e.d	4.2)	1.639
cv%	(15.0)	27.9

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation

*Graph for emergence (%) was drawn using untransformed data while the letters showing significance were given based on the transformed data.
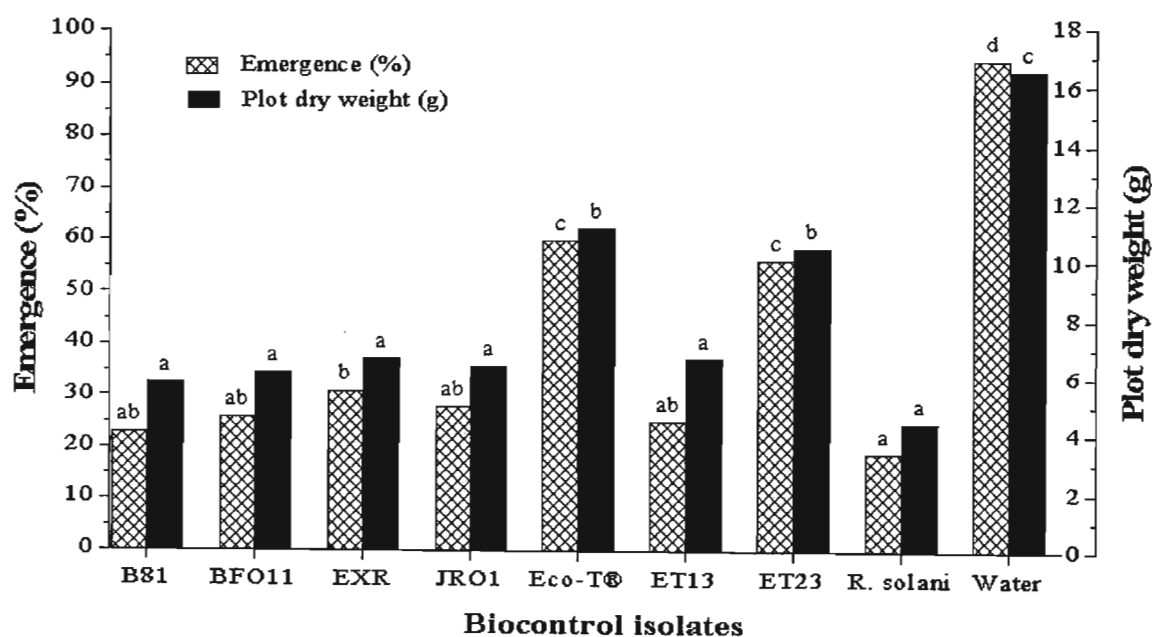
Fig. 5.2 Effects of treatment of maize seeds with *Trichoderma* and *Bacillus* isolates on *Rhizoctonia solani* in greenhouse, Trial 2

Table 5.3   Effects of treatment of maize seeds with *Trichoderma* and *Bacillus* isolates on *Rhizoctonia solani* in greenhouse, Trial 3

Treatments	Emergence (%)	Plot Dry Weight (g)
Bacillus Isolate B81	$25(30)^{ab}$	5.61^{ab}
Bacillus Isolate BFO11	$31(34)^{cd}$	6.30^{b}
Bacillus Isolate EXR	$36(37)^{d}$	7.00^{b}
Bacillus Isolate JRO1	$30(33)^{bc}$	6.35^{b}
Trichoderma Isolate Eco-T®	$58(50)^{e}$	10.45^{c}
Trichoderma Isolate ET13	$25(30)^{ab}$	5.74^{ab}
Trichoderma Isolate ET23	$57(49)^{e}$	10.62^{c}
Rhizoctonia solani only	$23(29)^{a}$	4.69^{a}
Water only	$90(71)^{f}$	15.75^{d}
F test	(<0.001)	<0.001
l.s.d	(3.1)	1.453
s.e.d	(1.5)	0.704
cv%	(5.2)	12.4

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation

*Graph for emergence (%) was drawn using untransformed data while the letters showing significance were given based on the transformed data.
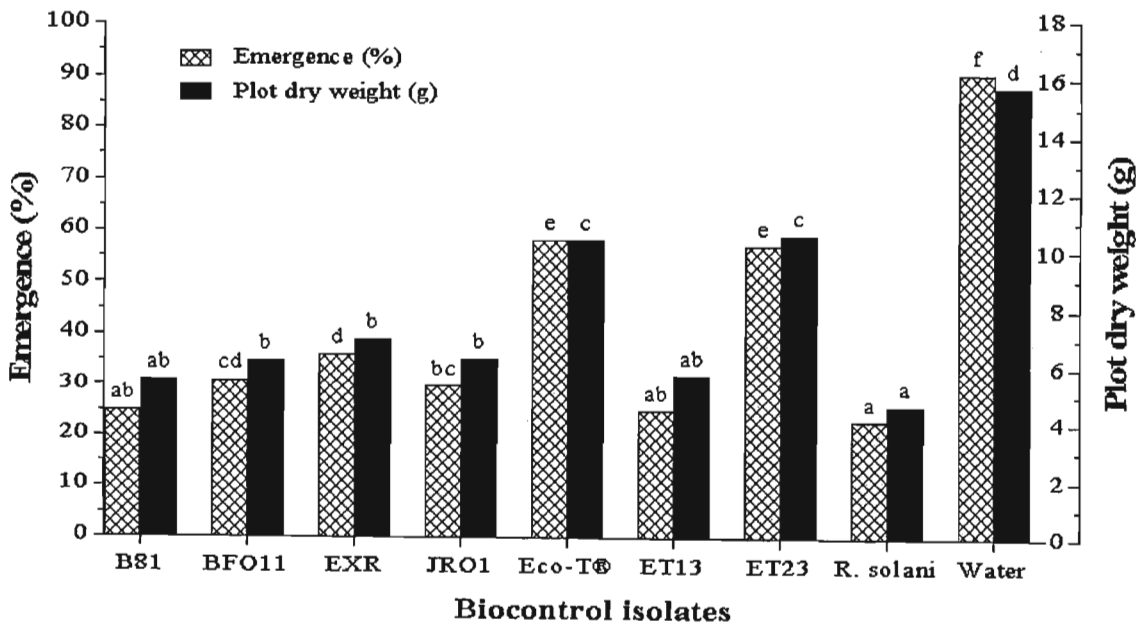
Fig. 5.3 Effects of treatment of maize seeds with *Trichoderma* and *Bacillus* isolates on *Rhizoctonia solani* in greenhouse, Trial 3

5.4 Discussion

5.4.1 Pathogenicity Test of *Fusarium* Isolates

Economically, stalk rots have been considered more important than any other diseases of maize, and *F. verticillioides* is regarded as one of the major maize pathogens (De Leon & Pandey, 1989; Drepper & Renfro, 1990). It causes kernel rot, root rot, seedling blight, stalk rot, ear rot and cob rot (Kommedahl & Windels, 1981). However, none of the *Fusarium* isolates obtained from infected maize kernels and cobs caused seed rot, root rot, seedlings blight or stalk rot when inoculated by drenching into soil or dipping wounded roots into the suspensions. Therefore, it was not possible to proceed with biocontrol experiments using any of these isolates which caused no visual symptoms of the disease.

Plants defend themselves in a variety of ways from plant pathogens. In general, they defend themselves against plant pathogens by a combination of two different ways: (1) structural characteristics that act as physical barriers and inhibit the pathogen from gaining entrance and spreading through the plant and (2) biochemical reactions that take place in the cells and tissues of the plant and produce substances which are either toxic to the pathogen or create conditions that inhibit growth of the pathogen in the plant (Agrios, 1997).

A combination of these defense mechanisms might had been involved in the variety of maize used in this experiment. The inability of the inoculum suspensions drenched into the roots of young maize cultures to cause infection on maize roots may be attributed to the presence of a thick cuticle that covers the epidermal cell walls, the structure of the epidermal cell walls, and the presence on the plant tissues made of thick-walled cells that hinder the penetration of the pathogen to the root cells (Agrios, 1997).

Although these pathogens did not cause visible symptoms, it does not mean they are not necessarily pathogenic. *Fusarium verticillioides* is also known as an endophytic symptomless pathogen and may infect maize without visual signs, and subsequently contribute to the total mycotoxin contaminants of maize (Bacon & Hinton, 1996; Yates *et al.*, 1997; Bacon *et al.*, 2001). However, the objective of the experiment was to find more

aggressive strains than isolates which cause symptomless infections only. *Fusarium* strains which cause these visual symptoms are economically more destructive since they do not only contribute to the accumulation of mycotoxins in kernels but also cause yield loss by causing seed rot reducing seedling stand, root rot and seedling blight reducing seedling vigor, and stalk rot causing death or lodging which makes harvesting difficult.

5.4.2   Greenhouse Biocontrol of *Rhizoctonia solani*

The effect of *R. solani* was strictly preemergence damping-off of maize as a result of seed rot. No postemergence damping-off was observed. This could partly be explained to the strength of maize roots to withstand infection once established since no postemergence damping-off was observed on the control treated with *R. solani* only.

Biocontrol agents applied as seed treatment to maize seeds caused improved seedling emergence (Table 5.1-5.3). Two *Trichoderma* isolates, *T. harzianum* Isolate Eco-T® and *Trichoderma* Isolate ET23, were most effective (P<0.001) in terms of the level of reduction in Rhizoctonia damping-off and increased plot dry weight relative to the control treated with the pathogen only. Many researchers have also demonstrated that *Trichoderma* spp. applied as seed treatment reduced damping-off caused by *R. solani* (Chet & Baker, 1981; Elad *et al*., 1982; Chet & Henis, 1983; Chet, 1990). However, the bacterial isolates showed variable and low levels of disease control compared to *T. harzianum* Isolate Eco-T® and *Trichoderma* Isolate ET23. Although it has been reported that *Bacillus* spp. reduce or control Rhizoctonia Damping-off (Asaka & Shoda, 1996; Kim *et al*., 1997), the high level of disease observed in this research may be attributed to the virulent nature of the strain, amount of inoculum used, slow germination or the high relative humidity, moisture and temperature in the greenhouse system favored more to the pathogen, or may be these *Bacillus* isolates were not very effective antagonists against *R. solani*. Fiddman & Rossal (1995) reported that in an *in vivo* test *B. subtilis* 205 controlled pre-emergence damping-off of oilseed rape as well as the non-inoculated control. However, post-emergence survival of seedlings was less due to the effect of temperature on the biocontrol efficacy of *B. subtilis* 205. This isolate was found to be more effective at lower temperatures.

In the challenge against Rhizoctonia damping-off in maize, the integration of biocontrol treatments and the use of maize cultivars with the ability to germinate and establish quickly

could result in better control since the longer the seed takes to germinate the greater the likelihood of *R. solani* attacking the maize seed.

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CHAPTER SIX

STUDIES ON THE BIOCONTROL OF THE PITCH CANKER FUNGUS (*FUSARIUM CIRCINATUM*)

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Abstract

The pitch canker fungus, *Fusarium circinatum*, is a major disease of pines, especially *Pinus patula*, in South African nurseries. In this work the effect of three *Trichoderma* isolates, *Trichoderma harzianum* Eco-T®, and *Trichoderma* Isolates ET23 and ET13, and four *Bacillus* isolates, BFO11, JRO1, B81 and EXR, was investigated on survival of *P. patula* seedlings. The effect of time of application of *T. harzinum* Eco-T® on survival of seedlings was also determined. Initially (4-8wk), application of the biological control agents improved survival of seedlings compared to the *F. circinatum* only treated control. However, after 12wk they no longer effectively reduced mortality of seedlings. Prior colonization and establishment of *T. harzianum* Eco-T® significantly improved disease control. There was a direct relationship between time of application of *T. harzianum* Eco-T® and seedling survival. Although there was significant reduction in disease incidence, owing to the virulent nature of the pathogen, the level of disease observed after 8wk was still high.

6.1   Introduction

Pathogenic species of *Fusarium* have worldwide distribution and are well-known pathogens in forest nurseries in many parts of the world (Bloomberg, 1981; Viljoen *et al.*, 1992). This group of fungi cause diseases such as seed decay, damping-off, root rot, and

stem cankers (Rathbun, 1922; Pawuk, 1978; Barnard & Blakeslee, 1980; Sutherland & van Eeden, 1980; Morgan, 1983; Huang & Kuhlman, 1990). *Fusarium* spp. are particularly damaging to conifer seedlings such as Douglas-fir and pine (Bloomberg, 1981; Huang & Kuhlman, 1990; James *et al.*, 1991) and are mainly associated with the root diseases.

Pitch canker of pine, caused by *Fusarium circinatum* Nirenberg and O'Donnell (=*F. subglutinans* (Wollenw. and Reinking) Nelson *et al.* f. sp. *pini* Correll *et al.*), was first reported in southeastern United States in 1946 (Hepting & Roth, 1946 *cited by* Gordon *et al.*, 2001), where it remains a chronic problem in plantations, seed orchards, and nurseries (Dwinell *et al.*, 1985). The disease was reported from California in 1986 (McCain *et al.*, 1987 *cited by* Gordon *et al.*, 1998), mainly from *Pinus radiata* D. Don planted in landscape settings (Correll *et al.*, 1991) and has since been reported in Mexico (Rodriquez, 1989 *cited by* Gordon & Okamoto, 1998), Japan (Muramoto & Dwinell, 1990) and South Africa (Viljoen *et al.*, 1994).

Currently there are no effective cultural, chemical or resistant varieties available to control this disease. Cultural methods have generally not been effective. Soil amendments which showed promise in controlling certain *Fusarium* diseases of agricultural crops have not yet proved to be adequately effective in the forest nursery (Smith, 1975). Three of the most important commercial species grown in South Africa are *P. patula* Schlechtend. & Cham. (45%), native to Mexico, and *P. elliottii* Englem. (27%) and *P. radiata* (9%), naturally occurring in the southern United States and California, respectively (Critchfield & Little, 1966; Hinze, 1993). These three species constitute 80% of all pines grown in South Africa and almost half of the total commercial forest investment (Hinze, 1993). However, Viljoen *et al.* (1995) have shown that *P. patula* and *P. radiata* are highly susceptible to the pitch canker pathogen, whereas *P. elliottii* is only moderately susceptible. Therefore, there is an urgent need to search for an effective and environmentally and ecologically sound control strategy. One of these strategies is the use natural enemies of the pathogen, which is an essential alternative to control the disease and promote ecological sustainability.

There are several reports on the control of *Fusarium* spp. by biological means using *Trichoderma* or *Bacillus* spp. (Marois *et al.*, 1981; Lock *et al.*, 1985; Sivan & Chet, 1985; Sivan *et al.*, 1987; Zhang *et al.*, 1996; Bacon *et al.*, 2001). Though there are very few reports on biological control of *Fusarium* pitch canker of pine by *Trichoderma* or *Bacillus*

spp. (Mitchell *et al.*, 2004) some researchers, however, have documented the use of ectomycorrhizal fungi to prevent *Fusarium* root rot of pine seedlings (Marx, 1969, 1972; Sinclair *et al.*, 1975, 1982; Chakravarty & Unestam, 1987a, 1987b; Farquhar & Peterson, 1991).

The objectives of this study were to evaluate the activity of the biological control agents, *Trichoderma* and *Bacillus* isolates, which showed relatively better performance against *F. oxysporum* f. sp. *conglutinans* (Wollenweb.) W.C. Snyder & H.N. Hansen on cabbage, and to determine the effect of time of inoculation of the biological control agent, *T. harzianum* Eco-T[®] Rifai, for the control of the pitch canker fungus.

6.2   Materials and Methods

6.2.1   Source of Plant Material[1]

Pine seedlings, *P. patula,* ranging in age from less than 2mo to 5mo were obtained from Sunshine Seedlings[2], Top Crop Nursery[3] and SAPPI Forest Products[4].

6.2.2   Microorganisms

Pathogen

Fusarium circinatum 3579 was provided by T.A. Coutinho[5]. The pathogen was preserved on silica gel at 4°C (Perkins, 1962; Windels *et al.*, 1988).

Biocontrol Agents

Three *Trichoderma* isolates, *T. harzianum* Eco-T[®6] and *Trichoderma* Isolates ET23 and ET13, and four *Bacillus* isolates, BFO11[7], JRO1[7], B81[8] and EX-R[9], which were previously

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[1] *P. patula* seedlings were obtained from the different seedling nurseries on the basis of availability.
[2] Sunshine Seedling Service, Scottsville 3209, Republic of South Africa
[3] Top Crop Nursery, P.O.Box 32, Cramond, 3220, Republic of South Africa.
[4] SAPPI Forest Products, Pietermaritzburg, Republic of South Africa.
[5] Prof. T.A. Coutinho, Department of Microbiology and Plant Pathology, Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, Pretoria, 0002.
[6] Plant Health Products (Pty) Ltd, P.O.Box. 207, Nottingham Road, Republic of South Africa.

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screened against *F. oxysporum* f. sp. *conglutinans* on cabbage (Chapter Two and Three) were used.

6.2.3 Preparation of Pathogen Inocula

Fusarium circinatum stored on silica gel was cultured onto Oat Meal Agar (OMA) and incubated at 25°C for 10-14d in the dark. Inoculum, consisting of both conidia and mycelia was obtained by flooding the agar plate with 15ml of sterile distilled water. Inoculum was counted using a Neubauer Improved Double Haemocytometer and adjusted to a concentration of 5×10^6 conidia ml^{-1} to cause satisfactory infection.

6.2.4 Preparation of Inocula of Biocontrol Agents

Trichoderma *Isolates*

A conidial suspension of the *Trichoderma* isolates was prepared from conidia collected from cultures grown on V8 agar medium. V8 agar plates were inoculated with agar blocks from half strength Potato Dextrose Agar (PDA) (MERCK) plates kept at 4°C and incubated for 7d at 25°C. Then 5ml sterile distilled water was pipetted into plates and conidia were collected by scraping the plate with cotton wool-tipped and curved sterile Pasteur pipette. The conidial suspensions of the *Trichoderma* isolates were then counted using a Neubauer Improved Double Haemocytometer and adjusted to 10^7 conidia ml^{-1}.

Bacillus *Isolates*

The four *Bacillus* isolates were grown in 250ml Erlenmeyer flasks each containing a 100ml Nutrient Broth (NB) (MERCK) for 4d at 30°C in a water bath shaker (Gesellschaft für Labortechnik, mbH, D-30938, Burgwedel, Germany) at 150 oscillations min^{-1}. Flasks were inoculated with the *Bacillus* isolates previously grown in Tryptone Soy Agar (TSA) (MERCK) for 48h. After 4d the *Bacillus* isolates were harvested by centrifugation in a Beckman J2-HS Centrifuge at 9000×g for 15min. The broth was decanted and bacterial pellets were resuspended in sterile distilled water. The bacterial cells were then counted

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using a plate dilution technique in a TSA medium and adjusted to a concentration of 10^7 c.f.u. ml^{-1} of water.

6.2.5   Biocontrol Evaluation

6.2.5.1 Biocontrol using *Trichoderma* and *Bacillus* Isolates

Both the biocontrol agents and the pathogen were applied as spore suspensions. Each pine seedling in a Speedling® 24 tray was treated with 4ml of a suspension of *Trichoderma* or *Bacillus* with a concentration of 10^7conidia ml^{-1} or 10^7 c.f.u. ml^{-1}, respectively, and 4ml of $5{\times}10^6$ conidia ml^{-1} of *F. circinatum*.

The experiment was repeated three times in which two of the three experiments were conducted with 3mo old seedlings obtained from Top Crop Nursery[10] while the third experiment was conducted with 5mo old seedlings obtained from SAPPI Nursery[11].

6.2.5.2   Application of *Trichoderma harzianum* Eco-T® Concurrent or Prior to Inoculation with *Fusarium circinatum*

The capacity of *T. harzianum* Eco-T®, registered for biocontrol of greenhouse grown plants, was examined over a time series when *F. circinatum* was inoculated. Seven treatments were used arranged in RCBD, replicated five times with each plot containing 24 seedlings. The treatments were set up as follows:

(i)   both the antagonist and the pathogen applied at the same time;

(ii)   pathogen applied 1d after the antagonist;

(iii)   pathogen applied 2d after the antagonist;

(iv)   pathogen applied 3d after the antagonist;

(v)   pathogen applied 4d after the antagonist;

(vi)   untreated control, to which no addition was made; and

(vii)   pathogen control, which was inoculated with spore suspensions of *F. circinatum* only.

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[10] Top Crop Nursery, Crammond, Republic of South Africa.
[11] SAPPI Nursery, Richmond, Republic of South Africa.

In this experiments less than 2mo old young seedlings obtained from Sunshine Seedlings were used. Each of the 24 pine seedlings in a plot was treated with 4ml of a suspension of *T. harzianum* Eco-T® with a concentration of 10^7 conidia ml^{-1} and/or 5×10^7 conidia ml^{-1} of *F. circinatum*.

6.2.6 Sterilization of Speedling® 24 Trays

Since the Speedling® 24 trays were previously being used by other researchers sterilization of the trays was necessary to eliminate the effect of other microbes. They were sterilized by dipping the trays in Plaz-Dip® and immediately air-dried. Plaz-Dip® is a 12% copper hydroxide suspension in PVA paint.

6.2.7 Plant Growing Medium

Pine seedlings were grown in a composted pine bark medium. It was used as a sole plant-growing medium for the entire study. Since composted pine bark is nutritionally poor and has poor water holding capacity irrigation was provided three times a day for 5min supplemented with NPK soluble fertilizer [3:1:3(38)].

6.2.8 Controls

On the biocontrol experiments there were two control treatments:
1. neither antagonist nor pathogen
2. pathogen only

6.2.9 Disease Assessment

Infected seedlings all appeared to show symptoms of disease exhibiting stem and needle discoloration (purpling/browning), early symptoms of wilt and drooping of the shoot. Unlike healthy plants, infected plants lacked white root tips and fine root hairs when lifted from trays. In advanced stages of the disease, infected plants were completely wilted with resinous material on their stem.

In the first experiment, all three trials were assessed for percentage of diseased plants at 4wk, 8wk and 12wk after application of treatments. While in the second experiment all three trials were assessed for percentage of diseased plans at 4wk and 8wk only for very high level of disease was observed on some of the plots after 8wk.

6.2.10 Statistical Analysis

Experiments were repeated three times, with treatments arranged in RCBD. All data were subjected to analysis of variance (ANOVA) using GenStat® Executable Release 6 Statistical Analysis Software to determine differences between treatment means. Percentage diseased seedlings was calculated on a per plot basis and transformed using angular transformation prior to analysis. All least significant differences were determined at P<0.05.

6.3   Results

6.3.1   Biocontrol using *Trichoderma* and *Bacillus* Isolates

In all three trials, application of biocontrol organisms yielded highly significant (P<0.001) suppression of disease after 4wk (Table 6.1-6.3; Fig. 6.1-6.3); and all *Bacillus* and *Trichoderma* isolates, except *Trichoderma* Isolate ET23 in Trial 2 (Table 6.2; Fig. 6.2), significantly increased survival of seedlings compared to the control inoculated with *F. circinatum* only. Eight weeks later in Trial 3 (Table 6.3; Fig. 6.3) all biocontrol organisms provided significant disease protection. However, all biocontrol organisms except *Bacillus* Isolate JRO1 and *Trichoderma* Isolate ET23 in Trial 1 and *Bacillus* Isolates EXR and BFO11 and *Trichoderma* Isolates ET23 and *T. harzianum* Eco-T® in Trial 2, significantly increased percentage of survival of seedlings compared to the control inoculated with the pathogen only (Table 6.1, 6.2; Fig. 6.1, 6.2). With the exception of *T. harzianum* Eco-T® in both Trial 1 and Trial 2 (Table 6.1, 6.2; Fig. 6.1, 6.2) and *Bacillus* Isolates B81 and JRO1 in Trial 2 (Table 6.2; Fig. 6.2), none of the other biocontrol agents significantly reduced percentage of diseased plants 12wk later (Table 6.1-6.3; Fig. 6.1, 6.3).

Although percentage of diseased plants was not significantly lower than the control plot, dry weights taken at the end of the experimental time (12wk) appeared to be significantly higher for most of the plots treated with biocontrol agents in Trials 1 and 2 (Table 6.1, 6.2; Fig. 6.1, 6.2). However, in Trial 3 none of the biocontrol agents significantly increased plot dry weight compared to the *F. circinatum* treated control (Table 6.3; Fig 6.3).

Table 6.1   Biocontrol of *Fusarium circinatum* using *Bacillus* and *Trichoderma* isolates on 3mo old *Pinus patula* seedlings, Trial 1

Biocontrol Isolates	Disease (%)			Plot Dry Weight (g)
	4wk	**8wk**	**12wk**	
B81	23(28)^{bc}	58(50)^{b}	93(76)^{bc}	11.67^{c}
BFO11	25(30)^{bc}	62(52)^{bc}	91(75)^{bc}	9.53^{ab}
EXR	25(30)^{bc}	66(54)^{bcd}	93(77)^{c}	9.44^{ab}
JRO1	35(36)^{c}	77(62)^{de}	91(75)^{bc}	9.59^{ab}
Eco-T®	18(25)^{b}	52(46)^{b}	80(64)^{b}	12.24^{bc}
ET13	27(30)^{bc}	64(53)^{bcd}	89(71)^{bc}	12.84^{cd}
ET23	34(36)^{c}	74(60)^{cde}	90(72)^{bc}	10.78^{bc}
F. circinatum only	54(47)^{d}	80(64)^{e}	96(82)^{c}	7.36^{a}
Water only	0(6)^{a}	0(6)^{a}	6(12)^{a}	15.55^{d}
F test	(<0.001)	(<0.001)	(<0.001)	<0.001
l.s.d.	(8.9)	(9.4)	(12.5)	2.924
s.e.d.	(4.3)	(4.5)	(6.1)	1.417
cv%	(20.5)	(12.9)	(12.8)	18.2

-Means with the same letter in the same experiment are not significantly different at $P<0.05$.

-Values in parenthesis represent transformed means using angular transformation.
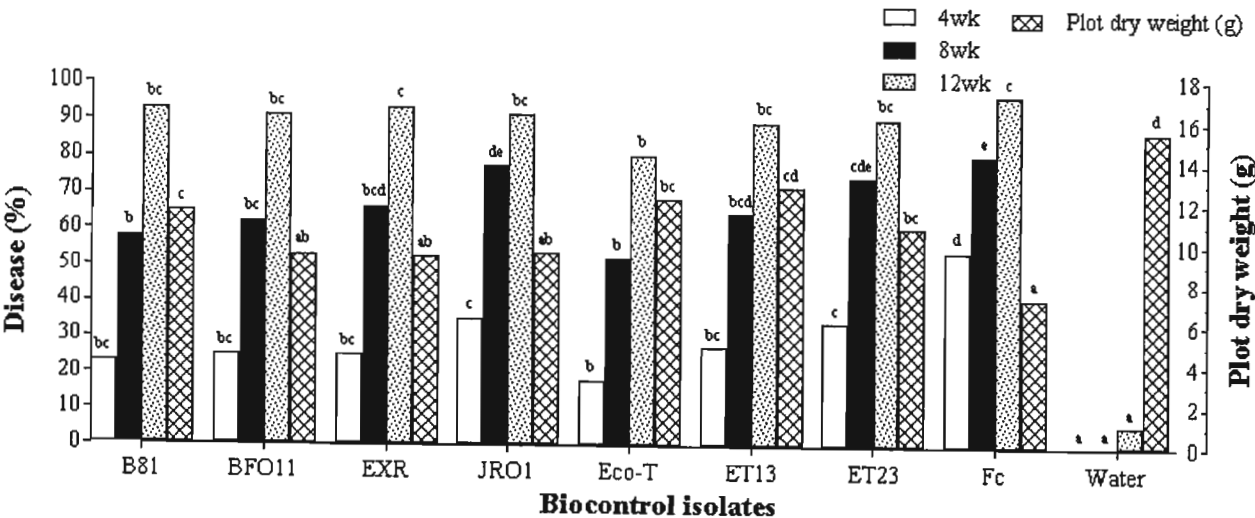
*Graph for disease (%) was drawn using untransformed data while the letters were given based on the transformed data.
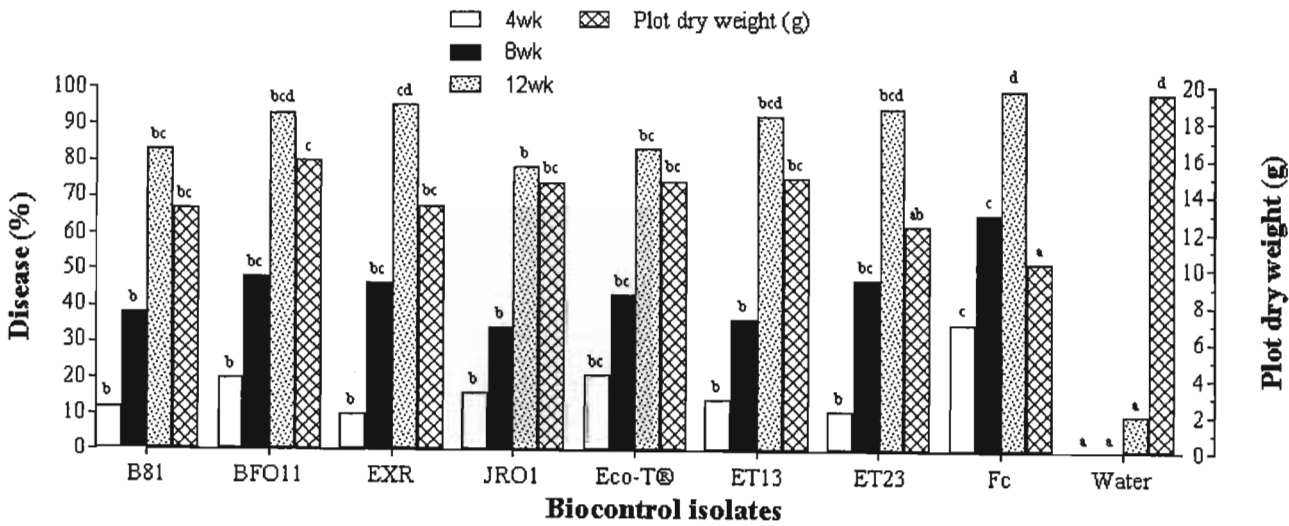
Fig. 6.1   Biocontrol of *Fusarium circinatum* using *Bacillus* and *Trichoderma* isolates on 3mo old *Pinus patula* seedlings, Trial 1.

Table 6.2 Biocontrol of *Fusarium circinatum* using *Bacillus* and *Trichoderma* isolates on 3mo old *Pinus patula* seedlings, Trial 2

Biocontrol Isolates	Disease (%)			Plot Dry Weight
	4wk	8wk	12wk	(g)
B81	12(19)^b	38(37)^b	83(67)^{bc}	13.35^{bc}
BFO11	20(26)^b	48(44)^{bc}	93(79)^{bcd}	15.96^c
EXR	14(21)^b	46(43)^{bc}	95(81)^{cd}	13.49^{bc}
JRO1	11(18)^b	34(35)^b	78(64)^b	14.74^{bc}
Eco-T®	10(18)	43(41)^{bc}	83(67)^{bc}	14.85^{bc}
ET13	16(23)^b	36(37)^b	92(75)^{bcd}	14.99^{bc}
ET23	21(27)^{bc}	47(43)^{bc}	94(78)^{bcd}	12.38^{ab}
F. circinatum only	35(36)^c	65(54)^c	99(87)^d	10.32^a
Water only	0(6)^a	0(6)	10(16)^a	19.58^d
F test	(<0.001)	(<0.001)	(<0.001)	<0.001
l.s.d.	(9.0)	(14.9)	(15.2)	2.798
s.e.d.	(4.3)	(7.2)	(7.3)	1.356
cv%	(28.3)	(27.1)	(15.3)	13.3

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

*Graph for disease (%) was drawn using untransformed data while the letters were given based on transformed data.
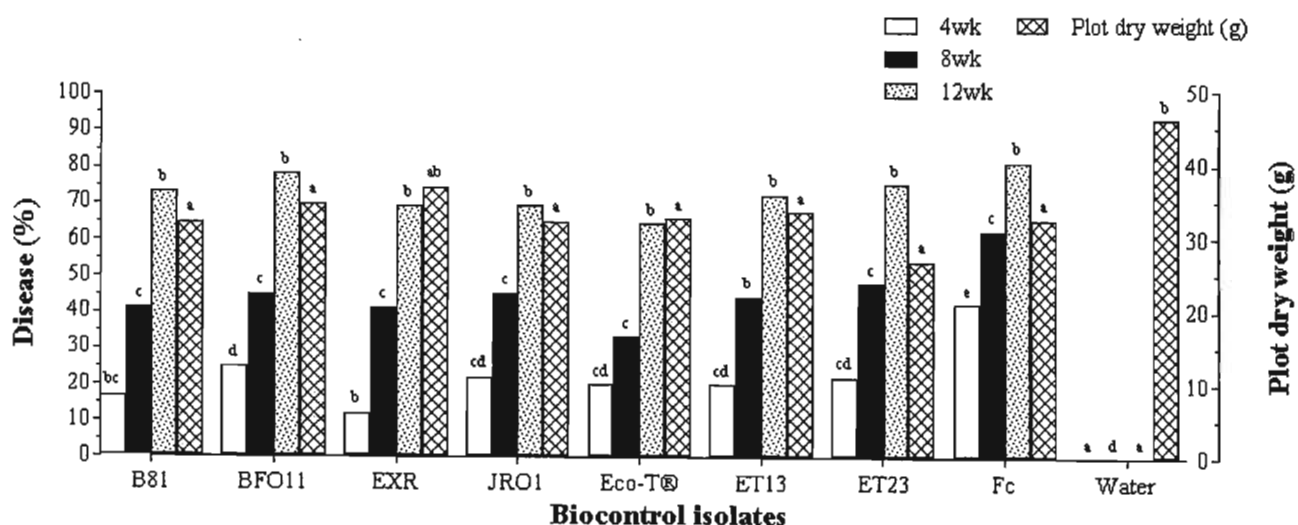
Fig. 6.2 Biocontrol of *Fusarium circinatum* using *Bacillus* and *Trichoderma* isolates on 3mo old *Pinus patula* seedlings, Trial 2

Table 6.3 Biocontrol of *Fusarium circinatum* using *Bacillus* and *Trichoderma* isolates on 5mo old *Pinus patula* seedlings, Trial 3

Biocontrol Isolates	Disease (%)			Plot Dry Weight (g)
	4wk	8wk	12wk	
B81	$17(24)^{bc}$	$41(40)^{c}$	$73(59)^{b}$	32.40^{a}
BFO11	$25(30)^{d}$	$45(42)^{c}$	$78(63)^{b}$	34.81^{a}
EXR	$20(27)^{cd}$	$41(40)^{c}$	$69(56)^{b}$	37.07^{ab}
JRO1	$22(28)^{cd}$	$45(42)^{c}$	$69(56)^{b}$	32.41^{a}
Eco-T®	$12(20)^{b}$	$33(35)^{b}$	$64(53)^{b}$	32.74^{a}
ET13	$22(28)^{cd}$	$44(41)^{c}$	$72(58)^{b}$	33.68^{a}
ET23	$20(27)^{cd}$	$48(44)^{c}$	$75(64)^{b}$	26.96^{a}
F. circinatum only	$42(40)^{e}$	$62(52)^{d}$	$81(64)^{b}$	32.62^{a}
Water only	$0(7)^{a}$	$0(7)^{a}$	$0(7)^{a}$	46.27^{b}
F test	(<0.001)	(<0.001)	(<0.001)	<0.001
l.s.d.	(5.2)	(4.5)	(11.0)	10.642
s.e.d.	(2.5)	(2.2)	(5.3)	5.156
cv%	(13.8)	(8.1)	(14.1)	21.2

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

*Graph for disease (%) drawn using untransformed data while the letters were given based on the transformed data.

Fig. 6.3 Biocontrol of *Fusarium circinatum* using *Bacillus* and *Trichoderma* isolates on 5mo old *Pinus patula* seedlings, Trial 3

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6.3.2 Application of *Trichoderma harzianum* Eco-T® Concurrent or Prior to Inoculation with *Fusarium circinatum*

Application of the biocontrol agent, *T. harzianum* Eco-T®, suppressed disease incidence for all inoculation times 4wk later compared to the control inoculated with *F. circinatum* only (Table 6.4-6.6). Although there was a slight decrease in the percentage of diseased plants with an increase in prior inoculation time of *T. harzianum* Eco-T® (Fig. 6.1-6.6), there was no significant difference between application at the same time, 1d, 2d or 3d prior to inoculation with *F. circinatum*. Application of the biocontrol agent 4d prior to inoculation with the pathogen significantly improved disease suppression as compared to concurrent, 1d, 2d or 3d in all three trials, except in Trial 1 (Table 6.4) as compared to 3d. A significantly high percentage of diseased plants was observed in the control treated with the pathogen only, ranging from 51 to 59% as compared to the untreated control where no disease was observed after 4wk.

After 8wk, application of *T. harzianum* Eco-T® 1d, 2d, 3d and 4d, and 2d, 3d and 4d significantly reduced the percentage of diseased plants compared to the control treated with the pathogen only in Trials 1 and 2 (Table 6.4; 6.5), and Trial 3 (Table 6.6), respectively. However, application of the antagonist together with the pathogen caused no significant reduction in disease level relative to the control treated with pathogen only. One percent of disease was observed in the control, where neither *F. circinatum* nor *T. harzianum* Eco-T® was inoculated, in all trials. This was probably caused by inoculum moved by water splash from neighbouring plots or by fungus gnats.

Plot dry weight taken at the end of the experiment was significantly higher in all three trials only when *T. harzianum* Eco-T® was applied 4d prior to *F. circinatum* (Table 6.4-6.6). Application of *T. harzianum* Eco-T® 3d before *F. circinatum* also resulted in significant difference in plot dry weight in Trials 2 and 3 (Table 6.5; 6.6) but not in Trial 1 (Table 6.4).

Table 6.4 Application of *Trichoderma harzianum* Eco-T® concurrent or prior to inoculation with *Fusarium circinatum* (*Fc*), Trial 1

Delay in *Fc* Application	Disease (%)		Plot Dry Weight (g)
	4wk	**8wk**	
0d	37(37)[c]	73(59)[de]	2.69[a]
1d	33(35)[c]	68(55)[cd]	2.91[ab]
2d	32(34)[c]	62(52)[c]	3.18[ab]
3d	30(33)[c]	62(52)[c]	3.57[b]
4d	16(24)[b]	50(45)[b]	4.67[c]
F. circinatum only	59(50)[d]	81(65)[e]	2.55[a]
Water only	0(6)[a]	1(7)[a]	5.68[d]
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(4.8)	(5.5)	0.828
s.e.d.	(2.3)	(2.7)	0.401
cv%	(11.6)	(8.8)	17.6

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.
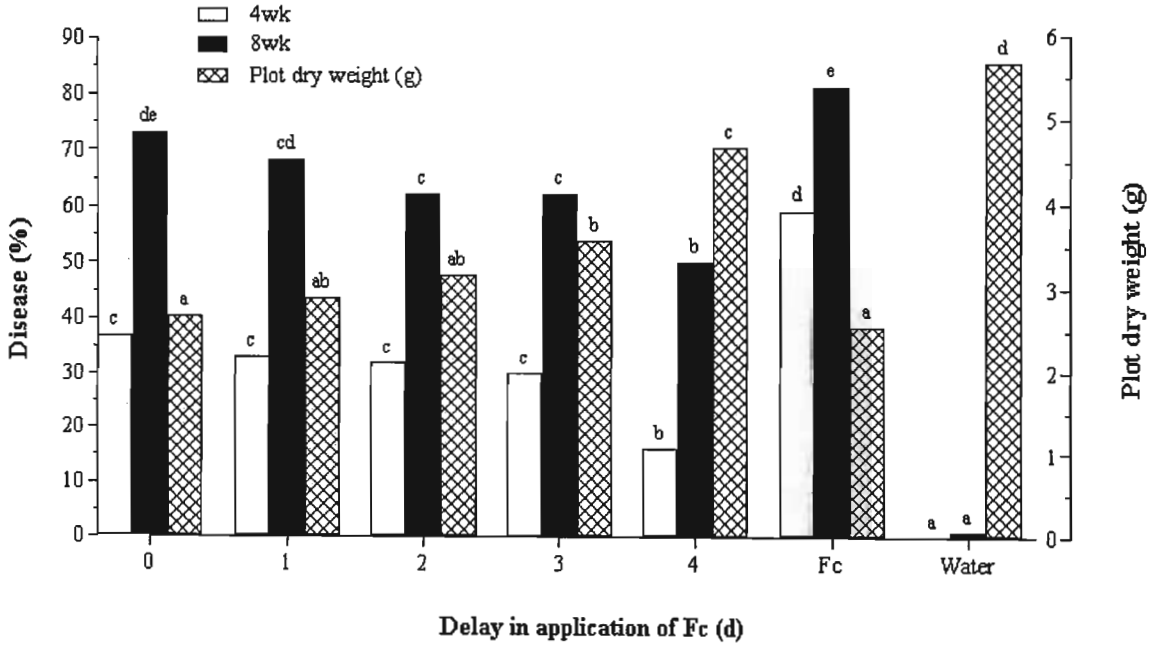
*Graph for disease (%) was drawn using untransformed data while the letters were given based on transformed data.
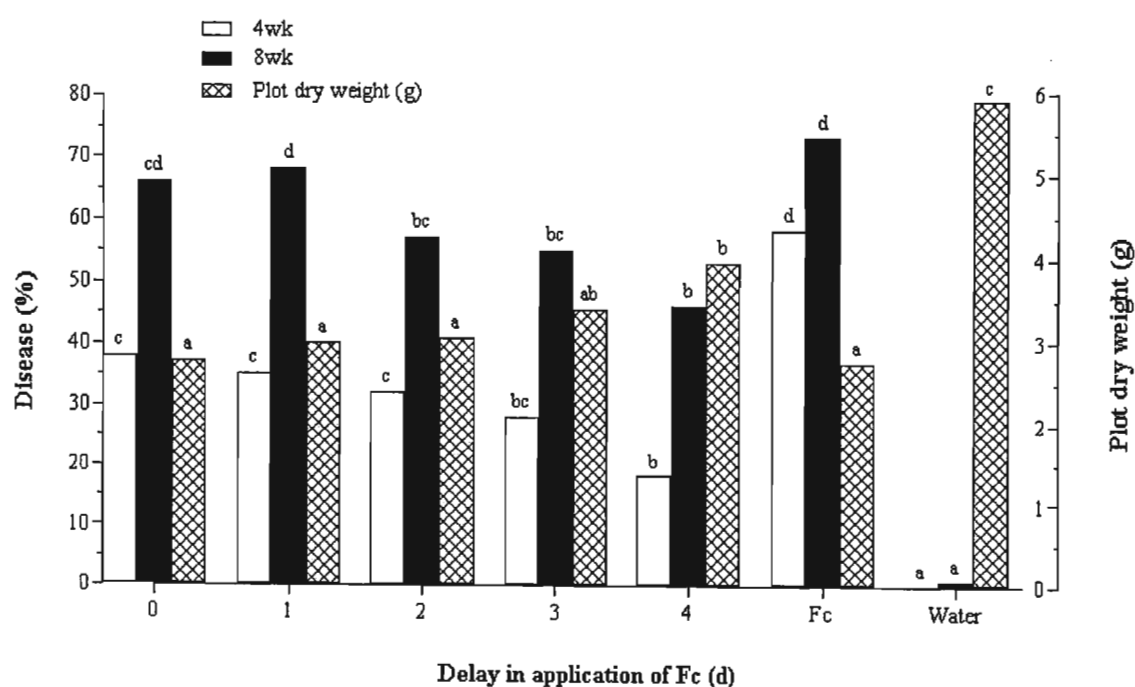
Fig. 6.4 Application of *Trichoderma harzianum* Eco-T® concurrent or prior to inoculation with *Fusarium circinatum* (*Fc*), Trial 1

Table 6.5   Application of *Trichoderma harzianum* Eco-T® concurrent or prior to inoculation with *Fusarium circinatum* (*Fc*), Trial 2

Delay in *Fc* Application	Disease (%)		Plot Dry Weight (g)
	4wk	**8wk**	
0d	38(38)^c	66(54)^{cd}	2.78^a
1d	35(36)^c	68(56)^d	3.00^a
2d	32(34)^c	57(49)^{bc}	3.05^a
3d	28(32)^{bc}	55(48)^{bc}	3.40^{ab}
4d	18(25)^b	46(43)^b	3.96^b
F. circinatum only	58(49)^d	73(59)^d	2.74^a
Water only	0(6)^a	1(7)^a	5.90^c
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(6.6)	(5.6)	0.746
s.e.d.	(3.2)	(2.7)	0.361
cv%	(16.1)	(9.6)	16.1

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

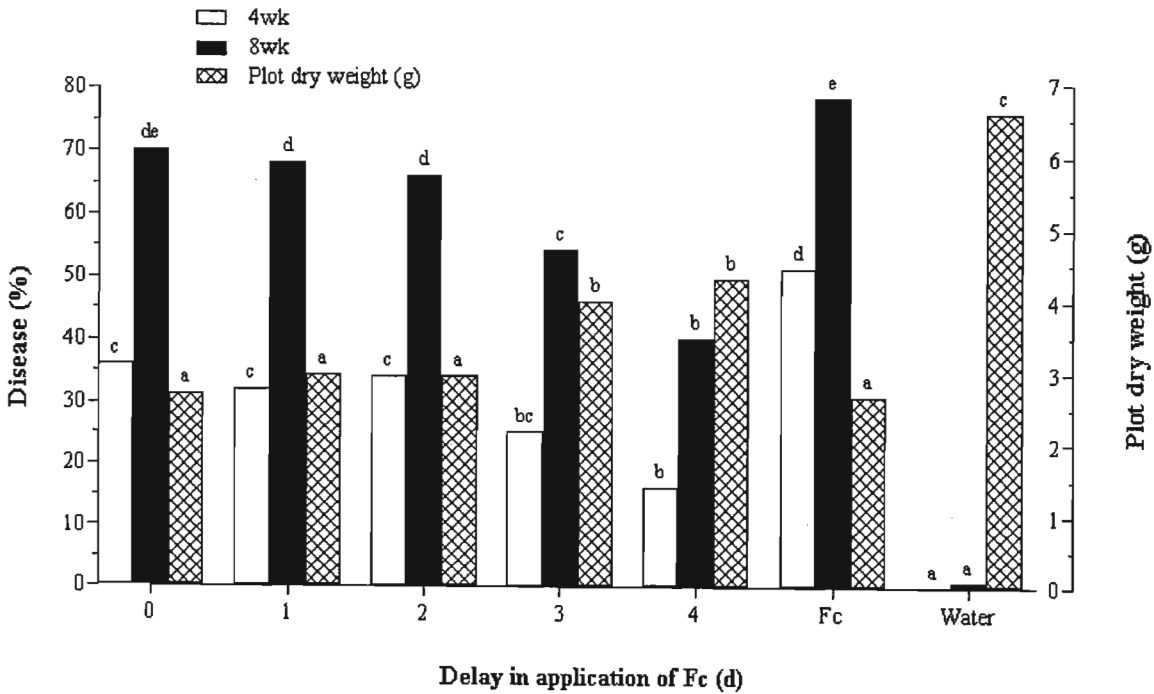*Graph for disease (%) was drawn using untransformed data.

Fig. 6.5   Application of *Trichoderma harzianum* Eco-T® concurrent or prior to inoculation with *Fusarium circinatum* (*Fc*), Trial 2

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Table 6.6 Application of *Trichoderma harzianum* Eco-T® concurrent or prior to inoculation with *Fusarium circinatum* (*Fc*), Trial 3

Delay in *Fc* Application	Disease (%) 4wk	Disease (%) 8wk	Plot Dry Weight (g)
0d	36(36)^c	70(57)^{de}	2.73^a
1d	32(35)^c	68(55)^d	3.00^a
2d	34(35)^c	66(55)^d	2.99^a
3d	25(30)^{bc}	54(47)^c	4.01^b
4d	16(24)^b	40(39)^b	4.33^b
F. circinatum only	51(45)^d	78(62)^e	2.69^a
Water only	0(6)^a	1(7)^a	6.60^c
F test	(<0.001)	(<0.001)	<0.001
l.s.d.	(6.1)	(4.9)	0.953
s.e.d.	(2.9)	(2.4)	0.462
cv%	(15.4)	(8.2)	19.4

-Means with the same letter in the same experiment are not significantly different at P<0.05.

-Values in parenthesis represent transformed means using angular transformation.

*Graph for disease (%) was drawn using untransformed data while letters were given based on the transformed data.

Fig. 6.6 Application of *Trichoderma harzianum* Eco-T® concurrent or prior to inoculation with *Fusarium circinatum* (*Fc*), Trial 3

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6.4 Discussion

6.4.1 Biocontrol using *Trichoderma* and *Bacillus* Isolates

The initial response to the application of both the fungal and bacterial biocontrol isolates was encouraging. This shows that survival of seedlings was positively influenced by the application of the biocontrol organisms. However, as time went by, most of the biocontrol isolates became less effective. After 8wk the percentage of control gained by the application of these biocontrol agents was disappointingly low, though statistically significant compared to the control treated with *F. circinatum* only.

Effective and efficient disease control using biological means relies on a number of factors. One of these factors that may grossly contribute to the efficacy of the biocontrol isolates used is the method of selection of the biocontrol microorganisms (Campbell, 1994). This may include the size of screen used and selection based on known mode of action.

In the initial steps of the whole project several *Trichoderma* and *Bacillus* isolates obtained from different sites were screened *in vitro* only against *F. oxysporum* f. sp. *conglutinans* on cabbage to minimize the cost and time incurred for screening such a large number of isolates. Although there is little information in the literature on the size of screens used in biocontrol studies, the number of isolates used in this project is considered rather small (Campbell, 1994). Powell & Faull (1989) estimated that only 5% of biocontrol agents (of all types) actually work in field trials. Therefore, when small numbers of isolates are used in the initial screening stage there is a chance of missing effective and efficient biocontrol microorganisms. However, *T. harzianum* Eco-T®, which caused better control than the other biocontrol isolates, was selected as of greater that 1000 isolates (M.D. Laing, 2004, pers. Comm.[12]) and was found effective against *Pythium* and *Rhizoctonia* spp.

Effective biocontrol microorganisms may also be missed when they are screened based on known mode of action such as antibiosis and mycoparasitism *in vitro*. Different biocontrol microorganisms exhibit different mechanisms of control, some of which may not be manifested *in vitro* though contribute much to the suppression of disease *in vivo*.

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Therefore, screening for potential biocontrol microorganism should be conducted *in vivo* in a condition which mimics the natural environment where the plants to be protected are grown. The mode of action of the biocontrol organism may be studied later after it is found to be effective against the pathogens tested. However, the use of a large number of different biocontrol organisms tested under various field conditions against different pathogens and host plants would be more complicated and lengthen the screening process. However, the use of a reasonable number of biocontrol isolates and testing them under natural conditions against different pathogens and host plants would pay back more than when laboratory conditions are used.

6.4.2 Application of *Trichoderma harzianum* Concurrent or Prior to Application of *Fusarium circinatum*

Root colonization and establishment by introduced biocontrol agents in the rhizosphere is essential for biocontrol of root pathogens. Increased population of the agents on the root should enhance disease control (Suslow & Schroth, 1982). Nutrients, rather than space, are thought to be limiting factors in competition in the rhizosphere colonization. Nutrients are believed to be most abundant in grooves between cells (Weller, 1983). Therefore, microorganisms (both pathogens and antagonists) compete for the colonization of these nutrient-rich sites. However, since the carrying capacity of the rhizosphere is limited, an introduced strain must displace the already established indigenous microorganisms if it is to be established and change the composition of the population (Bowen & Rovira, 1976; Weller, 1988). Prior root colonization and establishment promotes population increase and may in turn increase the combined effect of other mechanisms of control applied by the antagonist besides substrate competition and niche exclusion.

In this experiment prior establishment and colonization of nutrient-rich and potential infection courts for the pathogen by the antagonist, *T. harzianum* Eco-T®, resulted in significant decreases in the percentage of diseased plants. A clear trend was for control of disease correlated with increase in the time given to the antagonist to establish itself in the rhizosphere of the *P. patula* seedlings. This highlights the importance of prior establishment and colonization of the root area by the antagonist for effective disease control. Barrows-Broaddus & Dwinell (1985) reported that slash pine wounds treated with *Arthrobacter* Isolate A6 1d before inoculation were not significantly different from the

wounds inoculated with *F. moniliforme* Sheld. var. *subglutinans* Wollenw. & Reink. alone. However, wounds sprayed with *Arthrobacter* Isolate A6 7d or more before inoculation with *F. moniliforme* var. *subglutinans* significantly reduced disease and the number of conidia of the pitch canker fungus recovered from the wounds.

The results indicate that rhizosphere colonization and establishment are directly related to rhizosphere competence and time. A rhizosphere competent and fast growing antagonist is more likely to be an effective biocontrol agent. *Trichoderma harzianum* Eco-T$^®$ was by far the most effective when applied 4d prior to inoculation compared to other times of application. Although *F. circinatum* generally grows more slowly than *Trichoderma* spp., it overwinters as thick mycelial structures which are likely to be more active and fast growing compared to conidia when conditions become conducive for growth to resume. Therefore, in nurseries *Trichoderma* sp. applied as a conidial suspension may need a relatively long time to establish itself. This could provide a chance for the overwintering hyphae of the pathogen to colonize the infection courts on roots. It has been reported that formulations containing young, actively growing hyphae of isolates of *Trichoderma* spp. and *Gliocladium virens* J.H. Miller, J.E. Giddens, A.A. Foster, & von Arx added to soil and soilless mix attacked *Rhizoctonia solani* Kühn structures without being inhibited by soil fungistasis, as are conidia (Lewis & Papavizas, 1987; Lewis *et al.*, 1998).

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CHAPTER SEVEN

GENERAL OVERVIEW

Modern biological control, appropriately applied and monitored, is an environmentally safe and desirable form of long term management of pest species (Melland, 1992). At present, there are over 80 products for biological control of plant pathogens worldwide (Whipps & Davies, 2000), a significant improvement over the past decade. Most of these products are formulations either of the fungi *Gliocladium-Trichoderma* or the bacteria *Pseudomonas* and *Bacillus* (Paulitz & Bélanger, 2001).

Much of the research in this thesis focused on isolation and screening of biocontrol microorganisms against different pathogens on different crops, and improving their efficacy by using mixtures, and optimizing the timing of application prior to introduction of the pathogen. The research has confirmed the following:

1. Isolation of biocontrol organisms from the rhizosphere of the crop to be tested against is more likely to be effective.

2. The number of biocontrol microorganisms to be screened is an important factor that determines the success of finding effective ones.

3. Most of the biocontrol microorganism tested in the greenhouse significantly reduced percentage of disease incidence and severity of the cabbage yellows fungus.

4. Only *Trichoderma harzianum* Rifai Eco-T® and *Trichoderma* Isolate ET23 significantly reduced preemergence damping-off caused by *Rhizoctonia solani* Kühn on maize.

5. Application of biocontrol agents significantly reduced mortality of *Pinus patula* Schlechtend. & Cham. seedlings initially, although at later stages, they were no longer effective.

6. Earliness of application of *T. harzianum* Eco-T® against *Fusarium circinatum* Nirenberg and O'Donnell (=*F. subglutinans* (Wollenw. and Reinking) directly impacted upon the level of survival of *P. patula* seedlings. Prior colonization of

pine seedling roots by Isolate Eco-T® is important as post-infection control of *F. circinatum* was poor.

7.   None of the tested *Trichoderma* isolates were compatible with any of the *Bacillus* isolates. *Bacillus* Isolates B81 and BFO11 inhibited growth of *Bacillus* Isolates EXR and JRO1.

8.   Mixtures of *Bacillus* or *Trichoderma* isolates did not cause significantly better performance compared to when they were applied singly.

7.1   Isolation and Screening of *Trichoderma* and *Bacillus* Isolates as Potential Biocontrol Microorganisms

With development of selective media, isolation of *Trichoderma* spp. is now relatively easy (Askew & Laing, 1994). Similarly, *Bacillus* spp., unlike most bacteria, produce heat resistant endospores which make them easier to separate from other vegetatively growing bacteria or other less heat resistant microorganisms by heat treatment and plating them on agar media suitable for their isolation such as casein hydrolysate plus glucose.

However, although isolation of these microorganisms is simple, finding isolates which have the desired characteristics is not an easy task and involves chance. The chance of selecting an effective strain may be improved initially by first isolating the biocontrol microorganisms from the same environment in which they will be used (Weller, 1988). For instance, rhizosphere bacteria with the ability to provide biological control appear to comprise less than 10% of the total population of bacteria in the rhizosphere (Schroth & Hancock, 1981; Schroth & Hancock, 1982; Suslow & Schroth, 1982; Weller & Cook, 1986). In our case all *Trichoderma* and *Bacillus* isolates were isolated from the root area of the different test plants. This provided an opportunity to select for rhizosphere competent microbes.

Another most important stage in the search for effective and efficient biocontrol microorganisms is 'mass screening'. This initial stage of screening is critical for all subsequent stages, and depends on the use of an appropriate selection procedure to identify the right candidate (Whipps *et al.*, 1988; Campbell, 1994).

Screening of biocontrol microorganisms based on their *in vitro* interaction with the test pathogen, *Fusarium oxysporum* f. sp. *conglutinans* (Wollenweb.) W.C. Snyder & H.N. Hansen (*Foc*) was used in the initial stages of selection. *Trichoderma* and *Bacillus* isolates which exhibited antagonism towards the test pathogen were selected for further testing under greenhouse conditions using whole plants. This method of selection has been reported to have poor correlation with field activity of selected antagonists (Papvizas & Lumsden, 1980). However, it has merit when used in conjunction with suitable secondary screens, where it allows economical use of facilities and time (Campbell, 1989) and may contain useful characteristics (useful genes), such as antibiotic production, for later study (Andrews, 1992). Selection based on *in vivo* studies is usually the best method of selection because it involves real plants and field conditions where the biocontrol agents are eventually to be used. However, the main problem with plant tests *in vivo* is that they are more time consuming, and when more than one crop and pathogen are involved, the process becomes more lengthy and complicated.

7.2   Preliminary Selection under Greenhouse Conditions

The use of biocontrol is more prevalent in greenhouses and protected structures than in field crops, even though greenhouses account for only 0.02% of the area used in agriculture (Paulitz & Bélanger, 2001). This is because environmental conditions such as temperature, light, relative humidity and soil composition can be tightly controlled in favor of the biocontrol agent than to the pathogen. *Fusarium oxysporum* f. sp. *conglutinans*, like most *Fusarium* species, is more destructive when there are high temperature and low moisture conditions. Therefore, in our research conditions in the greenhouse were manipulated to favor the pathogen by reducing the irrigation period and creating high temperature conditions, causing stress on the plants to partially mimic natural field condition.

Of all the *Trichoderma* and *Bacillus* isolates tested more than two-third significantly reduced disease incidence and severity compared to the control treated with the pathogen only.

- The percentage of *Trichoderma* isolates which significantly suppressed disease incidence and severity was lower for seed treatments than for drench applications.

- *Trichoderma* isolates applied as seed treatments caused less reduction in disease incidence and severity than when applied by drenching. Quantitative estimation using *Trichoderma* selective media showed that the concentration of conidia coated onto the small cabbage seeds was 10-100 times lower than when applied by drenching although more than 500 times concentrations were used to coat the seeds.

- The percentage of isolates which suppressed disease and the level of control obtained were similar for both *Bacillus* and *Trichoderma* isolates when applied by drenching.

- Application of the biocontrol agents by drenching young cabbage seedlings in nurseries before being transplanted would actually be more effective because seed treatments proved to be relatively ineffective, and field application by drenching would be more difficult.

7.3 Greenhouse Tests of Selected Biocontrol Isolates against *Fusarium* sp. and *Rhizoctonia solani* on maize

Fusarium isolates, obtained from infected kernels and cobs of maize, did not cause any visual symptoms of disease when inoculated onto the roots of maize seedlings. As a result, biocontrol experiments using *Fusarium* diseases of maize was not possible.

Both *Trichoderma* and *Bacillus* isolates selected from preliminary tests using *Foc* on cabbage were tested against *R. solani* on maize applied as seed treatments.

- *Trichoderma* Isolate ET23 and *T. harzianum* Eco-T® significantly reduced preemergence damping-off caused by *R. solani* on maize. However, none of the *Bacillus* isolates significantly suppressed the disease.

7.4 Greenhouse Tests of Selected Biocontrol Isolates against *Fusarium circinatum* on *Pinus patula* seedlings

Both *Trichoderma* and *Bacillus* isolates selected from preliminary tests using *Foc* on cabbage were tested against *F. circinatum* on *P. patula* seedlings applied by drenching.

- Application of most of the *Trichoderma* and *Bacillus* isolates significantly reduced percentage mortality of pine seedlings after 4wk to 8wk. However, the efficacy of control of the biocontrol isolates reduced with time until they were no longer effective.

- *Trichoderma harzianum* Eco-T® reduced disease relatively better as compared to other *Trichoderma* or *Bacillus* isolates, especially in the first 4-8wk.

7.5   Effect of Application Time on the Biocontrol Activity of *Trichoderma harzianum* Eco-T®

Application of biocontrol microorganisms prior to the introduction of the pathogen gives the biocontrol agent the advantage to colonize the infection courts normally used by the pathogen, and consequently improves its disease control efficacy. Our findings on the biocontrol activity of *T. harzianum* Eco-T® applied together with or prior to *F. circinatum* inoculation on *P. patula* seedlings show that:

- The magnitude of disease suppression increased with an increase in prior inoculation time of the biocontrol agent.

- Application of *T. harzianum* Eco-T® 4d before inoculation with the pathogen was superior in increasing seedling survival than the other inoculation times.

- Reduction in seedling infection in the nurseries and mortality in the field can be obtained by seed treatment and timely application by drenching with biocontrol agents.

7.6   Improving Biocontrol using Mixtures of Biocontrol Agents

Introduction of single isolates of *Bacillus* or *Trichoderma* did not produce more than 50% disease reduction. Mixtures of biocontrol agents have been advocated as a means of improving the inconsistent and poor performance of single biocontrol agents in the field (Alabouvette *et al.*, 1979; Guetsky *et al.*, 2001, 2002). However, compatibility of the isolates to be added is critical before any combinations are made. Our research reveals the following facts on the compatibility between and among *Trichoderma* and *Bacillus* isolates:

- All *Bacillus* isolates severely inhibited growth of all *Trichoderma* isolates *in vitro*. *Trichoderma harzianum* Eco-T® was relatively more tolerant to the biochemical activity of the *Bacillus* isolates although it sustained severe inhibition.

- *Trichoderma harzianum* Eco-T® and *Trichoderma* Isolate ET23 colonized the agar plate equally and Environmental Scanning Electron Microscopic (ESEM) pictures show no degradation or lysis of mycelia but thickening of mycelia was observed. However, interaction of these two isolates with Isolate ET13 showed the formation of a thick mycelial mat at the point of contact. Samples taken from the interaction zone and viewed under the ESEM showed lysis of mycelium when Isolate ET13 was cultured with Isolate Eco-T® and Isolate ET23.

- *Bacillus* Isolate EXR was inhibited by all three Bacillus isolates while Isolate JRO1 was slightly inhibited by Isolates B81 and BFO11. Isolates B81 and BFO11 did not inhibit each other.

A certain degree of incompatibility may be tolerated as long as their combination results in better performance than when applied singly. Combinations of *Bacillus* or *Trichoderma* isolates were tested against Fusarium yellows of cabbage in the greenhouse. However, the results obtained from mixtures of *Bacillus* or *Trichoderma* were not significantly different from individual applications of the biocontrol isolates. This may be attributed to:

- Combinations of the biocontrol agents had no synergetic effect. Modes of actions involved by the different isolates were not different or they were not additive (Guetsky *et al.*, 2002).

- The slight antagonism observed *in vitro* might have contributed to the failure to show better performance.

7.7 Overall Conclusion

Results of this research present that *T. harzianum* Eco-T® and Isolate ET23 are promising biocontrol agents against soilborne plant pathogens such as *Foc, R. solani* and *F. circinatum,* although Isolate ET23 showed poor performance against *F. circinatum*. The losses caused by these pathogens can be reduced with the use of these biocontrol agents as part of sustainable and ecologically safe agricultural practices.

The fact that very few of the isolates managed to reduce diseases on the different plants may be attributed either to the procedure of selection used or to the source of isolates obtained. Some effective isolates might have been removed during the process.

Although the results obtained by mixtures of biocontrol agents were not satisfactory, it is logical that compatible biocontrol microorganism with different modes of action may have additive effects that increase the efficacy of disease control (Guetsky *et al.*, 2002).

7.8   Proposed Future Research Priorities

- More scientific knowledge on the sources of biocontrol agents to find more aggressive and effective biocontrol isolates.

- Contriving easier and economically feasible procedures of screening under field conditions, where biocontrol agents are to be eventually used, so that effective biocontrol agents would not be missed. For the successful production of biocontrol agents it is essential that realistic screens are used, with realistic environmental parameters such as temperature, soil microbial populations, and pathogen and host plants present.

- More scientific research on the mechanism of control employed by biocontrol microorganism under field conditions so that compatible and synergetic antagonists could be used in combination to obtained better efficacy of disease suppression.

- Greenhouse and field trials by integrating cultural practices, biocontrol agents, resistant cultivars and chemical fungicides (below lethal doses) are necessary.

7.9   References

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