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Utilisation of rhizosphere microflora in the biocontrol of root rot and growth enhancement of lettuce under hydroponic systems

by

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Declaration

I, the undersigned, hereby declare that the dissertation submitted for the degree of Master of Science, to the University of Pretoria, contains my own independent work. This work has hitherto not been submitted for any degree at any other University or Faculty

Leulseged Begashaw
August 2003

Dedicated to my mother, Belaynesh Addis

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PREFACE

Lettuce (*Lactuca sativa* L.), a member of the sunflower or compositae family is native to the Mediterranean basin (Harris, 1987). It is believed that lettuce was first domesticated along the shores of Egypt and since the 6th century BC this crop was cultivated by Persians. However, the worldwide introduction and cultivation of lettuce was relatively slow compare to other popular crops (Swiader *et al*., 1994). After tomato (*Lycopersicon esculentum* Mill), lettuce is possibly the most important salad vegetable crop (Harris, 1987). The United States ranks second in world production of lettuce after China (Harris, 1987). However, in many parts of the world, lettuce is still a minor crop. In South Africa, this crop has become more popular as production and consumption increases, since it is nutritious and a good source of various vitamins (Niederwieser, 2001).

Lettuce is one of the vegetable crops, which is currently cultivated in gravel bed hydroponic systems (Stanghellini and Rasmussen, 1994; Niederwieser, 2001). Hydroponics, the growing of plants without soil, has been used in commercial production of vegetable crops since the mid 1930's (Harris, 1987). Tomato, cucumber, spinach, lettuce and peppers are some of the major vegetable crops grown in hydroponic systems. Hydroponic cultivation is increasing in importance in South Africa because of the high production potential, high quality of the produce and efficient water usage (Niederwieser, 2001).

Pythium is an extremely common inhabitant of moist soil ecosystems and is generally regarded as an important pathogen in hydroponic systems since it produces motile spores known as zoospores (Van Der Plaats-Niterink, 1981; Rowe, 1986; Niederwieser, 2001). *Pythium* species are widely distributed throughout the world and act as parasites on a wide range of plants (Rowe, 1986). Stanghellini and Kronland (1986), indicated that various species of the genus *Pythium* have been circumstantially implicated as a cause of subclinical diseases. Yield losses caused by such diseases often go undetected since all plants in the field appear healthy but may already be affected by *Pythium* (Stanghellini and Kronland, 1986). It was also reported that *Pythium dissotocum* Drechsler reduces the yield of lettuce up to 35-54 % without showing any visible foliar symptoms. Jenkins and Averre (1983) reported that the

most prevalent among root infecting plant pathogens in North Caroline are *Pythium* spp., which can cause damage ranging from total losses to light or moderate root or stem damage.

Pythium spp. has the ability to cause growth reduction of different plants in soilless culture (Paulitz *et al*., 1992; Moulin *et al*., 1994; Rankin and Paulitz, 1994; Stanghellini and Rasmussen, 1994; Mc Cullagh *et al*., 1996; Menzies and Belanger, 1996; Paulitz, 1997; Utkhede *et al*., 2000; Zheng *et al*., 2000; Paulitz and Belanger, 2001). Due to the fact that *Pythium* spp. produces a motile zoospore and is easily spread in a re-circulating hydroponic system, it is difficult to control once the pathogen is introduced into the system (Stanghellini and Rasmussen, 1994). Chemicals such as metalaxyl and propamocarb have been used to control the zoospore spread. However, these products proved to be phytotoxic and resulted in unacceptable residues in plants (Rankin and Paulitz, 1994). To date, there is no reported resistance for *Pythium* spp. in lettuce for hydroponic systems (Zinnen, 1988). Surfactants have also been reported to control zoospores (Stanghellini and Rasmussen, 1994) in hydroponic systems. On the other hand the potential of biocontrol using growth-promoting microflora have not been explored and so far, little research have been done evaluating biocontrol of *Pythium* root rot in hydroponic systems. This dissertation therefore focused on developing an alternative disease management strategy for *Pythium* root rot of lettuce in hydroponic systems in South Africa.

The first chapter comprises a review of *Pythium* control in hydrophonically grown lettuce. A brief overview of lettuce plants is given to acquaint the reader with the host, the most economically important diseases in the hydroponic systems, their biology and possible control measures. The importance of *Pythium* in hydroponic system is briefly discussed to promote awareness of their presence and the extent of damage it causes. Chapter two describes collection and characterization of bacteria and fungi isolated from the rhizosphere of some grasses and sedges from a pristine environment at Nylsvley Nature Reserve, Northern province, South Africa. It evaluates *in vitro* antibiosis of the rhizosphere microflora against some common plant pathogens. Chapter three and four deals with first stage screening of selected rhizosphere antagonists for biological control of *Pythium* root rot and for growth promotion of lettuce in steam pasteurised Canadian peat moss and re-circulating gravel bed hydroponic system respectively in South Africa. The final chapter gives a general overview of the findings and discusses the potential of biocontrol in hydroponic systems.

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CHAPTER 1

LITERATURE REVIEW

1.1. LETTUCE

Cultivated lettuce (*Lactuca sativa* L.) belongs to the Asteraceae or sunflower family, sub-division Linguiflorae, in which the florets are strap-shaped and a milky juice (latex) is present in the stems and leaves. (Ware and McCollum, 1968; Swiader *et al*., 1994). The scientific name is derived from the Latin *lac* = milk and *sativus* = sown or planted (http//www.Hort.Purdue.Edu/rhodcu/hort410/lettuce//e0001.htm.). Lettuce is thought to be native to the Mediterranean area and inner Asia Minor where it was probably first domesticated along the shores of Egypt and subsequently became common in much of the Roman Empire (Swiader *et al*., 1994). Evidence of Cos lettuce has been traced back to 4,500 BC from an Egyptian tomb painting. Persians cultivated lettuce in the 6th century BC (Large, 1972). However, the worldwide introduction of lettuce was relatively slow (Swiader *et al*., 1994).

Lettuce is a hardy, annual, dicotyledonous, self-pollinating, diploid (2n=18) vegetable crop (Large, 1972; http//www.Hort.Purdue.Edu/rhodcu/hort410/lettuce//e0001.htm). It is usually regarded as a derivative of the wild *Lactuca scarita* L., which is a widely distributed weed. In Europe, there are over 100 species in the genus *Lactuca*. Chicory (*Chicorium intybus* L.) is also closely related to lettuce.

Four distinct types of cultivated lettuce are known (Swiader *et al*., 1994; http//www.Hort.Purdue.Edu/rhodcu/hort410/lettuce//e0001.htm), viz.:

1. Crisp head (iceberg types): Large, heavy, tightly folded heads; brittle or crisp textured; prominently veined leaves; wrapper leaves green; inner leaves whitish-yellow; predominantly out-door types.
2. Butterhead (bibb or Botson lettuce types): Soft leaves; smooth textured; varieties bred for both outdoor summer conditions and greenhouse winter conditions; summer butter heads larger and firmer than the winter type, winter butter heads smaller and less compact.
3. Cos (Romaine): Elongated leaves developing into large leaf-shaped heads, slower to bolt than other lettuce types; useful as a warm weather crop.
4. Leaf local marketing and home garden lettuce: Grown mostly in greenhouses in the winter.

The fourth type of lettuce is sometimes referred to, in a generic sense, as leaf lettuce to differentiate it from another type of lettuce known as stem lettuce, or celtuce (celery-lettuce) (*L. sativa* var. *augustana* L.) (Neild and Uhlinger, 1990). The latter is a perennial plant grown mainly for its thick succulent stems, or seed stalk. The stem is peeled and may be eaten raw like celery or it may be cooked (Swiader *et al*., 1994).

Lettuce is an important cool season crop and one of the easiest one to grow. As basic ingredient in salads, it is eaten more frequently than any other salad vegetable (Neild and Uhlinger, 1990). As with most salad vegetables, the amount of dry matter is low (4%) and generally contains 2.2% starch, 1.4% protein and 0.3% fat (Large, 1972). Although low in nutrients and energy, salad vegetables are excellent dietary sources of bulk and fiber (Swiader *et al*., 1994). According to history, emperor Caesar Augustus believed lettuce cured him from illness and he therefore created a stature in its honour (http://www.fmi.Org/news;letters/super-research/moreissues/Julyaug98 html).

Generally, China ranks the first in world production of lettuce followed by the United States (Neild and Uhlinger, 1990; http://www.fmi.org/newsletters/super-research/moreissues/julyaug98html). However under controlled environmental conditions where much of the crop is grown in enclosure structures, Europe is the second largest producer. In many parts of the world, lettuce nevertheless remains a minor crop (Swiader *et al*., 1994).

Iceberg lettuce is the second most popular fresh market vegetable after tomato. It exceeds 11 kg per capita consumption annually (Sanders, 2001). Leaf and head lettuce can mature within 40-50 and 70-75 days, respectively. A good head yield of lettuce is about 400-500 crates per acre and of the leafy type, 800-1000 crates per acre (Sanders, 2001).

Lettuce thrives best at relatively low temperatures and when climatic requirements are precise. Ample sunlight, uniform cool nights and plenty of moisture in the soil are essentials aspects for well-developed, solid heads (Swiader *et al*., 1994). The optimum temperature for growth of lettuce is 16 to 18 °C. At 21 to 27 °C, the plants flower and produce seed. Lettuce can tolerate a few days at higher temperatures (27 to 29 °C), provided that nights are cool (Sanders, 2001). This crop withstands light frost, but can be damaged by freezing temperatures (http://www.Aces.edu/department/ipm/lettuce.htm). It can be grown on a wide variety of soil types, provided the soil contains organic matter and that irrigation, drainage

and climatic conditions are appropriate. The largest commercial acreages are on muck soils, sandy loam and silt loam. Lettuce is, however, ideally suited to muck and does not grow well in dry soil (Swiader *et al.*, 1994; http://www.msue.msu.edu/msue/impl.mod03/017/449.html.).

1.2. HYDROPONICS

Importance

Soilless production of lettuce is advantageous due to the use of no or reduced amounts of fungicides. Hydroponic systems with 100% recirculating nutrient solutions eliminate problems associated with groundwater pollution with nitrates and phosphorus (Vestergaad, 1988). Hydroponics is the technology of growing plants in nutrient solution, either with or without artificial medium (sand, gravel, vermiculite, rock wool, perlite, peatmoss, coir or sawdust), which provides mechanical support to the root system (Stanghellini and Rasmussen, 1994; Jensen, 1997). The term hydroponics (i.e. “water working”) was coined by W. F. Gericke in 1930 (http://www.nfrec-sv.infas.ufl.Edu/hydroponics.htm).

Although the history of hydroponics can be traced to the 17th century, commercial use began in the early 1940s and is currently employed worldwide to grow flower, foliage, and bedding plants, in addition to high cash value vegetable crops (Stanghellini and Rasmussen, 1994). Western Europe is the center of hydroponic production (Zinnen, 1988). In combination with greenhouses, hydroponics is a high technology and capital-intensive. However, the technology is also very productive, conservative on water and land usage and more environmentally friendly. Zinnen (1988) described advantages of hydroponics over conventional growing of plants in soil. Firstly, in place of soil, chemically inert rooting media such as sand and rockwool provides mechanical support for plants. These media tend to vary less from batch to batch than soil, provide more consistent rooting conditions for the crop and can even be eliminated if some other forms of mechanical support, such as floating plastic pallets or a metal trough, are provided. Secondly, since nutrients are supplied exclusively in solution through the watering system and not by the rooting medium, the grower has more control over fertility and pH. Thirdly, the elimination of soil theoretically precludes disease caused by soilborne pathogens.

Hydroponic culturing methods are being used successfully to produce plants out of season in greenhouses and in areas where either soil or climatic conditions is not suitable for the crop. During World War II, for example, several U.S. Army units successfully produced vegetables hydroponically at various overseas bases (http://www.Thelettucefarm.com/history.htm). In arid regions, such as the Persian Gulf and the Arab oil producing states, hydroponic production of tomatoes and cucumbers is underway. These countries are also investigating additional crops that may be grown by this method, as they have limited arable land and fresh water (http://www.Thelettucefarm.com/history.htm). Also of interest is that the National Aeronautics and Space Administration (NASA) is working towards developing hydroponic systems that are a self-contained units capable of providing vegetables to space stations in orbit (Zinnen, 1988).

Although worldwide figures are not available, Eparvier *et al*. (1991) estimated that in 1991 roughly 4,000 ha were planted under hydroponic systems in northern Europe alone. In the United States, total greenhouse vegetable production (both soil and hydroponics) was about 220 ha in 1994 of which 75% was grown under hydroponic conditions in commercial facilities ranging in size from 232 m^2 to 16 ha. Major vegetable crops grown in hydroponic systems include tomato, cucumber, spinach, lettuce and peppers (Stanghellini and Rasmussen, 1994).

Systems lacking an inert substrate are called liquid hydroponic systems, whereas those employing a substrate such as sand, gravel, peat, perlite, vermiculite, rock wool, etc., are called aggregate hydroponics (Stanghellini and Rasmussen, 1994; Niederwieser, 2001). Hydroponic systems are also classified as either open or closed. In a closed system, the nutrient solution is recovered, replenished and recycled following its direct delivery to the root system. In an open system, the nutrient solution is not replenished or recycled, although it may be recovered or reused (Jenkins and Averre, 1983; Stanghellini and Rasmussen, 1994; Zinnen, 1998; Jensen, 1997). Both systems have certain advantages and disadvantages (Table 1.1). For instance, initial problems with nitrogen availability are one of the major disadvantages of open hydroponic systems while its advantage is less chance of spread of some diseases. In a closed hydroponic system, the major disadvantages include costly construction and maintenance, as well as rapid spread of some diseases (particularly these caused by *Pythium* spp.). The advantages are good root aeration and uniform watering and feeding of plants, especially in gravel culture systems (Niederwieser, 2001).

Table 1.1. Advantages and disadvantages of open and closed hydroponic systems (Summarised from Niederwieser, 2001)

Type	Advantages	Disadvantages
Open system		
Bag culture systems. Slabs, bags or pots of substrate placed on greenhouse floor with drip irrigation system.	Good root aeration. Less chance of spread of some diseases. Good lateral nutrient movement.	Initial problem with nitrogen availability. Coarse sawdust causes clogging of nutrient solution. Some sawdust could contain phytotoxic chemicals.
Closed system		
NFT* systems		
Thin film of re-circulating nutrient solution flowing through plastic channels containing plant roots without solid rooting media.	Lower production costs Lower labour cost with moveable system.	High initial capital investment.
Root mist system		
Plants are grown in holes in panels of expanded polystyrene or other light material. Roots are suspended in mid air. A misting system beneath the frame sprays the nutrient solution over the roots periodically.	None.	Uneven light in one of the sides during the day.
Gravel culture system		
Plants are grown in a gravel substrate without any calcareous material. Water is pumped into the beds, floods them to within several centimeters of the surface and drains back to the reservoir.	Uniform watering and feeding of plants. Good root aeration. Adaptable to many crops. Full automation possible. Can be used in non-arable areas where only gravel is available.	Costly to construct and maintain. Roots build up in gravel and block drainpipes. Some diseases (caused by *Pythium*) can spread through a cyclic system very rapidly.

* NFT = nutrient film technique.

1.3. DISEASES ASSOCIATED WITH HYDROPONIC SYSTEMS

Compared to the numerous and diverse root infecting pathogens associated with field-grown vegetable crops, relatively few have been reported on crops grown under hydroponic conditions. Stanghellini and Rasmussen (1994) refer to four viral, two bacterial and 20 fungal pathogens associated with root diseases of hydroponically grown vegetable crops (Table 1.2). Although this list, at first glance, appears ominous, relatively few of the pathogens have been associated with major or widespread economic losses in the industry.

With the exception of Fusarium wilt of tomato caused by *Fusarium oxysporum* (Schltdl. em. W.C. Snydere H.N. Hansen) f.sp. *radicis-lycopersici* (Sacc.) W.C. Snydere H.N. Hansen, most of the destructive root diseases in hydroponics have been attributed, either directly or indirectly, to the fungal genera *Pythium, Phytophthora, Plasmopara* and O*lpidium* (Table 1.2). *Pythium* and *Phytophthora* spp. are the most important cause of root diseases in hydroponic systems (http://www.plant-tech.co.zn/ login/propagation/rootdisease.htm). These two genera are also important pathogens in soil, field, orchard and nursery environments where they cause disease in plants as diverse as small seedlings and cuttings to mature orchard trees (Stanghellini and Rasmussen, 1994). In soil cultures, *Pythium* spp. are usually considered pathogens of younger plants. However, they have also become a serious problem in mature plants grown hydroponically (Rowe, 1986), where high humidity and succulence of plants allow infection of aerial stems as well as roots (Zinnen, 1988). Aerial infection is a greater threat to plants that grow for 2-3 months to produce a crop, e.g. cucumber, than short-term leafy crops such as lettuce (Zinnen, 1988; Stanghellini and Rasmussen, 1994).

What makes *Pythium* species important in hydroponic systems is their ability to produce zoospores. A zoospore is a motile, unicellular, propagative body measuring 3-12 µm in diameter and is favoured by an aquatic environment (Pauiltz, 1997; Paulitz and Belanger, 2001). Several *Pythiaceae*, in particular *Pythium,* are found in permanently wet soilless substrates, where conditions are conducive to their development and where they cause root losses (Blancard, 1994). *Pythium* spp. have a poor competitive ability in soil relative to other root-colonising organisms and often act only as primary colonisers and unspecialised pathogens (r-strategists) (Campbell, 1989), which use exudates from the germinating seed for saprotrophic growth (Rankin and Paulitz, 1994). However, in hydroponic systems, low numbers of competing microbes and efficient dissemination of zoospores through nutrient solutions increase the disease development potential (Campbell, 1989; Utkhede *et al*., 2000).

Table 1.2. Infectious agents isolated from roots of hydroponically grown vegetable crops (Adopted from Stanghellini and Rasmussen, 1994)

Pathogen	Spread by infested nutrient solution
Bacteria	
Clavibacter michiganense	Inconsistent
Ralstonia solanacearum	Yes
Fungi	
i. Non-zoosporic	
Colletotrichum coccoides	Inconsistent
Fusarium oxysporum f.sp. *lycopersici*	Inconsistent
F.oxysporum f.sp. *cucumerinum*	Inconsistent
F. oxysporum f.sp. *radicis-lycopersici*	No
Verticillium dahliae	Inconsistent
V. tricorpus	Ni*
Thielaviopsis basicola	Ni*
ii. Zoosporic	
Phytophthora crytogea	Yes
Phytophthora nicotianae	Yes
Phytophthora lactucae-radicis	Yes
Pythium aphanidermatum	Yes
Pythium debaryanum	Yes
Pythium dissotocum	Yes
Pythium intermedium	Yes
Pythium irregulare	Not tested
Pythium myriotylum	Yes
Pythium ultimum	No
Pythium sylvaticum	Not tested
Olpidium brassicae	Yes
Olpidium radicale	Yes
Viruses	
Lettuce big vein virus	Yes
Melon necrotic spot virus	Yes
Tomato mosaic virus	Yes
Cucumber mottle virus	Yes

* Not indicated

Zoosporic fungi, as opposed to non-zoosporic fungi, have been demonstrated experimentally to spread rapidly within a system via recirculating infested nutrient solutions (Table 1.2) (Stanghellini and Rasmussen, 1994). Zoospores have been implicated as the primary, if not sole infective propagules responsible for the spread of these pathogens via the recirculating nutrient solution.

In conclusion, pathogens of minor importance under field conditions can be of major economic significance in hydroponic systems. This is particularly true for *Pythium dissotocum* Drechs. This fungus was first identified as the cause of a destructive root rot of hydroponically grown spinach and has since been recognised as a major pathogen of hydroponically grown lettuce (Stanghellini and Kronland, 1986).

1.3.1. *Pythium* disease symptoms

When conditions for infection are optimal, *Pythium* spp. can be very destructive to feeder roots. Initially, infected feeder roots are typically light brown/orange (Lewies, 1998; Niederwieser, 2001) when holding well-washed roots against a white background. Infection progresses rapidly with darkening of the roots. Roots collapse or start to rot from the tip (terminal rot), leaving the vascular strands exposed. Aboveground plant parts show lesions on the stem, girdling the plant and causing stunting (Lewies, 1998). Various *Pythium* spp., besides causing extensive necrosis and a reduction in the feeder rootlet system, have been circumstantially implicated in so-called subclinical infection (Stanghellini and Kronland, 1986).

In many cases random individual plants show severe root symptoms amidst otherwise apparently healthy plants within a crop. *Pythium* has been consistently isolated from lettuce with diseased roots as well as from healthy lettuce root systems (www.plant-tech.co.zn/login/propagation/rootdisease.html). Stanghellini and Rasmussen (1994) reported that *P. dissotcum* causes symptomless infection of lettuce. This pathogen is particularly insidious because infected plants do not exhibit any root rot symptoms. The only indication of the presence of *P. dissotcum*, other than its isolation from roots, is a general retardation in the maturation rate of the plant (Stanghellini and Rasmussen, 1994). However, since all plants in a hydroponic system are uniformly infected, there is no reference point for diagnosis.

1.3.2. Distribution and pathogenicity of *Pythium*

Pythium spp. are ubiquitous (Mathews, 1931; Van der Plaats-Niterink, 1981). Different species can be found in the tropics and temperate or even colder regions and tropical species have also been isolated from greenhouse grown plants in temperate climates (Van der Plaats-Niterink, 1981). *Pythium* spp. occur most abundantly in cultivated soil near the root region in superficial soil layers. They occur less commonly in non-cultivated or acidic soils where *Trichoderma* is dominant and their presence have been restricted (Van der Plaats-Niterink, 1981). *Pythium* species have been recorded at depths of 0.75 and 335 cm, but not in a layer between 120 and 200 cm. They have also been isolated from soil from arable land, pastures, forests, nurseries, marshes, swamps and water. Dry sand areas, dry forests and salt marshes have generally low numbers of *Pythium* spp. (Van der Plaats-Niterink, 1981).

Pythium spp. can survive either saprophytically or parasitically. Their parasitic role often depends on external factors. When conditions are favourable for the fungus, but less so for the host, normally avirulent species can become pathogenic and cause fruit, root, or stem rot or pre- or post-emergence damping-off of seedlings. Young or watery tissue is preferentially attacked. Infection takes place when zoospores produce germ tubes or appressoria, which penetrate the plant by means of an infection peg (Van der Plaats-Niterink, 1981).

The availability of pectolytic and cellulolytic enzymes largely determines the pathogenic capacity, although phytotoxins and indolic growth factors can be involved. Infection depends on several factors such as inoculum density, soil water content, temperature, pH, light intensity, cation content and presence of other microorganisms (Van der Plaats-Niterink, 1981). Sufficient amounts or excess of water often favour infection and severity of attack (Stanghellini and Rasmussen, 1994). The influence of temperature depends on the species of *Pythium* involved. Infection of cucurbitaceous plants with *P. aphanidermatum* (Edson) Fitzp is most severe at temperatures between 30 and 35 ^{o}C. *P. myriotylum* Drechsler is most pathogenic at about 30 ^{o}C and *P. graminicola* Subramaniam infects cereals at about 25 ^{o}C. Temperatures below 23 ^{o}C are most favourable for infection with *P. ultimum* Trow, while *P. iwayamai* S. Ito (snow blight of cereals) only infects at low temperatures (Van der Plaats-Niterink, 1981).

Infection mostly takes place on the young roots, but leaves can also be affected. In susceptible plants, root exudates can cause an accumulation of zoospores and accelerate the encystment and germination thereof, especially in differentiating or injured roots. Inoculation with hyphal fragments can also cause infection, but at a slower rate (Van der Plaats-Niterink, 1981).

Any infective propagule, upon entry into a hydroponic nutrient solution, will eventually make contact with a root. The probability of root encounter is very high when one takes into consideration the density and confinement of roots in a hydroponic system, particularly those employing NFT (Jenkin and Averre, 1983; Stanghellini and Rasmussen, 1994). Once a zoosporic, root-infecting fungus has been introduced into a production facility, it will multiply and spread rapidly throughout the system. Methods of pathogen dispersal (Stanghellini and Rasmussen, 1994) include the following:

i. Self-dispersal (i.e. via zoospore motility).
ii. Dispersal resulting from recirculation of the nutrient solution.
iii. Root-to-root contact.

Zoospores readily pass through sand filters and the impellers of centrifugal pumps in a viable condition (probably as encysted zoospores). Additionally, many root pathogens are capable of growing via hyphae from infected to healthy roots (Stanghellini and Rasmussen, 1994).

The commonest source of infection in hydroponics is the planting of infected plant material, although it is not the only source. Surface water (dams, ponds, streams, etc.) has been shown to contain zoospores of *Pythium* spp. Invasion from this source seems to be the highest following heavy rainfall and run-off after a hot dry spell. Contamination of the system by dirt or debris carrying fungal spores or mycelium is also common (http://www.plant-tech.co.zn/login/propagation/rootdisease.html). Rankin and Paulitz (1994) pointed out that *Pythium* spp. can easily be introduced into hydroponic systems from infested water sources, contaminated soil, or naturally infested peat-based propagation media. Fungus gnats (*Bradysia* spp.) and shore flies (*Scatella stagnalis* Fallen) have been shown to be possible carriers, with viable zoospores of *Pythium* ingested by the root-feeding maggots and subsequently being disseminated by the adult fly (Rankin and Paulitz, 1994; http:/www.plant-tech.co.zn/login/propagation/rootdisease.html).

Zoospores of *Pythium* spp. are formed in a vesicle on infected roots and are eventually released. During the motile period, which can last up to 24 hours, zoospores locate a root

through a chemotactic mechanism, encyst, penetrate and infect the plant. Under optimal environmental conditions, these events can occur within five minutes. Subsequent to root infection, completion of the asexual life cycle (i.e. zoospore to zoospore) can occur within 12 hours depending on the particular species (Van Der Plaats-Niterink, 1981; Stanghellini and Rasmussen, 1994). The reproductive capacity of zoosporic fungi is enormous. For example, it has been calculated that about 40 sporangia of *Plasmopara lactucae radicis* Stang. & Gilbn. are produced on a 1 cm long segment of an infected lettuce root. Each sporangium produces approximately 100 zoospores. Thus, approximately 4 000 zoospores are produced per centimeter of infected root. The uniform infection of a single mature lettuce plant, which has about 2 000 cm of roots, can result in the production and release of about eight million zoospores (Stanghellini and Rasmussen, 1994).

The development of root disease in hydroponic systems may be due not only to the introduction of the casual organism into the system, but also to the condition of the crop and the nutrient solution. It was suggested that *Pythium* spp. could exist in NFT as saprophytes and that their growth (as saprophyte or parasites) depends on substrate availability. The substrate is the combination of root exudates and breakdown products of dead root tissue. Competition from other microorganisms in the substrate also affects growth of the pathogen (http:/www.plant-tech.co.zn/loin/propagation/rootdisease.html).

The importance of subclinical diseases caused by *Pythium* spp. became apparent in 1981, when commercial production of hydroponically grown spinach and lettuce was undertaken in a 0.5 ha greenhouse in Tucson, Arizona, USA (Stanghellini and Kronland, 1986). Within three months from the initial planting, commercial production of spinach was abandoned because of severe root rot caused by *P. aphanidermatum* and *P. dissotocum*. Lettuce plants growing under similar conditions as the diseased spinach plants did not appear affected. However, *P. dissotocum* was constantly isolated from lettuce feeder rootlets and although all rootlets were infected, they showed no recognisable shoot or root disease symptoms (Stanghellini and Kronland, 1980).

Stanghellini and Kronland (1986) found that *P. dissotocum* can cause up to 50% yield loss in lettuce even in the absence of root rot symptoms. The implications to growers are significant. Even when they produce an apparently healthy crop, with rot-free, white roots, growers may be losing half their yield potential to a pathogen they cannot see and can only identify and quantify by a competent mycologist (Zinnen, 1988). Jenkins and Averre (1983) also reported

that damage caused by this organism range from total loss to light to moderate root or stem damage.

1.4. DISEASE MANAGEMENT IN HYDROPONIC SYSTEMS

1.4.1. Background

Avoidance of root diseases was one of the motivating forces underlying the development of hydroponics (Zinnen, 1988; Stanghellini and Rasmussen, 1994). Although cultivation in hydroponics has resulted in a decrease in the diversity of root-infecting microorganisms compared to conventional culturing in soil systems, root diseases still occur and losses are even greater than in soil. In open field agriculture, the rapid development of a plant disease is generally regarded as a unique feature of above-ground infectious agents. However, the use of hydroponics (particularly closed systems) now imparts these same characteristics to root or below-ground infectious agents (Stanghellini and Rasmussen, 1994).

In field crops, soil temperature and moisture govern the incidence of root rots, with great fluctuations of these two parameters occurring throughout the growing season (Niederwieser, 2001). By contrast, both temperature and moisture are relatively constant throughout the growing season in a protected environment. Nutrient solution temperature is the single most important factor governing development of waterborne pathogens in hydroponics (Niederwieser, 2001).

Once a pathogen has become established, root rot can rapidly become a serious problem because of the:

i. Abundance of genetically uniform hosts.
ii. Physical environment with a more constant temperature and moisture regime.
iii. Mechanism for rapid and uniform dispersal throughout the cultural system (Stangehillini and Rasmussen, 1994; Zinnen, 1988).

Knowledge of the modes of pathogen introduction is a prerequisite for maintaining a pathogen-free environment. Additionally, accurate identification of the specific pathogen involved is essential for the selection or development of an appropriate control strategy, because no single method is applicable to all root-infecting pathogens (Stanghellini and Rasmussen, 1994). Jenkins and Averre (1983) pointed out that infection of vegetables in hydroponic systems limits the usefulness of this production system unless suitable preventive

or control measures are implemented. Hydroponic systems, therefore, offer opportunities for developing new biological and chemical control measures because the biocontrol agent or chemical can be added to the circulating medium at one point for distribution throughout the system.

1.4.2. Cultural and physical methods

1.4.2.1. Sanitation

Considerable effort is required to determine the actual effectiveness of sophisticated sanitation procedures, such as disinfecting growing trays and flushing nutrient solution systems (Stanghellini and Rasmussen, 1994). A non-circulating system may be appropriate for tomato and cucumber growers, but apparently is unsuitable for profitable production of leafy vegetables (e.g. lettuce) due to the added cost incurred by wasting of nutrients. Furthermore, there is no off-season to help reduce pathogen populations (Zinnen, 1988).

Removal of all infested plant debris, as well as disinfesting equipment and recycled aggregate substrates, is mandatory for the maintenance of a pathogen-free system (Stanghellini and Rasmussen, 1994). Niederwieser (2001) also added that the use of pathogen-free seed and seedlings is one of the most important disease prevention measures a grower can implement. Introducing contaminated seedlings into the system will immediately jeopardise the whole system, especially in the case of water-borne pathogens such as *Pythium* and *Phytophthora*. Pathogen-free greenhouse stock is therefore of utmost importance. The greenhouse should be housed in a facility physically separated from the production area and should not use the same nutrient solution employed in the production facility (Stanghellini and Rasmussen, 1994). However, Zinnen (1988) remarked that exclusion of inoculum has proved impractical and that sanitation procedures to reduce the inoculum load appear futile once the pathogen has established itself in a recirculating hydroponic system.

1.4.2.2. Manipulation of the physical environment

Environmental conditions constantly favourable for plant growth and development will often benefit the development and spread of pathogens (Menzies and Belanger, 1996). Hydroponic systems afford the opportunity of controlling environmental conditions to a certain degree. However, there is a trade-off between the requirement of the crop and that of disease

prevention (Niederwieser, 2001). Changing conditions in irrigation water/nutrient solution can influence conditions in the rhizosphere. There is, for example, evidence that the gravity flow rate in gravel bed systems can affect the severity of *Pythium* root diseases. A higher flow rate associated with a steeper incline tends to suppress the disease (Niederwieser, 2001).

Two of the most important environmental factors known to govern the life cycle of root-infecting pathogens and their disease cycles are temperature and moisture (Stanghellini and Rasmussen, 1994). Hydroponics provides a nearly constant saturated environment. Thus, the extent to which moisture in the root zone can be manipulated is too limited to have an impact on most *Pythium* spp. However, the temperature of the nutrient solution can be manipulated. If the temperature requirements of the root pathogen are known, nutrient solution temperatures can be raised or lowered to retard development of the organism (Stanghellini and Rasmussen, 1994; Menzies and Belanger, 1996).

1.4.2.3. Treatment of infested nutrient solution

Ultra violet (UV) irradiation and filtration of the nutrient solution, as well as amendment of the solution with potassium silicate, can reduce root decay and yield loss in some greenhouse-grown crops infected with *P. ultimum* (Rankin and Paulitz, 1994). The use of sterilisation systems is an attractive way to decrease the spread of pathogens in recirculating hydroponic systems though sterilisation technologies should not be seen as a way to correct poor management practices. Commercial sterilisation units based on pasteurisation, UV irradiation and ozone are available, with pasteurisation being the most popular in Europe (Menzies and Belanger, 1996).

i. Pasteurisation

Menzies and Belanger (1996) reported that pasteurising recirculating hydroponic solutions at 95°C for 30 seconds inactivates *Olpidium* spp. and *P. aphanidermatum.* The authors also illustrated that pasteurisation of nutrient solutions can increase yield when root pathogens have spread through the recirculating system.

ii. Ozone

According to Menzies and Belanger (1994) the recommended dosage for ozone treatment of water is 10 g $h^{-1}m^{-3}$ to achieve a redox potential of 754 mV in the solution. Ozone treatment of hydroponic solutions is equal in effectiveness to pasteurisation. The pH of the nutrient solution to be treated with ozone should be lowered to 4.0 to increase the stability and effectiveness of ozone as a sterilant. Ozone sterilisation of nutrient solutions was found to prevent a decrease in yield if root pathogens have spread through a recirculating system (Menzies and Belanger 1994).

iii. Ultraviolet (UV) irradiation

Ultraviolet irradiation has been tested for disinfesting hydroponic systems since the 1980's (Zhang *et al*., 2000). UV reduces the population of the target pathogen as well as the non-target microorganisms in hydroponic systems. In theory, a recirculating solution can be disinfested to any degree by manipulating the UV dosage. In reality, however, total disinfestation of recirculating solutions in a hydroponic system is extremely difficult to achieve (Zhang and Tu, 2000). For example, *Pythium* propagules, particularly oospores, can survive UV irradiation and multiply and accumulate in the rhizosphere. At a high UV dose, the accumulation of surviving propagules may be minimal over a short period (Zhang and Tu, 2000). Stanghellini *et al.* (1984) showed that spinach root rot caused by *P. apanidermatum* could be controlled by UV irradiation over a two-week period using a 90 J cm^{-2} UV dose. However, over an extended growing season, the accumulation of surviving propagules could be sufficient to cause root rot, especially when the non-target bacterial population starts to decline in the rhizosphere due to irradiation (Zhang *et al*., 2000).

In a perfect recirculation system, UV is effective as a pasteurisation treatment, but has not gained wide commercial acceptance due to the high cost of irradiation. Disease control is, however, difficult because of a number of factors that influence its effectiveness. UV sterilisation is, for example, incompatible with growing plants in peat as peat produces humic acid, which absorbs UV, making irradiation of the solution less effective. Sterilisation of hydroponic solutions with UV was found to increase yield in the presence of root pathogens spreading through the recirculating system (Menzies and Belanger, 1994).

iv. Filtration

Filtration of the nutrient solution to physically remove motile and encysted zoospores (10-12 µm in diameter) from the system has potential as a method of control (Goldberg *et al*., 1992; Runia, 1995; Menzies and Belanger, 1996). Goldberg *et al*. (1992) transplanted cucumber seedlings into separate hydroponic tanks to test the efficacy of filtration of zoospore-infested water for the control of *Pythium* root rot of cucumber. Each tank received water from a zoospore-infested source tank. The infested water was recirculated three times through a 20 µm filter or through a 20 µm filter followed by a 7 µm filter. Within 24 hours after the first recirculation cycle, 67% of the plants which received water filtered through a 20 µm filter were infected. None of the plants receiving water passing through the 20 µm and 7 µm filters were infected until one day after the third and final recirculation cycle. The fungus was recovered from the surface (0 mm deep) and middle (8 mm deep) of the 7 µm filter, but not from the inner core (16 mm deep). Thus, the 7 µm filter effectively removed the fungus from infected water.

1.4.3. Chemical methods

1.4.3.1. Fungicides

The addition of fungicides to recirculating nutrient solution obviously is an effective method of disease control (Stanghellini and Rasmussen, 1994). Propamocarb (Stanghellini and Rasmussen, 1994) and metalaxyl (Stanghellini and Rasmussen, 1994; Rankin and Paulitz, 1994; Zinnen, 1988; Olsen and Young, 2001) have been reported to provide a high degree of disease control. Niederwieser (2001) also reported fungicides such as furaxyl, fosetyl Al and K-phosphonate being active against pythiaceous fungi.

Zinnen (1988) pointed out that metalaxyl is a fungistatic chemical, which can control root rot in experimental tanks, but it is not registered for commercial use. Stanghellini and Tomlinson (1987) also indicated that no effective fungicides are registered for control of *Pythium spp*. in hydroponically grown vegetables. In South Africa, Niederwieser (2001) noted that not a single fungicide is registered for use against *Pythium* on hydroponically grown crops. New registrations allowing addition of fungicides to the nutrient solution might be difficult due to pytotoxicity and the possibilities of unacceptable residues on the plants (Rankin and Paulitz, 1994). A metalaxyl drench (10 µg a.i. ml^{-1}) controlled plant mortality from *P.*

aphanidermatum infection, but the pathogen could still be recovered from treated plants. Furthermore, strains of *Pythium* resistant to metalaxyl have already been discovered (Zinnen, 1988).

According to Stanghellini and Rasmussen (1994), the reasons for the lack of registered products are numerous. Firstly, most fungicides have a lag period between application and harvesting and most commercial hydroponic facilities harvest daily. Secondly, the limited acreage of hydroponics does not warrant the cost of registration. Thirdly, the probability that strains of the pathogen resistant to the chemical could develop is very high.

1.4.3.2. Synthetic surfactants

Surfactants are amphiphathic molecules that can modify the properties of a liquid medium at a surface or interface by reducing the surface tension (Stanghellini and Miller, 1997). Tomlinson and Faithful (1979) pioneered the intentional use of synthetic surfactants for control of zoosporic pathogens. Continuous application of Agral, a non-ionic liquid surfactant containing 90% (v/v) alkyl phenolethylene oxide condensate, to hydroponic nutrient solutions, control some diseases caused by zoosporic agents. For instance, *P. aphanidermatum* was isolated from roots of cucumber plants before, but not after, addition of Agral at rate of 20 µg ml^{-1} to the nutrient solution (Stranghellini and Tomlinson, 1987). Over a range of concentrations tested, Agral had little or no effect on either the rate of mycelial growth or the germination of zoospore cysts and sporangia. However, concentrations of 20 and 25 µg ml^{-1} were completely inhibitory to vesicle formation and zoospore production by *P. aphanidermatum, P. dissotocum, P. tracheiphilum* Matta and *P. intermedium* de Bary. The lytic effect on both zoospore and vesicles, i.e., fungal structures surrounded only by a plasma membrane, suggests that the mode of action of Agral may reside in alteration of the integrity and/or permeability of the plasma membrane (Stanghellini and Rasmussen, 1994). This hypothesis is supported by the fact that Agral had little or no effect on mycelial growth or direct germination of zoospore cysts and sporangia (Rankin and Paulitiz, 1994; Stanghellini and Rasmussen, 1994).

Although Agral is the only surfactant discussed here, toxicity to zoospores and vesicles is not exclusive to the compound. Previous work by Tominson and Faithfull (1979) showed that among ten surfactants tested *in vitro*, seven (including Agral and other anionic, cationic and nonionic types) were toxic to zoospores of *Olpidium brassicae* (woronin.) P.A. Dang.

1.4.4. Biological methods

1.4.4.1. Background

In terms of public perception, the negative aspects of pesticides seem to outnumber their benefits. Reports of pesticide residues in food, soil, river and groundwater systems undermine consumer trust and strengthens the perception that pesticide residues on food pose a threat to human health (Campbell, 1994; Glinck and Bashan, 1997; Walsh *et al*., 2001). Thus, the increasing concern is that modern methods of crop production have an overall negative impact on the environment and society (Becker and Schwinn, 1993; Glick, 1995; Whipps, 1997). In response, stricter legislation has been introduced with the consequence that many older pesticides have been withdrawn from the market. The increased requirements for toxicological data are reflected in the considerably higher costs of modern fungicides compared to their predecessors (Becker and Schwinn, 1993).

Concern resulting from increased pathogen resistance to pesticides, and the lack of reliable chemical control, or resistant plant varieties, has further reduced the number of disease control options available (Becker and Schwinn, 1993). The imminent withdrawal of methyl bromide for soil fumigation in many countries has added impetus to the development of alternative control strategies (Becker and Schwinn, 1993; Stanghellini and Miller, 1997). Thus, as Becker and Schwinn (1993), Menzies and Belanger (1996), Whipps (1997) and Stanghellini and Miller (1997) have pointed out, biological control is one of the potential alternatives for the actual and perceived problems inherent to agrochemicals.

Biological control of plant diseases, in its widest sense, is any means of controlling disease, or reducing the amount or effect of pathogens that rely on biological mechanisms or organisms other than man (Campbell, 1989). It includes:

i. Crop rotation and some tillage systems and fertiliser practices which affect microbes.
ii. The direct addition of microbes antagonistic to pathogens and/or favourable to the plant.
iii. The use of chemicals to change the microflora.
iv. Plant breeding, as it is known that change the plant genome, which may affect disease.
v. The utilisation of phyllosphere and rhizosphere microflora (Campbell, 1989).

A more narrow approach is to restrict biological control to the artificial introduction of antagonistic microflora into the environment to control the pathogen. This is derived from the

entomologists' approach to biological control of insect pests by the introduction of predators to prey on a particular pest (Campbell, 1989). However, the definition of biocontrol is continuously being adapted, broadened and changed to suit the needs of researchers and their perspectives of where the focus and strategies of research should be (Korsten and Jeffries, 2000). A general broad definition of biological control is the reduction of inoculum density or disease-producing activities of the pathogen or parasite in its active or dormant state, by one or more organisms, accomplished naturally or through manipulation of the environment, host, or antagonist, or by mass introduction of one or more antagonists (Baker and Cook, 1974; Korsten and Jeffries, 2000).

1.4.4.2. Biological control using resistant varieties

The first line of defence against a plant pathogen is the use of resistant cultivars. Accurate identification of the pathogen to species level is mandatory for the selection of appropriate cultivars. Unfortunately, few lettuce cultivars are resistant to most *Pythium* species (Stanghellini and Rasmussen, 1994). Disease-resistant cultivars specifically, developed for hydroponic cultivation, are not readily available as seed companies have little incentive to produce such cultivars for a limited market (Zinnen, 1988). Because hydroponic facilities are highly mechanised, cultivars must be selected for synchronous and high germination, rapid and uniform growth, cosmetic quality and pleasant taste. Without these features a cultivar is of little use to a grower who must have a crop on time every day.

1.4.4.3. Natural products

i. Amendment with silicone

Chérif and Belanger (1992) stated that several workers reported a reduction in severity of powdery mildew and significant yield increase of cucumber and other crops through amendment with silicone (Si). In their research, they evaluated the amendment of a recirculated nutrient solution with potassium silicate as a means to control *P. ultimum* infection in long English cucumber. Supplying the solution with 100 to 200 ppm of Si significantly reduced mortality, root decay, and yield losses attributed to infection by this pathogen. Further work showed that amending nutrient solutions with 100 ppm silicone dioxide (SiO_2) also reduced the severity of *P. aphanidermatum* on cucumber grown in NFT (Chérif and Belanger, 1992). The mode of action in reducing the severity of infection is,

however, not known. It appears as if Si acts systemically as it enhances resistance in the aerial as well as the underground parts of the plant (Chérif and Belanger, 1992). From an economic point of view, it is difficult to critically assess the economic benefits of using soluble Si in general (Menzies and Belanger, 1996). This is because a yield increment has not been shown with cucumber when soluble Si was added to the hydroponic solution in the presence of *Pythium* spp.

ii. Chitosan

Chitosan is a natural bioactive substance that inhibits fungal growth and also activates defense mechanisms in plants (El Ghaouth *et al*., 1994). It is a nontoxic β-(1,4)-glucosamine polymer obtained from the chitin of fungal walls and arthropod exoskeletons that have been chemically deacetylated to provide more than 70% free amino groups. The polycationic nature of chitosan provides the basis for its physio-chemical and biological function. Chitosan inhibits a number of pathogenic fungi, including several soilborne pathogens such as *F. oxysporum*, *Rhizoctonia solani* J.G. Kühn and *Pythium paroecandrum* Drechsler (Benhamou, 1992; El Ghaouth *et al*., 1994). El Ghaouth *et al*. (1994) cultivated cucumber plants in the presence of chitosan (100 or 400 µg ml^{-1}) in a hydroponic system. The treatment resulted in the control root rot caused by *P. aphanidermatum* and triggered several host defence responses, including the induction of structural barriers in root tissue and the stimulation of antifungal hydrolases (chitinase, chitosanase, and β-1, 3-glucanase) in both the roots and leaves. Chitosan did not cause any apparent phytotoxicity to the cucumber plants. The interplay of the antifungal and eliciting properties of chitosan makes it a potential antifungal agent for the control of root rot of cucumber caused by *P. aphanidermatum.*

1.4.4.4. Biological control using rhizosphere microflora

The term rhizosphere was introduced in 1904 by the German scientist Hiltner to denote the region of the soil that is subjected to the influence of plant roots (Subba, 1986). Rhizosphere soil is characterised by greater microbial activity than the soil away from plant roots. The intensity of such activity depends on the distance to which exudation from the root system can migrate (Subba, 1986). Plants grown in hydroponic systems are known to develop a natural population of microflora on their root surfaces, which is also present in the re-circulating nutrient solutions. Large amounts of bacteria (10^5 to 10^6 cfu) have been shown to develop

within 20 hours of planting a crop and can remain at a stationary level for 12 weeks (Berkelmann *et al*., 1994).

According to Weller (1988), microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since the rhizosphere provides the frontline defence for roots against attack by pathogens. Pathogens encounter antagonism from rhizosphere microorganisms before and during primary infection and also during secondary spread on the root. In some soils described as suppressive to pathogens, microbial antagonism of the pathogen can lead to significant disease control. Although pathogen-suppressive soils are rare, those identified are excellent examples of the full potential of biological control of soilborne pathogens (Whipps, 1997).

Previous research has demonstrated that fungal isolates collected from the rhizospheres of cultivated crops such as pepper (*Capsicum sp.*), wheat (*Triticum aestivum* L.), maize (*Zea mays* L.) and zoysia grass (*Zoysia tenuifolia* Willd. ex Trin.) enhanced the growth of a variety of crop plants (Meera *et al*., 1994). The rhizosphere fungal species belonged to the genera *Fusarium, Penicillium, Rhizopus* and *Trichoderma*. However, most of the isolates from the zoysia grass rhizosphere and some from rhizospheres of other crop plants did not sporulate and were therefore termed "sterile". These sterile, saprophytic fungal isolates promoted plant growth and suppressed soilborne diseases such as damping-off caused by species of *Fusarium* and *Pythium* root rot caused by *Fusarium*, *Rhizoctonia* and S*clerotium* and take-all caused by *Gaeumannomyces graminis* (Sacc.) Arx & D. L. Olivier var. *tritici* J. Walker in a number of crop plants (Meera *et al*., 1994). Simultaneous inoculations of soil with plant growth-promoting fungi (PGPF) and pathogenic fungi or incorporation of PGPF into the soil before infestation with pathogenic fungi decreased the severity of certain soilborne diseases (Meera *et al*., 1994).

A considerable number of bacterial species, mostly associated with the plant rhizosphere, are also able to exert a beneficial effect upon plant growth (Rodriguez and Fraga, 1999). Some of these plant growth-promoting rhizobacteria (PGPR), particularly *Pseudomonas* spp. and *Bacillus* spp., significantly suppressed disease and increased yields of crops in field trials performed by Glick and Bashan (1997) and Glick *et al*. (2001). Their use as biofertilisers or control agents for crop improvement has been the focus of numerous investigations for a number of years (Zhou and Paulitz, 1993, Benizri *et al*., 2001; Glick *et al*., 2001). For instance, application of different species of *Pseudomonas*, originally isolated from the

rhizosphere of cucumber, to plant culture media such as nutrient solution or rock wool reduced root and stem rot of cucumber in both laboratory and greenhouse experiments (Zhou and Paulitz, 1993). It is also stated that microorganisms isolated from hydroponic systems have antagonistic activity against plant pathogens. Some of the organisms isolated include species of fungi such as *Alternaria*, *Botrytis*, C*olletotrichum*, *Fusarium*, *Mucor*, *Nectria*, *Penicillium, Phoma*, *Pythium*, *Rhizopus* and *Trichoderma*, as well as Actinomycetes and species in the bacterial genera *Bacillus*, *Bacterium*, *Micrococcus* and *Pseudomonas* (http://www.sardi.sa.gov.au/hort/floricul/ssf_lit.htm). Some of these bacteria also promoted the growth of cucumber and induced pathogen resistance (Zhou and Paulitz, 1993).

Introduction of soil rhizosphere organisms as antagonists to the rhizosphere is difficult if the soil is already fully occupied. Thus, introducing the antagonist into the soil immediately after fumigation or steaming, or establishing antagonists in the rhizosphere/rhizoplane at the time of rooting of cuttings, germination of seeds or transplanting of seedlings, may be useful strategies to pre-empt the colonisation potential of infection sites (Linderman *et al.*, 1983).

1.4.4.5. Mode of action of biological control

Understanding the mechanism through which biocontrol of plant diseases occur is critical to the eventual improvement and wider use of biocontrol methods (Fravel, 1988). Several modes of action of microbial biocontrol agents have been identified, none of which are mutually exclusive (Whipps, 1997). These can involve direct interactions between the antagonist and pathogen, viz. competition, antibiosis, biosurfactant, etc. Indirect interaction is also known where the plant itself responds to the presence of the antagonists, resulting in induced resistance and/or plant growth promotion (Glick and Bashan, 1997; Whipps, 1997; Tilak *et al*., 1999). These mechanisms have been discussed and reviewed in several papers (Dekker, 1963; Baker, 1968; Gottlieb, 1976; Blakeman and Fokkema, 1982; Leong, 1986; Fravel, 1988; Liu *et al*., 1995; Meera *et al*., 1995; Whipps, 1997; Ramamoorty *et al*., 2001; Zehnder *et al*., 2001; Zhang *et al*., 2001) and books (Baker and Cook, 1974; Cook and Baker, 1983; Campbell, 1989).

i. Competition

Competition for space or specific infection sites on roots and seeds by plant growth promoting microflora leads to niche exclusion. It has been suggested as a mode of action for control of

numerous soilborne pathogens, but relatively few studies have provided unequivocal evidence of this hypothesis (Weller, 1988; Campbell, 1989; Whipps, 1997; Tilak *et al*., 1999). Microorganisms may compete for nutrients and while one organism (because of better uptake mechanisms or extracellular enzymes) obtains most of its nutrients and therefore grows better, the other has insufficient nutritional capacity and subsequently dies or is excluded from the niche. Microbes compete for both carbon and nitrogen sources. Competition is also possible for oxygen, space, and in the case of autotrophs, light. An essential point of the definition is the deprivation of one of the organisms. If there are excess nutrients, there is no competition (Campbell, 1989).

Competition for iron, mediated by antagonistic microorganisms producing iron-chelating moieties, termed siderophores, has been conclusively demonstrated as a mode of action for biocontrol in soils where iron is limited (Glick and Bashan, 1997; Whipps, 1997). Characteristically, these soils have a pH of 7 or above (Glick and Bashan, 1997). Several species of bacteria have been shown to be active biocontrol agents by competing for iron, but the most widely recognised are fluorescent pseudomonads. Pseudomonads produce a range of siderophores including pseudobactins and pyoverdines, which are fluorescent, as well as non-fluoresecent phyochelins and salicylic acid (Weller, 1988). However, it is the fluorescent siderophores, which have a very high affinity for iron, that are generally implicated in biocontrol (Whipps, 1997). These potent iron chelators are thought to sequester the limited supply of iron that is available in the rhizosphere, to a form that is unavailable to pathogenic fungi and other deleterious microorganisms, thereby restricting their growth (Weller, 1988; Whipps, 1997).

Using Tn5 mutagenesis, siderophore production by *Pseudomonas* spp. has been shown to be important in the control of *Pythium* spp. For example, pyoverdine production was responsible for control of *P. ultimum* damping-off of cotton and wheat (Whipps, 1997). Pyochelin production was involved in the control of *Pythium* damping-off of cucumber, as too little pyoverdine was produced in time to prevent the pathogen from invading the germinated seedling (Whipps, 1997).

ii. Rhizosphere colonization / competence

The term colonisation (in this case a root) refers to the colonisation of the root (internal or surface) as well as the rhizosphere, by introduced microorganism (Weller, 1988).

Colonisation of a plant can only occur from inoculum either resident in the environment or introduced by wind, water, animals, man, etc. (Campbell, 1994). One of the key features exhibited by many biocontrol agents of soilborne plant pathogens is an ability to colonise seeds or roots. By establishing in the infection court, the biocontrol agent is able to exhibit a range of direct or indirect biocontrol mechanisms in key ecological niches and thus prevent or delay infection by the pathogen (Benizri *et al*., 2001). In some cases, most notably for those antagonists acting through induced resistance, such spermosphere or rhizosphere competence is a pre-requisite for effective biocontrol (Whipps, 1997). Bacteria have potential advantages over fungi in biocontrol of plant disease (Baker, 1989). Amongst these advantages is the ability of some bacteria to colonise the rhizospheres of roots. Usually, an inter arrival at a primary resource already occupied by a resident species will not find a vacant niche, and has to compete for space and resources (Weller, 1988; Campbell, 1989).

iii. Antibiosis

Current evidence supports the concept that antibiosis is the dominant mechanism of disease suppression by introduced strains that produce secondary metabolites (Fravel, 1988; Glick and Bashan, 1997). Molecular techniques and direct isolation have demonstrated unequivocally that antibiotics are produced in the spermosphere and rhizosphere and play a major role in the suppression of soilborne pathogens (Tilak *et al*., 1999). Antibiotics are generally considered to be metabolites that can inhibit microbial growth. They are secondary metabolites produced by antagonists when nutrients become limiting and are frequently phloroglucinols. The phenolic broad-spectrum antibiotic is one of the major determinants for biocontrol activity of PGPR. The antibiotics pyoluteorin (plt), pyrrolnitrin (prn), penazine-1-carboxylic acid (PCA) and 2-4 diacetyl phloroglucinol (phl) are currently a major focus of research in biological control (Tilak *et al*., 1999).

Antibiotics are low molecular weight (<1 KDa) compounds (Whipps, 1997). However, much of the growth inhibition effect can be demonstrated *in vitro* following the production of some large enzymes and peptides. These molecules may be involved in parasitism, or through the release of small molecules such a siderophores, which are involved in competition rather than directly affecting microbial growth (Whipps, 1997). Consequently, some care must be exercised when applying the term antibiosis as a mode of action of an antagonist when based solely on *in vitro* observations (Whipps, 1997).

iv. Biosurfactants

Recently, Stanghellini and Miller (1997) discovered a novel antimicrobial metabolite, a biosurfactant, which presents a new antagonistic mechanism for biological control of zoosporic plant pathogens. Biosurfactants of diverse chemical structures are produced by several microorganisms. Isolates from the bacterial genera *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Rhodococcus*, *Acinetobacter* and *Corynebacterium* have been reported to produce surfactants. Some fungal genera, including *Candida* and *Torulopsis* are also known to produce surfactants.

Stanghellini and Miller (1997) pointed out that the production of biosurfactants in nature is not clear, although several have already been chemically characterised (e.g., rhamnolipid, surfactin and trehalose lipid). The following potential roles have been proposed for biosurfactants:

i. Enhancement of bioavailablity and biodegradation of slightly soluble organic carbon sources such as petroleum hydrocarbons.
ii. Use as an aid in attachment and detachment of bacteria to surfaces.
iii. Use in antibacterial defence mechanisms.
iv. Function as a virulence factor in the pathogenesis of specific microorganisms in both plants and animals.
v. Use as an aid in the colonisation of leaf surfaces.
vi. Most recently, because of their capacity to bind metals, a role in metal uptake or in reducing metal toxicity.

v. Induced systemic resistance

Induced protection of plants against pathogens by biotic or abiotic agents has been reported since the 1930s (Ramamoorthy *et al*., 2001). Several terms have been used to describe the phenomenon of induced resistance, e.g. “systemic acquired resistance (SAR)”, “translated resistance” and "plant immunisation” (Ramamoorthy *et al*., 2001). A number of papers have discussed and reviewed induced systemic resistance including Weller (1988), Ahmed and Baker (1987), Meera *et al*,. (1994), Liu *et al*., (1995), Meera *et al*., (1995), Tilak *et al*., (1999), Press *et al*., (2001), Zehnder *et al*., (2001) and Zhang *et al*., (2001).

Induced resistance is defined as enhancement of a plant's defensive capacity against a broad spectrum of pathogens and pests that is acquired after appropriate stimulation (Glick and Bashan, 1997). The resulting elevated resistance due to an inducing agent upon infection by a pathogen is called induced systemic resistance (ISR) or SAR. The induction of systemic resistance by rhizobacteria is generally referred to as ISR, and that by other agents as SAR (Campbell, 1989; Whipps, 1997; Ramamoorthy *et al*., 2001; Zehnder, *et al*., 2001).

The biotic inducers of SAR include virulent pathogens, non-pathogens and elicitors derived from fungal cell wall metabolites. Abiotic agents acting as elicitors are salicylic acid (SA), ethylene, dichloro-isonicotinic acid and benzothiadiazole (Ramoorthy *et al*., 2001). The utilisation of natural PGPR as inducers of plant defence responses may increase their applicability and offer a practical way to induce immunisation (Ramammorthy *et al*., 2001). Inducers of systemic resistance have also been shown to promote the growth of many crop plants (Meera *et al*., 1994).

vi. Plant growth promotion

Over the last 25 years, there have been an increasing number of reports on promotion of plant growth following treatment of seeds, roots, cuttings, soil or artificial growth medium with bacteria and fungi (Whipps, 1997), particularly species of *Pseudomonas* and *Trichoderma* (Table 1.3). Growth promotion has been expressed in various of ways, but most commonly as an increase in germination, emergence, fresh or dry mass of roots or shoots, root length, yield and flowering. Indeed, the term plant growth promoting rhizobacteria (PGPR) has been coined specifically to describe bacteria which colonise roots and have the ability to stimulate plant growth (Glick and Bashan, 1997; Whipps, 1997; Benizri *et al*., 2001). The bacteria that provide some benefit to plants are of two general types, those that form a symbiotic relationship with the plant and those that are free-living in the soil, but are often found near, on, or even within, the plants roots. Beneficial free-living soil bacteria are usually referred to as PGPR or by a group of workers in China, as yield increasing bacteria (YIB) (Glick, 1995).

Growth promotion has frequently involved application of known biocontrol agents, but even so, the modes of action involved in the plant growth promotion observed have not always been clear. In soil containing a major pathogen such as *Pythium*, the growth promotion effect may well reflect biocontrol acting through mechanisms such as competition for nutrients and space, antibiosis (De Souza and Raaijmaker, 2000) and production of siderophores

(Ramamoorthy *et al*., 2001). Other important mechanisms include production of lytic enzymes such as chitinase and 1,3-glucanases, which degrade chitin and glucan present in the cell walls of fungi, hydrogen cyanide (HCN) production and degradation of toxins produced by phytopathogens (Baker, 1989; Tilak *et al*., 1999; Ramamoorthy *et al*., 2001).

Direct effects are commonly thought to be mediated by production of plant hormones such as auxins, cytokinins or gibberellins. It is difficult to obtain unequivocal evidence for their production in non-sterile soil, although several studies carried out in sterile conditions have implied their involvement (Whipps, 1997).

Associative nitrogen fixation may also occur with *Azospirillum*, *Azotobacter*, *Bacillus* and possibly some *Pseudomonas* spp. to increase plant growth directly. Production of vitamins, conversion of non-utilisable material to a form that can be used by the plant, and improved availability and uptake of some minerals may also contribute to the growth promotion phenomenon (Glick and Bashan, 1997; Whipps, 1997). Thus, PGPR can affect plant growth directly as well as indirectly. The direct promotion of plant growth by PGPR for the most part entails either providing the plant with a compound that is synthesised by the bacterium or facilitating uptake of certain nutrients from the environment. Indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms (Glick, 1995).

Table 1.3. Published reports on plant growth promotion following the application of bacteria and fungi to seeds, roots or growth media

Commodity	Disease	Pathogen	PGPR/F	Mode of application	Mode of action	Reference
Cucumber	Root rot	*Pythium aphanidermatum*	*Pseudomonas fluorescens* *P. corrugata,* *Serratia plymuthica*	Bacterial suspension.	Disease suppression.	McCullagh *et al.*, 1996.
Cucumber	Angular leaf spot Anthracnose	*Pseudomonas syringae* pv. *lachrymans* *Colletotrichum orbiculare*	*Bacillus pumilus* WRF, *Curtobacterium flaccumfaciens* ME1, *Bacillus subtilis* GBO3	Seed coating.	Induced systemic resistance.	Raupach and Kloepper, 2000.
Floricultural and horticultural crops	NI	NI	*Trichoderma harzianum*	Add condial suspension or peat-bran mixture to propagative beds and rooted cuttings.	NI	Chang *et al.*, 1986.
Loblolly and Slash pine	Post-emergence damping-off and seedling rot.	*Fusarium* sp. *Phytophthora* sp. *Pythium* sp. *Rhizoctonia* sp.	12 bacterial strains	Drenching bacterial suspension.	Direct or indirect.	Eneback *et al.*, 1998.
Lettuce	NI	NI	*Rhizoctonia solani* (non-pathogenic)	NI	NI	Sneh *et al.*, 1986.
Lettuce	NI	NI	*Streptomyces griseoviridis*	NI	NI	Tahuonen and Lahdenpera, 1988.
Lettuce	NI	NI	*Pseudomonas* spp.	NI	NI	Van Peer and Schippers, 1989.
Lettuce	NI	NI	*Trichoderma viride*	NI	NI	Coley-Smith *et al.*, 1991.

Table 1.3. cont…

Commodity	Disease	Pathogen	PGPR/F	Mode of application	Mode of action	Reference
Lettuce	NI	NI	*Trichoderma* spp.			Ousely *et al*., 1993; Ousely *et al*., 1994.
Lettuce, cabbage, onion	NI	NI	*Pseudomonas aeruginosa* R75, *P. cepacia* R85, *P. fluorescens* R111.	Mix with soil.	NI	Germida and de Freitas, 1994
Lettuce	NI	NI	*Pseudomonas putida, P. fluorescens*	NI	Phosphorus solublisation.	Rodriguez and Fraga, 1999.
Groundnut	Root canker	*Rhizoctonia solani* AG-4	*Bacillus subtilis*	Wettable formulation apply on seed.	Direct or indirect.	Turner and Backman, 1991.
Tomato	NI	NI	*Pseudomonas fluorescens*	Drench suspension on peat media.	Direct or indirect.	Gagne *et al.*, 1993.
Tomato	Tomato mottle virus	Virus	*B. amyloliquefaciens* 937b, *B. subtilis* 937b, *B. pumilus* SE34	Seed treatment with powdered spore formulation.	Induce resistance.	Murphy *et al*., 2000.

PGPR/F = plant growth promoting rhizobacteria or fungi.

NI= not indicated.

1.4.4.6. Biocontrol of *Pythium* in hydroponic systems

Soilless substrates lack the microbial diversity and biological "buffering" found in natural soils. In natural soils, soilborn pathogens such as *Pythium* spp. are limited by antagonism from other microorganisms and are subjected to nutrient competition and fungistasis (Cook and Baker, 1983; Paulitz, 1997). Thus, if *Pythium* is introduced into a natural soil, its spread is much slower than when introduced into a substrate that is essentially sterile. One of the commonest problems in hydroponic systems is infestation by *Pythium* spp. Most growers do not realise that *Pythium* is an 'opportunistic' fungus representing a typical r-strategist that often takes advantage of low population pressures from other microbes (Goldberg and Stanghellini, 1991).

Biological control would seem to be ideally suited for soilless systems. If biocontrol is to be successful anywhere, it will be in a closed structure with a soilless system (Paulitz, 1997; Utkhede *et al*., 2000). One of the reasons for the failure of biocontrol in field agriculture has been the lack of consistency, often caused by unfavourable environmental conditions. Biocontrol agents are living organisms and are sensitive to temperature, moisture and pH. However, the environmental conditions in greenhouses are more uniform and can be adjusted to make conditions favourable for the growth of the biocontrol agent and unfavourable for the pathogen. Biocontrol agents can easily be added to nutrient solutions in a hydroponic system and be dispersed (Paulitz, 1997; Niederwieser, 2001).

There are, however, some disadvantages inherent to greenhouse inoculation experiments. One of the drawbacks is that they do not reflect exactly what happens in a commercial greenhouse. Most systems start out clean, but the pathogen is introduced at a later stage, probably at low population densities, in a random fashion, and possibly several times during the cropping cycle. In most experiments, the biocontrol agents are added to the soilless system at the start, before adding a massive dose of pathogen inoculum (Paulitz, 1997). Under these conditions, the biocontrol agents cannot completely control disease, so most biocontrol treatments have disease severities midway between the inoculated non-protected control and the non-inoculated healthy control (Paulitz, 1997). In hydroponic systems growers expect complete control of particularly soilborne diseases. Experiments should be performed with the introduction of a low inoculum density of the pathogen. Under these conditions, the biocontrol agent may prevent the explosive build-up of

pathogen populations in the system by delaying or reducing its establishment and keeping its population density below the economic threshold (Paulitz, 1997; Paulitz and Belanger, 2001).

Despite the many advantages of biocontrol in a hydroponic system, a review of literature from 1985 to the present, produced relatively few reports dealing with this method of disease control in soilless systems, particularly when compared to the expansion of research activities focusing on developing biocontrol systems for above-ground diseases. Most of the studies in hydroponic systems involve rhizobacteria such as *Pseudomonas* and *Bacillus*. For example, *Pseudomonas* reduced cucumber root colonisation by *P. aphanidermatum* (Moulin *et al*., 1994) and *Pythium* diseases on cucumber in a closed rock wool system (Postma *et al*., 1995). *Trichoderma* spp. have been the most widely tested fungal biocontrol agents and have been reported active against *Pythium ultimum* on cucumbers (Wulff *et al*., 1998).

Goldberg and Stanghellini (1991) found control of *Pythium* provided by introduced bacterial antagonists in hydroponic systems to be inconsistent. Rankin and Paulitz (1994) significantly reduced diseases caused by *P. aphanidermatum* in rock wool-grown cucumber with an isolate of *Pseudomonas corrugata* and *P. fluorescens*, but could not increase yield comparable to the healthy control. McCullagh *et al*. (1996), on the other hand, reported significant control of *Pythium* root rot in cucumber, as well as increased yield, by the above two *Pseudomonas* species.

1.5. CONCLUSION

The hydroponic industry is becoming important due to increasing urbanisation and less arable land available close to cities, in area where the climate is not conducive to vegetable production and the increasing demand by consumers for better quality produce (Niederwieser, 2001). However, due to the nature of soilless culture, root pathogens, particularly those producing motile zoospores, can easily be established. *Pythium* spp. are therefore the major root rot pathogens of hydrophonically grown crops worldwide (Niederwieser, 2001).

Control of *Pythium* in hydroponic systems is difficult once the pathogen has established itself. Control measures relying on sanitation require sophisticated and laborious procedures. Chemical control is limited by the lack of registered products. Similarly, disease resistant cultivars

specifically developed for hydroponic conditions are not available. Even surfactants reportedly effective in controlling *Pythium* spp. (Stanghellini *et al.*, 1996), have inherent limitations. The most noticeable of these is the fact that they have no effect on fungal structures possessing a cell wall (e.g. hyphae, sporangia and encysted zoospores).

For commercial production, yield reductions caused by soilborne diseases have to be limited to ensure that losses remain below the economic threshold level. Some success has been achieved with biological control of soilborne pathogens in hydroponic systems. Biological control may therefore offer an alternative approach to disease management in soilless systems. Ample scope exists for future development of biological control in hydroponic systems, particularly with biocontrol agents adapted to the aquatic environment. As stated by Linderman *et al*., (1983): "Investigators of biological control of plant pathogens need to develop tenacity and avoid becoming discouraged and frustrated. Perhaps the challenge to persist may come from the knowledge that somewhere there is a working biocontrol system for nearly all plant diseases. The challenge is to find it, make it work, and then understand it".

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CHAPTER 2

ANTAGONISTIC ACTIVITIES OF SOILBORNE MICROORGANISMS ISOLATED FROM THE NYL FLOOD PLAIN IN SOUTH AFRICA

ABSTRACT

Bacteria and fungi were isolated from the rhizosphere of grasses and sedges at Nylsvley Nature Reserve in South Africa. About 150 rhizobacteria and 49 rhizosphere fungi were isolated from ten different sites at Nylsvley Nature Reserve. The rhizobacterial isolated were mostly Gram negative (72%) and rod shaped (73%). The dominant fungal genera were *Trichoderma, Aspergillus*, *Penicillium*, *Neosartorya* and *Fusarium*. Out of the 49 fungal isolates, *Trichoderma* spp. (26%) were the most dominant followed by *Aspergillus* spp. (24%). The isolated fungi were evaluated *in vitro* by means of the dual culture assay against a range of plant pathogens for general screening namely *Colletotrichum gloeosporioides*, *Pythium irregulare*, *Penicillium digitatum*, *Fusarium solani* and *Geotrichum candidum*. The bacterial isolates were also screened *in vitro* against selected test pathogens i.e. *C. gloeosporioides*, *Geotrichum* and *Fusarium* species. All rhizosphere fungal isolates showed positive antagonism (mycelium inhibition) against *G. candidum* (100%) and *F. solani* (100%). The rhizobacterial isolates showed positive antagonism against *G. candidum* (71%) and *C. gloeosporioides* (76%). These findings indicate that the rhizosphere microbial populations of some grasses and sedges harbour potential biocontrol agents with potential to control root diseases.

2.1. INTRODUCTION

The interest in using microorganisms to achieve the dream of low input sustainable agriculture and to circumvent expensive and possibly environmentally deleterious agricultural chemicals is increasing. It also focuses new attention on finding useful and efficient microorganisms from the rhizosphere in biological control of plant pathogens (Benizri *et al*., 2001). The rhizosphere is the root zone where interactions between soil, microorganisms and the plant takes place (Bowen and Rovira, 1999; Benizri *et al*., 2001). It is a region of intense microbial activity, driven by root exudates (Bowen and Rovira, 1999). Benizri *et al*. (2001) divided the zone into three parts: the ecto-rhizosphere (adhering soil), which is defined as the

soil adjacent to living roots; the rhizoplane or root surface; the inner area of the root (rhizodermal and cortical cells).

The rhizosphere is able to support high microbial activity due to its high carbon concentration provided by rhizodeposition (Lynch and Whipps, 1990). Rhizodeposits are usually considered to be secretions (compound actively released as a result of metabolic processes), exudates (compounds released by autolysis of older rhizodermal cells) and sloughed off cells (Lynch and Whipps, 1990). The varying types and quantities of these compounds have been postulated to act as key factors influencing the density and diversity of rhizospheric microorganisms (Baudoin *et al*., 2001).

A number of different bacteria may be considered to be plant growth promoting rhizobacteria (PGPR) including *Azotobacter*, *Azospirillum*, *Pseudomonads*, *Acetobacter*, *Burkholderia*, *Enterobacteria* and *Bacilli* (Glick and Bashan, 1997). There are also fungi that have growth promotion and biocontrol activity, including *Phoma*, *Trichoderma* and *Penicillium* species as well as non-sporulating fungi isolated from zoysia grass (*Zoysia tenuifolia* Willd. ex Trin.) (Meera *et al*., 1994; Shivanna *et al*., 1994; Meera *et al*., 1995; Shivanna *et al*., 1996).

Various reports deal with enhanced biological control and stimulation of plant growth by means of microorganisms derived from the rhizosphere of natural environments (Myatt *et al*., 1992; Paulitz *et al*., 1992; Shivanna *et al*., 1994; Shivanna *et al*., 1996; Landa *et al*., 1997; Bowen and Rovira, 1999; Tilak *et al*., 1999). For instance, studies revealed that several fungi including species of *Phoma*, *Trichoderma* and *Penicillium* and non-sporulating fungi isolated from zoysia grass (*Zoysia tenuifolia* Willd. ex Trin.) rhizosphere promoted plant growth and suppressed soilborne fungal diseases in a number of crop plants (Shivanna *et al*., 1994; Shivanna *et al*., 1996). Moreover, efforts to isolate, select and apply plant growth promoting rhizobacteria (PGPR) for control of specific soilborne fungal pathogens have been reviewed (Kloepper 1993; Glick and Bashan, 1997).

This chapter describes the isolation and identification of bacteria and fungi from the rhizosphere of some grasses and sedges in the Nylsvley Nature Reserve, an area within the Nyl flood plain. It also describes the *in vitro* screening of the isolates for their antagonistic activity against a number of selected phytopathogenic fungi.

2.2. MATERIALS AND METHODS

2.2.1. Isolation of microorganisms

Isolation of microorganisms was conducted along the Nyl flood plain of Nylsvley Nature Reserve, an area of about 16000 ha extending in a north-north easterly direction from Kranskop Mountain near Nylstroom towards Potgietersrus in Limpopo Province (Fig 2.1, 2.2). The Nylsvley Nature Reserve is located in extensively undulating to flat terrain between 1050 and 1080 m.a.s.l. The climate is semi-arid with a mean annual temperature and rainfall of and 18.6 ^{0}C and 587 mm, respectively. The dominant grass species in flooded sections is *Oryza longistaminata* A. Chev. & Roehr. Various other grasses are also present, particularly the emergent *Panicum schinzii* Hack., *Setaria sphacelata* (Schumach.) Moss and Leersia, *Sporobolus* and *Acroceras* spp. Dominant sedges include *Cyperus*, *Schoenoplectus* and *Eleocharis* spp. Trees such as *Acacia caffra* (Thunb.) Willd., *A. karroo* Hayne, *A. nilotica* (L.) Willd. ex Del. subsp. *kraussiana* (Benth.) Brennan and *A. tortillis* (Forsk.) Hayne subsp. *heteracantha* (Burch.) Brenan are scattered throughout the area. Agricultural activities in the region around the Nyl Flood Plain are diverse and include the cultivation of field crops, vegetables, subtropical and deciduous fruit (Barnes*, et al*., 2001).

In January 2001, root zone soil was collected from 10 randomly selected sites in Nylsvley Nature Reserve (Fig. 2.1). Some of the sites were at the edge of the swampy area and others in the dry grassland (Table 2.1, Fig. 2.2). Three samples were taken to a depth of ca. 25 cm at each site, pooled in a plastic bag and transported in an ice-chest to the laboratory at the University of Pretoria.

At the laboratory, each composite sample was mixed thoroughly and 50 g sub-sample serially diluted in Ringer's solution (Johnson and Curl, 1972; Truelove, 1986). The dilutions were plated in duplicate on tryptic soy agar (Difco) and the medium of Martin (1950) for the enumeration of bacteria and fungi, respectively. Plates were incubated in the dark at 28 ^{0}C for six days and the number of colonies counted at each plate. Representatives of each morphologically distinct bacterial and fungal colony on each dilution plate were isolated. Fungal isolates were maintained in sterile water at room temperature and bacterial isolates in nutrient broth (Merck) containing 15% glycerol at -70 ^{0}C. After dilution plating, the various

soil samples (with the roots contained in them) were dried to constant mass at 60 ^{0}C, which was used for calculating the cfu g $^{-1}$ of soil and root.

Fungi were identified according to references in Hawksworth *et al*. (1995), whereas bacteria were characterised according to pigmentation, cell and colony morphology, Gram staining and Hugh-Leifson's test according to Schaad *et al*. (2001).

2.2.2. Antagonistic action

Isolates of the phytopathogenic fungi, *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. (accessions 500 and 503), *Fusarium solani* (Mart.) Appel & Wollenw., *Geotrichum candidum* Link, *Penicillium digitatum* (Pers.: Fr.) Sacc. and *Pythium irregulare* Buisman were selected from the culture collection of the Department of Microbiology and Plant Pathology, University of Pretoria. The isolates were revived on potato-dextrose agar (PDA) (Difco) for three days at 28 ^{0}C.

Antagonistic activity of the fungal isolates from Nylsvley Nature Reserve was determined by placing a 7-mm-diameter plug from the periphery of an actively growing colony of the isolate and the pathogen 4 cm from each other on the surface of a 90 mm-diameter PDA plate and incubated the plates at 25 ^{0}C. An uninoculated control (not challenged with rhizosphere fungal isolates) was included. Growth of the pathogens was measured in mm after five, seven, 10 and 14 days, and the percentage growth inhibition and relative growth rate calculated according to the formulae (Paulitz *et al*., 1992; Landa *et al*., 1997; Bevivino *et al*., 1998):

$$\text{Percentage growth inhibition} = \frac{R_1 - R_2}{R_1} \times 100$$

$$\text{Relative growth ratio} = \frac{R_2}{R_1}$$

Where R_1 is the radial distance grown by fungus with out the isolate (control value) and R_2 is the distance grown on a line between the inoculation position of the fungal phytopathogens and the antagonist isolate (inhibition value).

The bacterial isolates were screened against three of the pathogens, viz. *C. gloeosporioides* isolate 503, *F. solani* and *G. candidum*. Each bacterial isolate was streaked in triplicate along

three equidistant curved lines at the periphery of a 90-mm-diameter STD1 agar plate and incubated in dark at room temperature for 48 hours. A 7-mm-diameter plug from the edge of an actively growing culture of a pathogen was then placed centrally in each plate (Fig 2.3), and the plates re-incubated at room temperature in the dark. Growth of the pathogen was measured after five, 10 and 15 days and the extent of inhibition calculated as above. Each experiment was replicated three times in a completely randomized design (CRD).

2.2.3. Statistical analysis

Data of the antagonist screening experiments were subjected to analysis of variance (ANOVA). When a significant ($P < 0.05$) F-test was obtained from the treatment, separation was accomplished by the least significance difference ($LSD/CD_{0.05}$ and $LSD/CD_{0.01}$).

2.3. RESULTS

2.3.1. Isolation of microorganisms

Rhizosphere fungi were more abundant in swampy areas compared to open dry grasslands (Table 2.1). Total fungal counted ranged from $2.14x10^2$ to $2.2x10^3$ cfu g^{-1} in the rhizosphere. Rhizobacterial isolates seemed more or less uniformly distributed in the Nylsvley Nature Reserve. County ranged between $1.7x10^6$ and $2.0x10^7$ cfu g^{-1} (Table 2.1). Based on macroscopic characters such as pigmentation, size and shape of the colony, more than 150 rhizobacteria and 49 fungi were isolated. Of the 49 rhizosphere fungal isolates that were collected, most belonged to the genera *Aspergillus*, *Fusarium*, *Neosartorya*, *Penicillium* and *Trichoderma* (Table 2.2). *Trichoderma* (26%) was the most abundant followed by *Aspergillus* spp. (24%) (Fig. 2.4).

Most rhizobacterial isolates were Gram-negative (72%), while only 16% were Gram-positive and the rest of the isolates were variable (12%). Most isolates were rod shaped (73%), Only a few cocci, coccobacilli, diplocci and tetrade isolate were found among the total number of rhizobacterial isolates (Fig. 2.5). The Hugh-Leifson oxidation-fermentation medium revealed that most of the Gram-negative rods were strictly aerobic or had an oxidative metabolism (41%), whereas 31% were facultative or had a fermentative metabolism.

2.3.2. Antagonistic action

Most of the rhizosphere fungal isolates significantly inhibited the *in vitro* mycelium growth of the phytopathogens, albeit to differing degrees (Appendix 1-6). Of the 49 rhizosphere fungal isolates tested against *F. solani* and *P. irregulare,* 39 and 38 isolates consistently inhibited the mycelium growth of the pathogens respectively (Figure 2.6 and Table 2.3). The general trend in the fungus to fungus dual culture assay were that, out of the total 49 indigenous rhizosphere fungal isolates, 100% of the isolates revealed positive antagonism against *G. candidum* and *F. solani* (Fig. 2.6).

In the bacterium to fungus *in vitro* antagonism assay, most of the indigenous rhizobacterial isolates showed a clear inhibition zone towards the phytopathogens (Fig. 2.3). In dual culture assays against *G. candidum* and *C. gloeosporioides*, the bacterial isolates differed significant ($P < 0.01$) in the extent of growth inhibition of this pathogen in all dates of evaluation (Appendix 8 and 9). However, the rhizosphere bacterial isolates exhibited less *in vitro* mycelium inhibition against *F. solani* (Appendix 7). In general, out of the total indigenous rhizobacterial isolates, 71 and 76% of the isolates showed positive antagonism towards *G. candidum* and *C. gloeosporioides* respectively (Fig. 2.7).

2.4. DISCUSSION

Not all organisms in the rhizosphere can be isolated by a single technique or by using one culture media. Soil-dilution and plate counting methods are commonly used even if it reveals only relative numbers of viable propagules (bacteria, fungal spores, mycelial fragments, microsclerotia) (Curl and Truelove, 1986). These techniques also favor the isolation of abundantly sporulating genera such as *Trichoderma*, *Aspergillus*, *Penicillium* and *Neosartorya* (Curl and Truelove, 1986), which agree with our results. Traditional plating methods for bacteria and fungi recover only a small percentage of organisms seen by direct counting under the microscope. Part of the discrepancy between plate counts and total counts is that total counts often do not discriminate between live and dead cells (Curl and Truelove, 1986). Another major reason why organisms that are isolated on commonly used media give a biased picture of the rhizosphere microflora, both qualitatively and quantitatively, is that these media are not suitable for the majority of soil microorganisms as they select for relatively fast-growing species. The majority of soil microorganisms are oligotrophs and characterised

by their small size (especially under low-nutrient conditions), complex shapes and often extremely slow growth rates (Poindexter, 1981; Koch, 1990).

In the present study, it was discovered that the density of micoflora that were isolated from open grasslands was generally more abundant than at the edge swampy area. This could be explained by the fact that in an area of low moisture content increased exudation of various compounds can be found which can result in a higher microbial population density (Curl and Truelove, 1986).

During this study, it was indicated that Gram negative, rod shaped bacteria with an oxidative metabolism is found in higher proportions than their counter parts. This is in agreement with findings by Rovira and Brisbane (1967) that applied numerical taxonomy to 195 rhizosphere isolates. The rhizosphere bacteria of wheat and clover were found to be Gram negative, poleomorphic species that multiply rapidly. Miller (1990) found that incubation temperature has a profound effect on the organism isolated. Many Gram negative organisms frequently have temperature optima between 20 ^{o}C and 30 ^{o}C in laboratory media whereas Gram positive organisms have temperature optima below 20 ^{o}C. It seems paradoxical that most isolated soil bacteria live optimally at 25 ^{o}C, while the predominant field soil temperatures are usually between 10 and 15 ^{o}C during the growing season (Bowen and Rovira, 1999). This could indicate selective enrichment of selected groups and that these species isolated may not represent the true rhizosphere population. Pertaining to rhizosphere fungi, it appears if the Nyl Flood Plain harbours a fairly unique population of soil fungi as none of the *Aspergillus*, *Fusarium* and *Penicillium* isolates could be identified to species level according to Raper and Fennell (1965); Domsch *et al*. (1980); Nelson *et al*. (1983); Pitt (1985); Klich and Pitt (1988); and Burgess *et al*. (1994).

The results obtained in the antibiosis *in vitro* screening assay indicated that the Nylsvley Nature Reserve soils supported indigenous rhizosphere bacteria and fungi that antagonise the major plant pathogens used for general screening. In a fungal-fungal dual culture plate assay, all the rhizosphere isolates inhibited the mycelium growth of *G. candidum* and *F. solani*. The percentage mycelium growth inhibition obtained in this study appears to be higher than the level of inhibition obtained with *P. Megasperma* f.sp. *medicaginis* Dreschsler from the study by Myatt *et al*. (1992) using Chickpea rhizosphere microflora.

Results from the dual cultures bioassays suggest that production of antifungal substances by these rhizosphere bacteria and fungi may be involved in the inhibition of mycelium of the fungal pathogens (Landa *et al*., 1997). From the result obtained, it is important to note that the antagonistic activity of indigenous rhizosphere bacterial and fungal isolates were effective against a diverse range of pathogens assayed in this dual culture experiment. *In vitro* screening for antibiosis is frequently used to select prospective antagonists, even though the *in vitro* activity may not be related to biocontrol in the field or greenhouse (Fravel, 1988). However, an extensive survey of antagonists by Broadbent *et al*. (1971) summarised the opposing viewpoints on the value of *in vitro* assays. In their study, 3,500 microorganisms were screened *in vitro* for antibiosis. Approximately 40% of these inhibited one or more of the nine tested pathogens on agar and of these, only 4% were effective biocontrol agents in soil. The authors noted that, while some microorganisms inhibited pathogens on agar they also did so in soil.

In the dual culture experiment, the rhizosphere fungal and bacterial isolates showed a lower percentage positive antagonism towards *P. digitatum* and *Fusarium* sp. respectively. Often microbial antagonists provide different results under *in vitro* assays (Spadaro *et al*., 2002). Variation in sensitivity even amongst isolates of the pathogen may also affect the usefulness of an *in vitro* assay (Fravel, 1988). For instance, Jones and Pettit (1987) reported considerable variation in sensitivity to gliotoxin among anastomosis group of *R. solani*. It is not surprising that the assay conditions affect results of antibiosis *in vitro* since the production of amongst other antibiotics is greatly affected by environmental factors (Castoria *et al*., 1997; Tilak *et al*., 1999; Castoria *et al*, 2001; Spadaro *et al*., 2002).

In conclusion, selection of organisms based only on *in vitro* assays can result in missing some potentially useful organisms. When the selection was based on *in vitro* tests regardless of the mode of action, more than half of the organisms did not show such inhibition of the pathogen when subsequently tested and would therefore not have been selected for *in vivo* test (Renwick *et al*., 1991). There is general agreement with the ability of the potential biocontrol agent to operate in the presence of the host plant and diseases under glasshouse or field conditions (Deacan, 1991; Andrews, 1992). Despite this, the dual culture test for inhibition is often the first part of screening, simply because of the ease with which it can be done and the impression (albeit false) that useful organisms are selecting; since the organism so obviously inhibits pathogen on the agar that it is hard to believe that it will not continue to do so in the

field (Campbell, 1994). Therefore, the rhizosphere fungal and bacterial isolates that showed spectrum of antagonism against previously mentioned pathogens were further evaluated for growth promotion and biocontrol activity *in vivo* conditions.

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Table 2.1. Total counts of rhizosphere microflora collected from different sampling sites at Nylsvley Nature Reserve

Site no.	Description of the collection site	Dry weight of soil and roots	Microbial concentration (cfu/g of soil and roots)[1]	
			Fungi	Bacteria
1	Sedges in Swampy area	15.9	$1.04x10^3$	$5.53x10^6$
2	*Cyprus* spp. grass under acacia canopy	46.63	$2.14x10^2$	$5.35x10^6$
3	Dry grass land under open sky (*Oryza longistaminata*)	44.67	$4.5x10^2$	$4.9x10^6$
4	Swampy and birds hide (*Cyprus* spp.)	27.14	$1.7x10^3$	$4.1x10^6$
5	*Oryza longistaminata* grass at the edge of swampy area	47.55	$1.5x10^3$	$5.7x10^6$
6	*Cyprus* spp at the edge of swampy area	22.69	$2.2x10^3$	$2.2x10^6$
7	*Brassica* spp. at the edge of swampy area	18.64	$1.6x10^3$	$3.2x10^6$
8	Sedges, reeds at hide in swampy area	31.74	$1.1x10^3$	$1.7x10^6$
9	*Oryza longistaminata* grass	39.72	$1.6x10^3$	$2.6x10^6$
10	Dry grass savanna land (*Panicum* and *Setaria*)	14.64	$1.0x10^3$	$2.0x10^7$

[1] Colony forming units per dry weight of roots with adhering soil.

Table 2.2. Identification of fungal isolates collected from the rhizosphere of plants growing in the Nylsvley Nature Reserve flood plain

Isolate no.	Collection Site number	Genus and / or Species isolated
1	6	*Penicillium* sp.
2	6	*Penicillium* sp.
3	3	Sterile
4	1	*Aspergillus* sp.
5	1	*Aspergillus* sp.
6	1	*Aspergillus* sp.
7	4	*Penicillium* sp
8	6	*Penicillium* sp.
9	4	*Aspergillus* sp.
10	1	*Aspergillus* sp
11	8	*Aspergillus* sp.
12	2	*Trichoderma harzianum* Rifai
13	2	*T. harzianum*
14	4	*Neosartorya fischeri* (Wehner) Malloch & Cain
15	1	*Aspergillus* sp.
16	8	*Pencillium* sp.
17	8	*Pencillium* sp.
18	9	*N. fischeri*
19	2	*T. harzianum*
20	6	*T. hamantum* (Bonord.) Bain.
21	9	*Fusarium* sp.
22	4	*Fusarium* sp.
23	6	*Trichoderma* sp.
24	4	*Aspergillus* sp.
25	5	*Aspergillus* sp.
26	6	*Fusarium* sp
27	6	*Penicillium* sp.
28	4	*N. fischeri*
29	6	*Trichoderma* sp.
30	5	*T. harzianum*
31	2	*Aspergillus* sp.
32	5	*Aspergillus* sp.
33	8	*Aspergillus* sp.
34	1	*N. fischeri*
35	3	*T. harzianum*
36	5	Sterile
37	3	*T. harzianum*
38	10	*Pencillium* sp.
39	6	*N. fischeri*
40	6	*T. harzianum*
41	5	*T. harzianum*
42	4	*T. harzianum*
43	5	*T. harzianum*
44	10	*Penicillium* sp.
45	6	*N. fischeri*
46	1	*N. fischeri*
47	10	*N. fischeri*
48	8	Sterile
49	6	Sterile

Table 2.3. Effect of different rhizosphere fungal isolates on the mycelium growth of common plant pathogens

Plant pathogens	**Rhizosphere fungal isolates[1] (%)**			
	Growth inhibition after 5 days (%)[2]		**Growth inhibition after 14 days (%)[2]**	
	< 30	**30 – 100**	**< 30**	**30 - 100**
Pythium irregulare	0.00	100.00	20.40	79.60
Penicillium digitatum	51.02	48.98	71.42	28.58
Geotrichum candidum	83.68	16.32	8.16	91.84
Colletotrichum gloeosporioides-503	36.73	63.27	28.57	71.43
Colletotrichum gloeosporioides-500	75.51	24.49	28.57	71.43
Fusarium solani	73.46	26.54	0.00	100.00

[1] Calculated out of the total 49 fungal isolates.

[2] Inhibition of mycelium growth of the pathogen which was incubated at room temperature (25 ^{o}C); each value represents a mean of three replicates on Petri dishes.

Table 2.4. Effect of different rhizosphere bacterial isolates on mycelium growth of common plant pathogens

Pathogens	Rhizosphere fungal isolates[1] (%)			
	Growth inhibition after 5 days (%)[2]		Growth inhibition after 15 days (%)[2]	
	< 10	10 - 100	< 10	10 - 100
Geotrichum candidum	49.64	50.35	76.59	23.41
Colletotrichum gloeosporioides-503	65.73	34.27	38.47	61.53
Fusarium solani	57.75	42.25	94.37	5.63

[1] Calculated out of the total 142 bacterial isolates.

[2] Inhibition of the mycelium growth of the pathogen that was incubated at room temperature (25°C); each value represents a mean of three replicates of Petri-dishes.

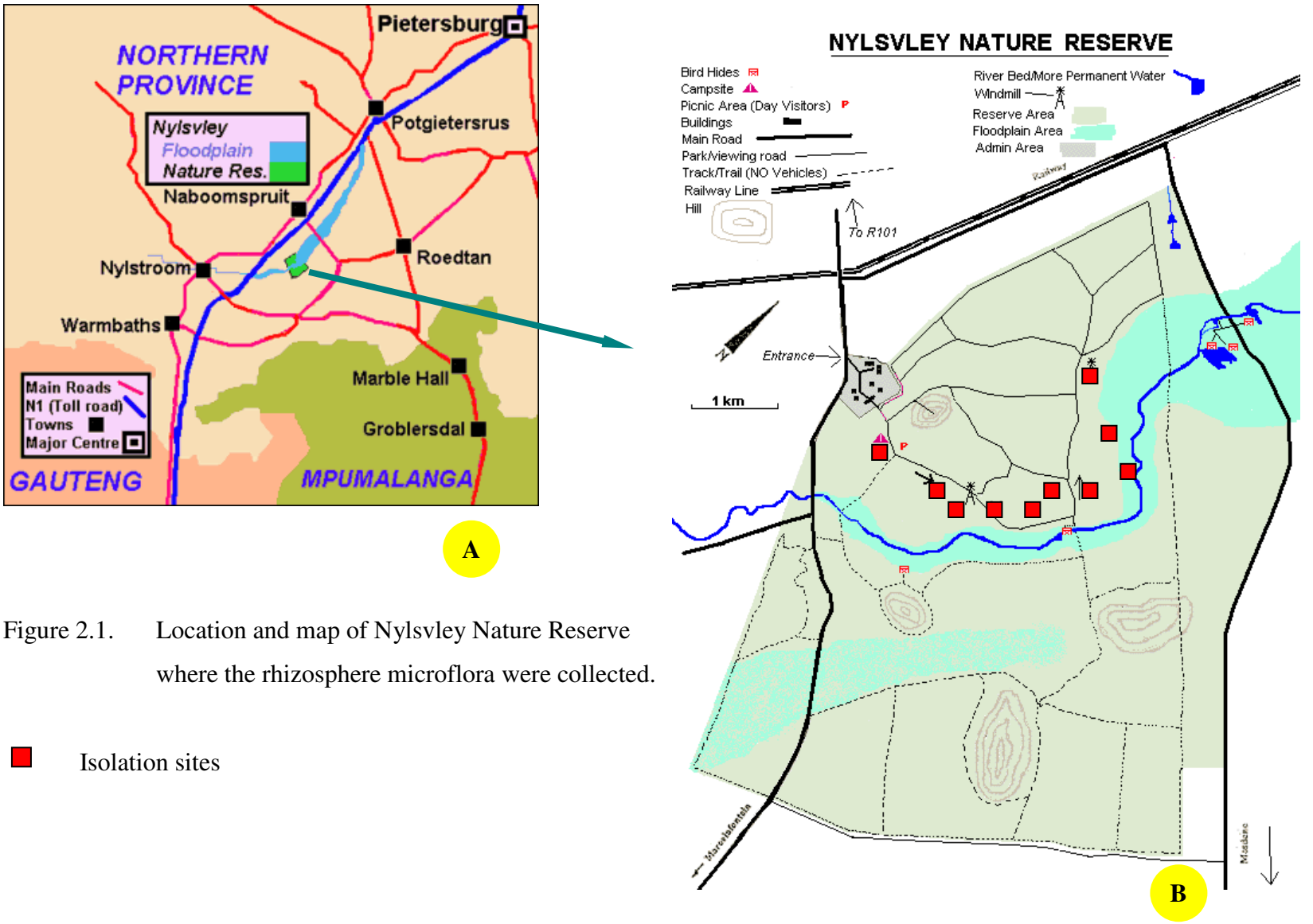

Figure 2.1. Location and map of Nylsvley Nature Reserve where the rhizosphere microflora were collected.

■ Isolation sites

Figure 2.2. The general appearance (A) and savanna grassland (B) dominated by long grass (*Oryza longistaminata* L.) in the Nylsvley Nature Reserve flood plain.

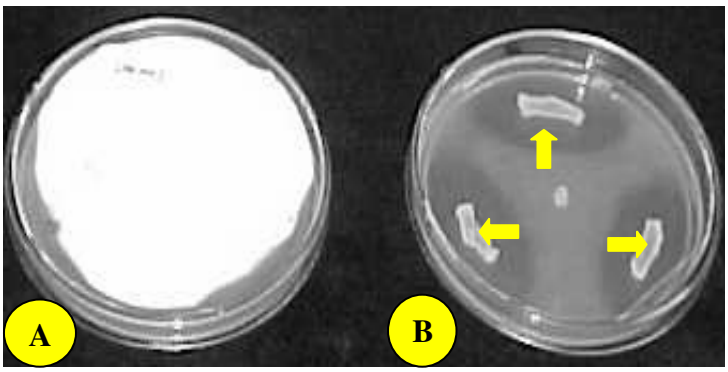

Figure 2.3. *In vitro* mycelium inhibition by rhizobacterial isolate, A: *Fusarium solani* not challenged by rhizobacterial isolate and B: *Fusarium solani* challenged with bacterial isolate 43B in a dual culture.

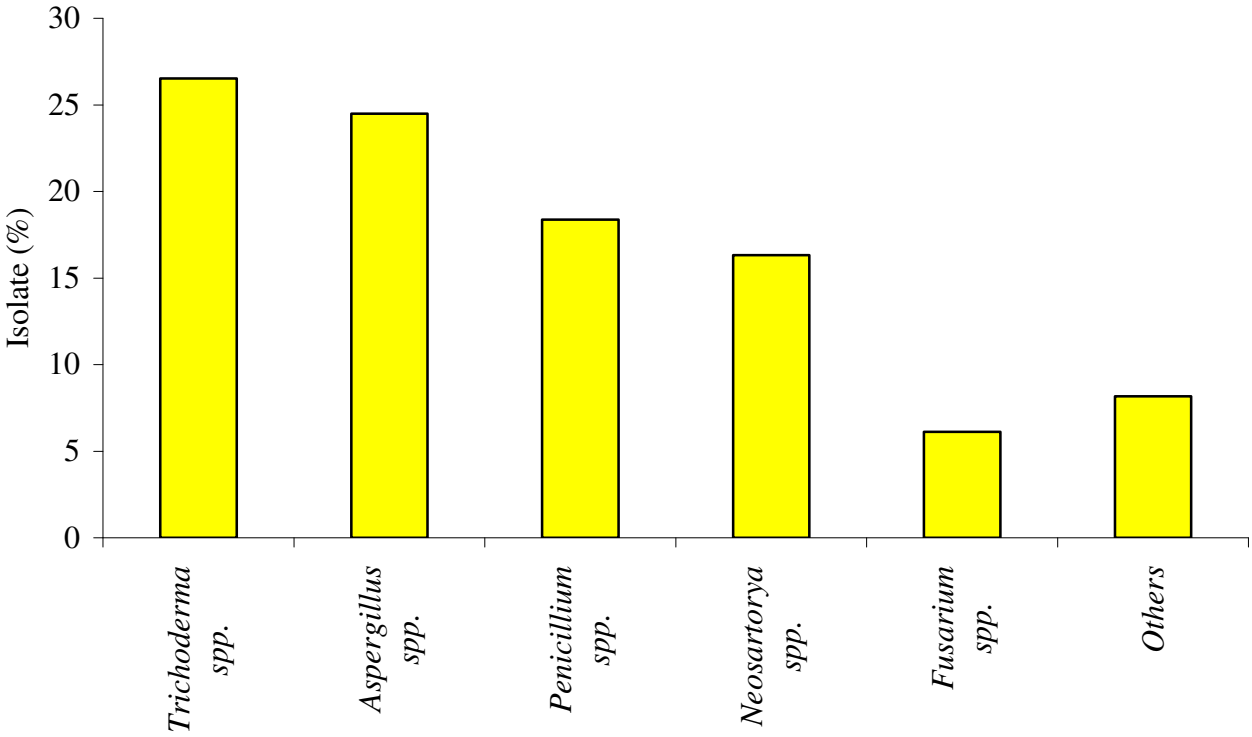

Figure 2.4. The proportion of rhizosphere fungal genera isolated from Nylsvley Nature Reserve.

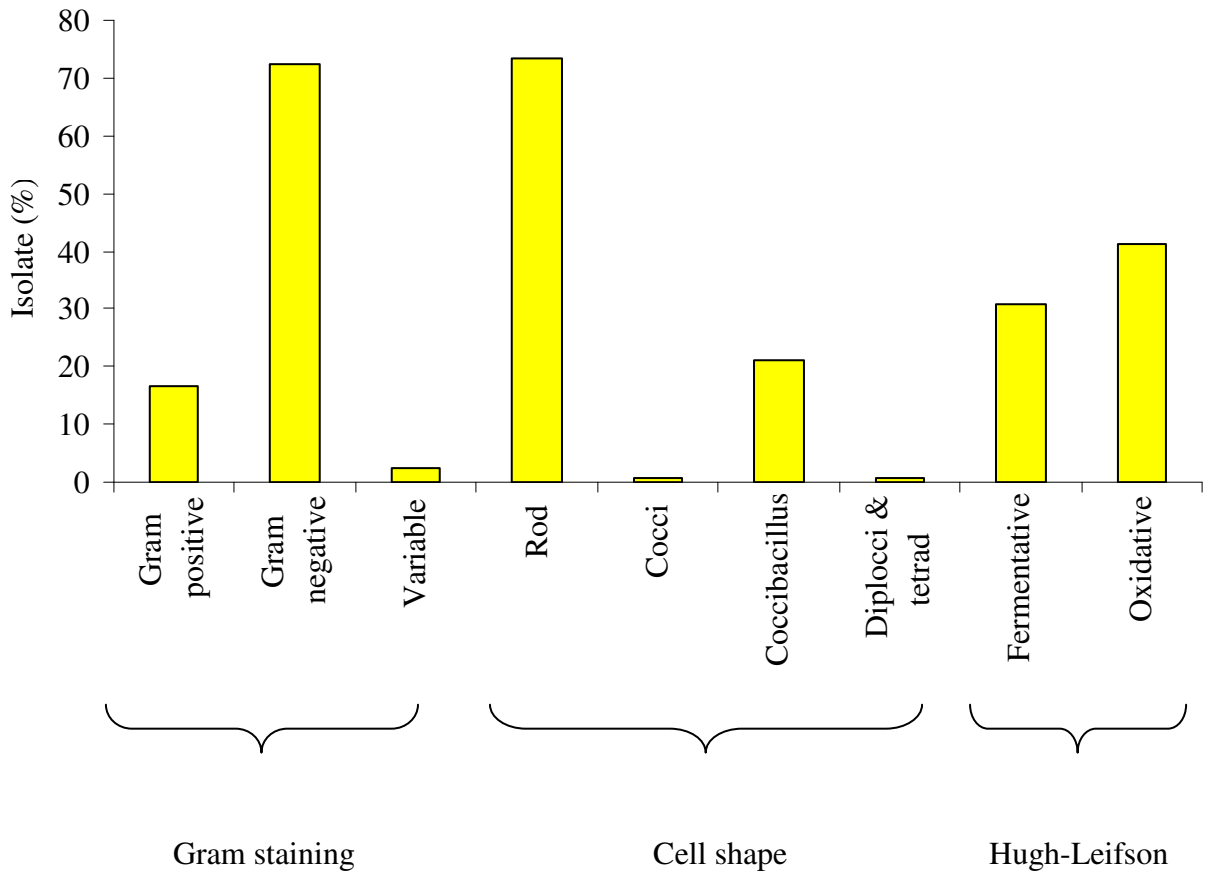

Figure 2.5. Gram staining, Morphological and physiological characteristics of bacteria isolated from the rhizosphere of grass and sedges grown in the Nylsvley Nature Reserve.

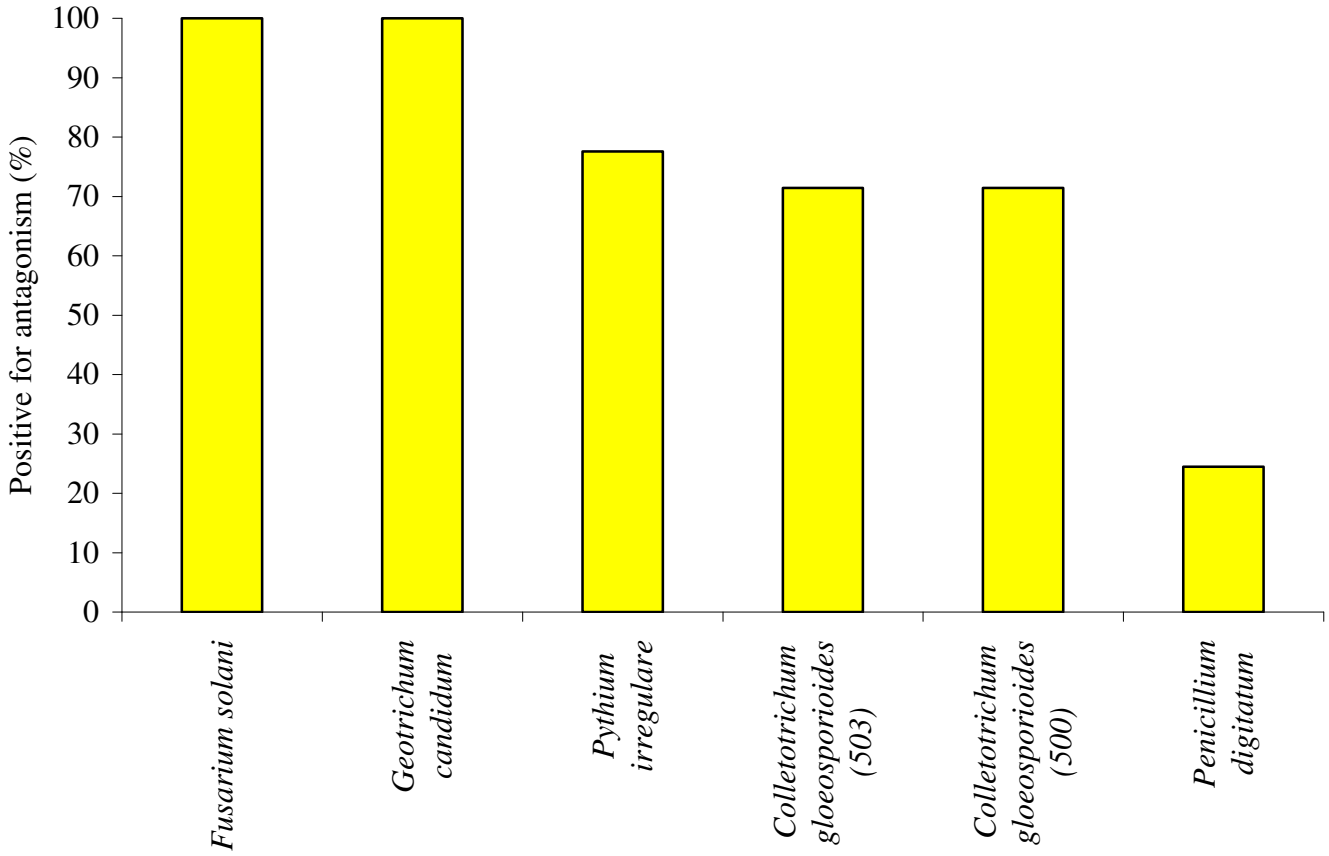

Figure 2.6. Antagonism of rhizosphere fungal isolates against the six phytopathogens tested for *in vitro* inhibition in the dual culture assay.

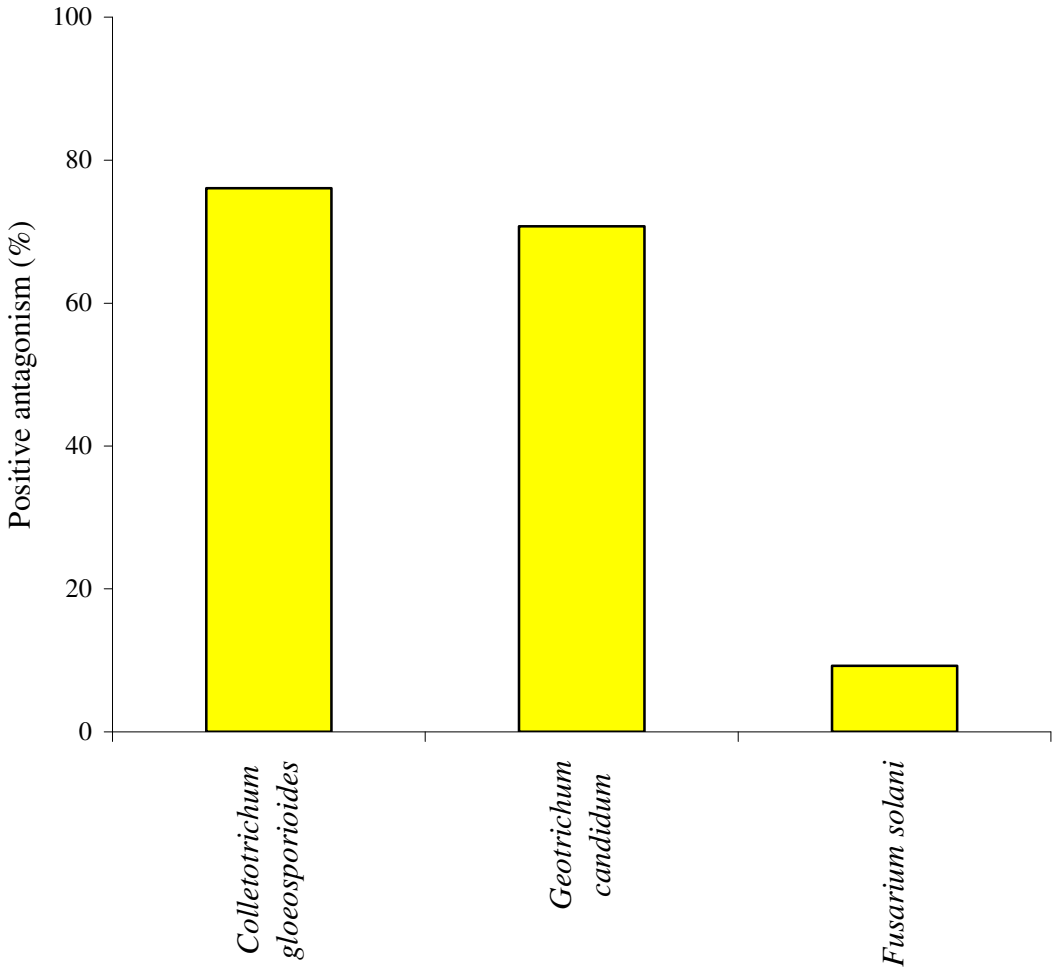

Figure 2.7. Antagonism of rhizosphere bacterial isolates against the three phytopathogens tested for *in vitro* inhibition in the dual culture assay.

CHAPTER 3

PRELIMINARY SCREENING OF RHIZOSPHERE ISOLATES FOR GROWTH PROMOTION OF LETTUCE SEEDLINGS AND BIOCONTROL OF ROOT ROT CAUSED BY *PYTHIUM* GROUP-F UNDER GREENHOUSE CONDITIONS

ABSTRACT

The microflora isolated from rhizosphere soil in the Nylsvley Nature Reserve were preliminary screened for growth promotion and biocontrol of *Pythium* root rot of lettuce under greenhouse conditions in a seedling tray system. In the first growth promotion experiments, four commercial products and 44 rhizosphere fungal and bacterial isolates were tested. Of all the treatments, the commercial product Bactolife™ and ten rhizobacterial isolates, viz. 87B, 114B, 57B, 68B, 107B, 91B, 20B, 4B, 90B and 24B resulted in improved fresh leaf weight in comparison with the non-inoculated control. In the second growth promotion experiment, most rhizosphere isolates and the commercial product Bactolife™ resulted in increased fresh leaf and root mass compared to the non-inoculated control. One commercial biocontrol product, Bactolife ™ and 21 rhizosphere fungal and bacterial isolates were screened for biocontrol of *Pythium* root rot. The bacterial isolate 43B caused a significant increase in fresh root mass compared to isolates 4B, 32B, 90B, 114B, 57B and 9B. The treatments with isolates 87B, 76B, 51B, 9B and Bactolife™ prevented root infection by *Pythium*. However, only isolate 68B showed significant root infection compared to the *Pythium* inoculated control.

3.1. INTRODUCTION

Agricultural production in greenhouses and other protected structures offers a unique niche for the development and use of biocontrol agents (Paulitz and Belanger, 2001). On the other hand, some diseases, which are of minor importance or even unknown in the field, may become a serious limiting factor in crop production in the greenhouse (Menzies and Belanger, 1996). From a phytopathological point of view, the high labour, high technological inputs in greenhouse production systems provide unique opportunities for disease control, especially by means of avoidance of infection. However, constant favourable environmental conditions for plant growth and development can also benefit the development and spread of pathogens. Thus, the grower is faced with the challenges of maintaining environmental conditions

optimal for plant growth, but not for disease development, a balance that is often difficult, if not impossible, to maintain.

The unique situation in greenhouses is firstly that most pathogens cannot be excluded from the greenhouse environment. Airborne spores enter through doors and screens; soilborne pathogens enter through dust, contaminated soil or shoes, tools or equipment and many pathogens are introduced on seeds or contaminated propagating plant material (Zinnen, 1988). Zoosporic pathogens enter through irrigation water and insects carry fungal inoculum. Although the temperature, light and fertiliser regimes are optimised for maximal plant growth, these conditions may also be favourable for the pathogen (Zinnen, 1988). Disinfected soil or soilless substrates such as peat or rockwool lack microbial diversity and biological buffering present in a natural soil ecosystem. In this biological vacuum, soil borne pathogens such as *Pythium* can grow and spread rapidly (Zinnen, 1988; Menzies and Belanger, 1996).

Some conditions that favour disease development also favour the management of disease control, particularly by means of biological control agents (Linderman *et al*., 1983). Environmental conditions such as temperature and relative humidity can be effectively controlled in the greenhouse. Like the pathogen, biocontrol agents are also sensitive to environmental fluctuations or extremes and unfavourable conditions in the field, which has been cited as a reason for failure or inconsistent performance. Conditions in the greenhouse can be more effectively optimised for biocontrol agents (Paulitz, 1997). Another reason why biocontrol has found a niche in greenhouse applications is because of the absence of registered fungicides. High registration and development costs and the lack of return on investments act as deterrents to chemical companies in registering products for the relatively small greenhouse market (Paulitz and Belanger, 2001).

Experiments previously conducted with certain sterile and sporulating fungi isolated from zoysia grass (*Zoysia tenuifolia* Willd. ex Trin.) and *Trichoderma* spp. enhanced growth remarkably in a variety of crops (Hyakumach *et al.*, 1992; Shivanna *et al*., 1994). Further, a diverse array of rhizobacteria, including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Klebsiella*, *Enterobacter* and *Serratia* has been shown to promote plant growth (Olmedo *et al*., 2000). Growth promotion has been expressed in a variety of ways but most commonly as an increase in germination, emergence, fresh or dry mass of roots or shoots, root length, yield and flowering (Whipps, 1997). The purposes of the present chapter were the

screening of rhizosphere microflora for biocontrol of *Pythium* root rot and growth promotion of lettuce seedling under greenhouse conditions.

3.2. MATERIALS AND METHODS

3.2.1. Host and pathogen

Butterhead lettuce (*Lactuca sativa* L. var. *capitata* L.) was used throughout these experiments. Two-week-old seedlings were obtained from Hydrotech (Pretoria) in a polystyrene seeding tray. An isolate of *Pythium* group-F was obtained from a previous study (Gull, 2003). Stock cultures of the fungus were maintained as described in chapter two and fresh cultures were grown on V8-juice agar medium.

3.2.2. Greenhouse conditions

Polystyrene seedling trays, 67 cm long and 23 cm wide, containing 300 wells / tray (5 x 5 cm width and 6.5 deep) were used for these experiments. The wells were filled with steam pasteurised Canadian peat moss. Each treatment comprised three replicates of five plants/replicate (total of 15 plants/treatments). In order to avoid cross contamination between wells and to provide sufficient aeration for healthy growth of the seedlings, alternative rows were left empty and each alternate row of lettuce seedlings was treated with either the isolate or a commercial product. The plants were watered at ten-minute intervals for roughly two-minutes by means of automated micro irrigation system with overhead emitters. Inoculation with the pathogen was done 4-5 days after application of the rhizosphere isolates or commercial products in the biocontrol experiment. In the growth promotion experiments, rhizosphere isolates or commercial products were applied without *Pythium* inoculation. Fresh mass of roots and shoots as well as plant height were recorded after a month. Plant height was measured from soil level and percentage root infection was determined by random excising ten root segments ca. 2 mm long from each seedling and plating on BNPRA medium selective for *Pythium* (Masago *et al*., 1977) as modified by Botha and Coetzer (1996). Colonies developing from the root segments were transferred water agar supplemented with 30 g ml^{-1} β-sitosterol (Bates and Stanghellini, 1984), and incubated for three days at 25^0C before identification by microscopic examination.

3.2.3. Inoculum preparation

i. Fungi

Six fungal isolates that were obtained from the rhizosphere of some grasses and sedges of the Nylsvley Nature Reserve (Chapter 2) were screened for their effectiveness as biocontrol agents of root rot and growth promotion of butterhead lettuce. Fungal isolates were grown for five to seven days on Potato dextrose agar (Difco). Five mm diameter culture discs were transferred to 250-ml flasks containing 100 ml malt extract broth (Merck, BioLAB). The isolates were incubated without shaking at 28 ^{o}C for 15 days in darkness. The fungal mat was separated from the culture filtrate by filtering through a few layers of cheesecloth. The mycelia were thoroughly washed with sterile distilled water to remove the remaining culture medium from the mycelial mat and placed on Whatman no.1 filter papers to remove excess moisture. Seven gram of the mycelial mat was added to 100 ml of sterile water (Meera *et al*., 1994) and blended in a Waring blender for three minutes.

ii. Bacteria

The rhizobacterial isolates were streaked on STD1 agar (Merck, BioLAB) and incubated for 48 hours. Bacterial cells were collected with a sterile swab, mixed with Ringer's (Merck) and vortexed for two minutes. The final cell concentration was adjusted to 2 x 10^{6} cfu $^{-1}$ ml using a Petroff-Hauser counting chamber.

iii. Commercial products

Efficacy of the rhizosphere bacteria and fungal isolates was compared to four commercial biocontrol products, namely Bactolife A+B (Bactec, Newcastle), Extrasol, *Streptomyces grisecoviridis* and Tricoflow T (Hygrotec, Pretoria). A fungicide Fongarid (Novartis, Kempton park) was included as a standard. Five ml of each product was applied as side dressings to each seedling.

iv. *Pythium* inoculum

For inoculation with *Pythium*, zoospores were induced according to the method used by Paulitz *et al.* (1992). An isolate of *Pythium* group-F was cultured at 26 ^{o}C on V8-juice agar

plates 48 hours. The culture was cut into 1 cm wide strips and half of the strips were transferred to empty Petri dishes and flooded with 20-25 ml of sterile distilled water. After 30-60 minutes, the water was replaced with the same amount of fresh sterile water. The plates were incubated for 18 hours at 35 ^{o}C under fluorescent light to induce sporulation and subsequently exposed to 20 ^{o}C overnight to stimulate zoospore release. The concentration of zoospores was adjusted to 8 x 10^4/ml using a Haemocytometer.

3.2.4. Experiments

i. Growth promotion experiments

Based on results of the *in vitro* screening for antibiosis, 44 rhizosphere bacterial and six fungal isolates showing broad-spectrum antibiosis against previously mentioned phytopathogens (Chapter 2) were selected for large scale screening for growth promotion of lettuce seedlings (Table 3.1). Additionally, two *Trichoderma* species from the previous work of Adandanon (2001) and four commercial biocontrol products viz., Bactolife™ (10ml/100l of water), Extrasol™ (0.1g/l), Tricoflow™ (0.01 g/l) and *Streptomyces grisecoviridis* (1 ml/l) were included for comparison. Five ml of the rhizosphere isolates' inoculum or commercial products was applied for each plant and sterile water was used as control. Of the 44 rhizosphere isolates, 18 isolates which showed growth promotion activity were again tested in growth promotion final experiment (Table 3.3). A completely randomised experimental design with three replications was used in all experiments.

ii. Biocontrol experiments

The best performing 18 rhizobacterial and two fungal isolates from the large scale screening experiment (3.2.4.i) of growth promotion were selected for further evaluation (Table 3.2). In this experiment, isolate BSB (*Bacillus subtilis*) which previously showed biocontrol and growth promotion activity (unpublished data), a fungicide, Fongarid (250 g furalaxyl /kg a.i.) 1.6 g/l of water and the commercial biocontrol product Bactolife ™ (10ml/100l of water) were included for comparison. The selected fungal and bacterial isolates were challenge inoculated with *Pythium* group-F to determine their efficacy as potential biocontrol agents. According to a pilot study done previously to determine the concentration of zoospores, 8 x 10^4/ml zoospores were effective in reducing the growth and infection of the root of lettuce seedlings

at temperature (24 ^{o}C) similar to greenhouse condition (unpublished data). In all the experiments, this concentration of zoospore was applied four days after application of the rhizosphere isolates or commercial biocontrol products. Each plant in the treatment received 5ml of inoculum of rhizosphere isolates, commercial biocontrol products and *Pythium* zoospore suspension as a side dressing on Canadian peat moss once.

3.2.5. Statistical analysis

Data were analysed statistically according to the GLM procedure and means separation according to Duncan's multiple range test at 1 % level.

3.3. RESULTS

In the first growth-promotion experiments, the four bacteria 97B, 92B, 121B, 76B and 106B showed a tendency of improved growth in terms of plant height compared to the untreated control, although these effects were not statistically significant (Table 3.1). The same trend existed for fresh shoot weight. Bacterial isolates 57B, 87B, 114B, 57B, 68B, 107B, 91B, 26B, 4B, 90B and 24B showed a tendency of improved shoot weight compared to the untreated control although this was not statistically significant. Although most of the treatments resulted in improved root weight in comparison with the untreated control, only the treatment isolate 66B rendered a statistically significant effect (Table 3.1). In the second growth promotion experiment most rhizosphere isolates as well as Bactolife^{TM} A +B showed a tendency of improved plant growth in comparison with the untreated control, although these effects were not statistically significant (Table 3.3).

In the biological control experiment there were some significant differences amongst treatments, but none of the treatments differed significantly from the non-inoculated and *Pythium* inoculated control. Amongst the 22 rhizosphere isolates evaluate treatments with isolates 87B, 76B, 51B, 9B and Bactolife^{TM} A + B showed zero incidence of *Pythium* in the roots of treated plants compared to a 0.16% infection in the inoculated untreated control (Table 3.2) although these were not statistically significant.

3.4. DISCUSSION

It is a common phenomenon that the biocontrol treatments in screening experiments show a certain trend which might not be statistically significant, but can still be used for screening purposes. Similarly, in the present study many of the rhizosphere isolates and some of the commercial products did show growth-promoting effects although these effects were not statistically significant ($P < 0.01$). A similar observation was made by Romeiro *et al.* (2000), who showed that among 26 rhizobacteria evaluated for tomato plants, two rhizobacteria showed better growth promotion than control plants for all parameters evaluated although statistically not significant. It is also probable that the experimental system comprising lettuce seedlings grown in small cells for a period of one month was not ideal for optimum expression of growth-promoting effects. The procedure was however adopted because it provided for a large number of small experimental units which would be ideal for screening a large number of isolates. It is possible that the use of larger cellea seedling trays, providing for a greater root volume and a longer growth period (six to eight weeks) which might render more significant results.

The same constraints apply to the biocontrol experiments where effects were present although not statistically significant. Furthermore, in the biocontrol experiment the low incidence of *Pythium* in the inoculated control contributed to non-significant effect. Although the inoculation procedure was appropriate, it is conceivable that the Canadian peat used as a substrate in which the lettuce seedlings were grown, might have had a suppressive effect on the *Pythium* zoospores applied. A suppressive effect of organic substrates is a common phenomenon (Hoitink *et al*., 1991). It might therefore be advisable to use an inert non-organic substrate such as vermiculite in future screening experiments, and *Pythium* inoculum should be applied repeatedly. The results obtained during this study were however sufficient for selecting the most promising isolates for further testing.

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Table 3.1. Effect of different rhizosphere microorganisms and commercial biocontrol products on shoot and root growth of lettuce grown in Canadian peat in seedling tray under greenhouse conditions

No.	Treatments	Plant height (cm)[a]	Fresh shoots weight /plant (g)[a]	Fresh root mass/plant (g)[a]
1	Streptomyces	134.96 b	3.36 ba	1.14 b
2	Bactolife A+B	144.30 ba	3.92 ba	1.10 b
3	Tricoflow	136.86 b	3.68 ba	1.14 b
4	Extrasol	146.63 ba	3.44 ba	0.92 b
5	19F	136.20 b	3.30 ba	0.71 b
6	2F	129.63 b	3.14 ba	0.81 b
7	9F	141.76 ba	3.80 ba	1.26 b
8	*Trichoderma* sp.	133.83 b	3.42 ba	0.92 b
9	18F	135.20 b	3.31 ba	0.96 b
10	47F	126.30 b	3.02 b	1.13 b
11	*Trichoderma* sp.	136.30 b	3.48 ba	0.82 b
12	3F	129.96 b	3.22 ba	1.11 b
13	125B	124.06 b	3.34 ba	1.04 b
14	87B	133.40 b	3.93 ba	0.93 b
15	114B	143.10 ba	4.01 ba	1.08 b
16	57B	144.16 ba	4.63 a	1.06 b
17	72B	136.30 b	3.77 ba	1.17 b
18	68B	138.40 b	4.51 ba	1.02 b
19	93B	142.33 ba	3.42 ba	0.94 b
20	107B	139.30 b	4.22 ba	1.08 b
21	111B	124.86 b	3.26 ba	0.92 b
22	91B	136.10 b	3.96 ba	0.89 b
23	47B	135.43b	3.65 ba	1.08 b
24	105B	135.53 b	3.26 ba	0.89 b
25	49B	120.06 b	3.48 ba	0.97 b
26	43B	142.30 ba	3.71 ba	1.15 b

Table 3.1 cont…

No.	Treatments	Plant height (cm)	Fresh shoot weight/plant (g)	Fresh root mass/plant (g)
27	51B	133.76 b	3.82 ba	1.22 b
28	89B	137.73 b	3.77 ba	1.12 b
29	19B	123.30 b	3.66 ba	1.05 b
30	20B	138.50 b	3.90 ba	0.95 b
32	92B	147.3 ba	3.28 ba	0.81 b
33	121B	149.83 ba	3.80 ba	0.87 b
34	76B	147.63 ba	3.78 ba	1.18 b
35	4B	129.76 b	3.94 ba	1.15 b
36	66B	130.40 b	3.42 ba	3.20 a
37	59B	123.63 b	3.36 ba	1.68 b
38	97B	177.43 a	3.27 ba	1.04 b
39	83B	121.53 b	3.30 ba	1.10 b
40	147B	142.60 ba	3.62 ba	0.90 b
41	32B	143.30 ba	3.74 ba	1.21 b
42	106B	149.20 ba	3.76 ba	1.21 b
43	79B	139.87 b	3.40 ba	0.95 b
44	90B	133.53 b	4.12 ba	0.99 b
45	100B	125.97 b	3.29 ba	0.99 b
46	13B	137.87 b	3.78 ba	1.15 b
47	50B	125.50 b	3.09 ba	0.94 b
48	24B	140.53 b	4.34 ba	1.05 b
49	Control	145.87 ba	3.82 ba	0.98 b

In each column values followed by the same letters are not significant different according to Duncan's multiple range test, $P \leq 0.01$.

[a] Each value is the mean of three replicate rows containing five lettuce seedlings in each, evaluated once a month after inoculation with different rhizosphere microflora and biocontrol commercial products.

Table 3.2. Fresh shoot and root weight of lettuce as affected by *Pythium* group-F inoculation and treatment with different rhizosphere microflora

No.	Treatments	Plant height (cm) [a]	Fresh shoot weight/plant (g) [a]	Fresh root weight/plant (g) [a]	Root infection (%) [b]
1	BSB	168.17 ba	5.41 a	0.77 ba	0.16 b
2	87B	188.83 ba	5.75 a	0.76 ba	0.00 b
3	76B	199.17 ba	5.65 a	0.65 ba	0.00 b
4	4B	213.33 a	6.24 a	0.58 b	0.33 b
5	97B	185.33 ba	5.74 a	0.71 ba	0.16 b
6	32B	194.33 ba	4.63 a	0.58 b	0.33 b
7	106B	193.67 ba	5.84 a	0.70 ba	0.83 ba
8	90B	180.17 ba	5.95 a	0.64 b	0.83 ba
9	24B	191.67 ba	6.2 a	0.71 ba	2.33 ba
10	114B	199.67 ba	6.25 a	0.58 b	1.16 ba
11	57B	199.67 ba	5.59 a	0.64 b	1.33 ba
12	68B	170.83 ba	5.01 a	0.79 ba	3.16 a
13	107B	175.17 ba	6.3 a	0.76 ba	1.33 ba
14	91B	170.83 ba	6.6 a	0.91 ba	1.50 ba
15	43B	169.50 ba	6.23 a	1.06 a	0.50 b
16	51B	178.17 ba	6.88 a	0.91 ba	0.00 b
17	20B	187.33 ba	6.06 a	0.75 ba	1.16 ba
18	121B	150.5 b	5.86 a	0.93 ba	0.16 b
19	35F	157.83 b	6.18 a	0.76 ba	0.33 b
20	*Trichoderma* sp.	196.67 ba	5.37 a	0.70 ba	1.83 ba
21	9B	179.17 ba	5.69 a	0.6 b	0.00 b
22	Bactolife A+B	164.83 ba	6.8 a	0.87 ba	0.00 b
23	*Pythium* alone	162.00 ba	5.81 a	0.8 ba	0.16 b
24	Fongarid	156.71b	5.02a	0.41b	1.27ba
25	Control	160.17 b	4.75 a	0.79 ba	0.00 b

In each column values followed by the same letters are not significant different according to Duncan's multiple range test, $P \leq 0.01$.

[a] Each value is the mean of three replicate rows containing five lettuce seedlings in each, evaluated one month after inoculated with different rhizosphere microflora and commercial products.

[b] Each value is the mean of three replicate Petri dishes with ten citrus leaf disk in each, evaluated 3-6 days after incubated *Pythium* selective media.

Table 3.3. Effect of different rhizosphere microflora on growth of shoots and roots of lettuce

No.	Treatments	Fresh shoot weight (g) [a]	Fresh root weight (g) [a]
1	BSB	5.41 a	0.93 ba
2	87B	5.75 a	0.76 ba
3	76B	5.65 a	0.90 ba
4	4B	6.24 a	0.72 ba
5	97B	5.74 a	0.56 b
6	32B	4.63 a	0.66 ba
7	106B	5.84 a	0.69 ba
8	90B	5.95 a	0.70 ba
9	24B	6.23 a	0.66 ba
10	114B	6.25 a	0.75 ba
11	57B	5.59 a	0.73 ba
12	68B	5.01 a	0.93 ba
13	107B	6.33 a	0.88 ba
14	91B	6.63 a	0.80 ba
15	43B	6.23 a	0.84 ba
16	51B	6.88 a	1.13 a
17	20B	6.06 a	0.88 ba
18	121B	5.86 a	0.96 ba
19	35F	6.18 a	0.81 ba
20	*Trichoderma* sp.	5.37 a	0.88 ba
21	9B	5.01 a	00.86 ba
22	Bactolife A+B	6.80 a	1.04 ba
23	Control	4.75 a	0.70 ba

In each column values followed by the same letters are not significant different according to Duncan's multiple range test, $P \leq 0.01$

[a] Each value is the mean of three replicate rows containing five lettuce seedlings in each, evaluated one month after inoculated with different rhizosphere microflora and commercial products.

CHAPTER 4

EFFICACY OF DIFFERENT RHIZOSPHERE BACTERIAL AND FUNGAL ISOLATES AND COMMERCIAL PRODUCTS ON GROWTH PROMOTION AND BIOCONTROL OF *PYTHIUM* ROOT ROT OF LETTUCE IN A RE-CIRCULATING GRAVEL BED HYDROPONIC SYSTEM

ABSTRACT

The efficacy of 12 rhizobacteria and one fungal isolate as well as two commercial biocontrol products, viz. Biostart and Bactolife A + B were evaluated in a re-circulating hydroponic system for growth promotion of lettuce and biocontrol of *Pythium* root rot. Rhizobacteria isolates 24B (*Bacillus stearotermophilus*), BSB (*B. subtilis*), 57B (*B. cereus*) and 87B (*Proteus penneri*) significantly enhanced the fresh leaf weight of lettuce. Isolate *B. subtilis* consistently enhanced the fresh leaf and root weight by 29.82 and 24.31% compared to the untreated control. In the biocontrol experiments, treatments with rhizobacteria isolate 91B (*P. penneri*) and 43B (*B. pumilus*) significantly increased fresh leaf weight and suppressed *Pythium* root infection of lettuce. Isolate 91B (*P. penneri*) and 121B (*P. penneri*) significantly decreased the incidence of *Pythium* after the 1st and 2nd week of inoculation respectively in the re-circulating nutrient solution. The combination of rhizobacteria *B. subtilis* and *B. pumilus* showed a synergistic effect as reflected in increased fresh leaf weight and total biomass per plant whilst suppressing root rot caused by *Pythium* group-F. Isolate *B. subtilis* increased fresh leaf weight and total biomass whilst reducing the percentage yield reduction due to *Pythium* infection. It also significantly lowered *Pythium* incidence after the 1st week of inoculation, reducing it to zero at the 2nd and 3rd weeks. Treatment with *B. subtilis* reduced *Pythium* root infection in greenhouse experiments. These results demonstrate that various rhizobacterial isolates have growth-promoting effects as well as biocontrol capabilities against *Pythium* root rot on lettuce plants in a re-circulating gravel bed hydroponic system.

4.1. INTRODUCTION

Root diseases caused by *Pythium* spp. are a particularly acute problem in re-circulating nutrient systems because these systems offer an ideal environment for root pathogens to infect and spread (Gold and Stanghellini, 1985). Once these pathogens are introduced in these systems, their control is very difficult and sometimes the growers are forced to destroy the

crop. *Pythium* spp. can infect the tiny feeder roots of lettuce produced in hydroponic systems and cause yield reduction without visible disease symptoms. Thus, it is frequently impossible for commercial growers to determine if their lettuce crop is suffering from *Pythium* root rot (Utkhede *et al*., 2001).

Pythium can be easily introduced into soilless systems through infected water or movement of contaminated soil and plants from outside the system, soil on workers' shoes and also by fungus gnats (Paulitz *et al*., 1992). The lack of competing microorganisms in soilless culture exacerbates the problem, since *Pythium* species are pioneer colonizers and do not compete well with other microbes. Favourable moisture conditions, a susceptible succulent host and a mechanism for the rapid dispersal of the pathogen throughout the culture system increase the severity of *Pythium* disease in hydroponic culture (Zinnen, 1988).

Although fungicides have shown some promise in controlling *Pythium* in hydroponics (Gold and Stanghellini, 1985; Paulitz and Belanger, 2001), it is usually not a financially viable option to register these products for use in hydroponic systems. Phytotoxicity and residue problems must also be overcome. In addition, small industries often face a challenge in terms of not having new chemical products registered for use on their crops due to the chemical companies perceived small profit margins for such products. Surfactants that lyse zoospores may offer alternative means of disease control (Stanghellini and Tomlinson, 1987; Stanghellini *et al*., 1996). Manipulation of light, temperature and nutrient composition may also reduce the severity of disease (Gold and Stanghellini, 1985). Disinfestation of irrigation water by methods such as ultraviolet irradiation (Stanghellini *et al*., 1984), ozonation, chlorination, chlorine dioxide treatment (Mebalds *et al*., 1997) and filtration (Goldberg and Stanghellini, 1991) is an essential element in combating *Pythium* infection in hydroponic systems.

Enhancement of plant growth by plant growth promoting rhizobacteria or fungi (PGPR/F) can result in more than one mechanism operating to combat disease development and include biocontrol through competition, production of antibiotics or siderophores and increased nutrient availability through nitrogen fixation or organic and inorganic phosphate solubilisation (Olmedo *et al*., 2001). Under hydroponic conditions, inoculation responses were observed in all tested plant species inoculated with PGPR such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium* and *Pseudomonas* spp. These responses included increases in shoot dry weight and improved quality of the root system; reflected in an increase

in dry weight, total root length and root surface area (Caletti, 2000). However, little or no research has been done in South Africa to examine the potential of biocontrol organisms and PGPR/F in hydroponics. Closed, environmentally controlled greenhouses are more suitable for biocontrol than open fields, because of the uniform environmental conditions in the greenhouse and ease of introducing a large inoculum of the biocontrol agent. Antagonistic rhizosphere-colonising bacteria could be introduced into the nutrient solution as a potential control measure for *Pythium* root rot in soilless cultures (Rankin and Paulitz, 1994).

In this chapter the objective was to screen and evaluate rhizosphere-colonising bacteria and fungi isolated from a pristine environment (Nylsvley Nature Reserve) for biocontrol of root rot and growth promotion of hydroponically grown lettuce.

4.2.MATERIALS AND METHODS

The most promising twelve isolates of rhizobacteria and one fungal isolate (Appendix 10) that were previously screened in a seedling tray system (Chapter 3) were subsequently tested in a re-circulating gravel bed hydroponic system in greenhouses for their growth promoting ability and possible biocontrol of Pythium root infection on lettuce plants. Selected commercial biocontrol products viz. Biostart™ (Microbial Solutions, Kya Sand,) and Bactolife™ A+B (Bactec, Newcastle) were included in these experiments for comparative purpose. The re-circulating gravel bed hydroponic system (Fig. 4.9) comprised of fourteen units, each with a 100 l reservoir supplying nutrient solution to three troughs, 13 cm wide, 10 cm deep and 250 cm long. The troughs had an incline of 1:3 and were filled with 9.5 mm diameter granite chips to a depth of 5 cm. The entire system was sterilised by flushing first with 40% formaldehyde diluted at the rate of 10 ml/l water and subsequently with 10% sodium hypochlorite diluted at the rate of 10 ml/l water, followed by rinsing with tap water. A nutrient solution consisting of 0.9 g Agrasol™ O' 3:2:8, 0.6 g calcium nitrate monohydrate and 0.3 g Micromix™ l^{-1} tap water was circulated through the troughs by means of an IDRA® 300 lh^{-1} submersible pump returning to the reservoir by gravity flow. The nutrient solution was replaced once a week and the pH and electric conductivity were maintained at 6.9 and 2.10 δ respectively.

Butterhead lettuce seedlings were grown in steam-pasteurised Canadian peat moss. At the four-leaf stage, sixteen seedlings were transplanted into each trough with 20 cm spacing between plants, except for the biocontrol experiments where each trough was planted with four pre-inoculated and 12 uninoculated plants. Four to five treatments with three replicates

(each trough representing a replicate) were included in each experiment. Each experiment was replicated three times in a completely randomized design (CRD).

4.2.1. Biocontrol experiment

The rhizobacterial and fungal isolates and commercial biocontrol products were evaluated in five greenhouse experiments for biocontrol of *Pythium* group-F, are listed in Appendix 10. Six rhizobacterial isolates and various combinations of these isolates were included in this study (Table 4.2). The most promising isolates were subsequently compared in a final experiment.

Inoculum of the fungal isolate was prepared from a 7-day-old culture grown on potato dextrose agar (PDA, Difco). Three 10 mm diameter mycelial discs from the growing margin of the culture were transferred to a 250 ml flasks containing 100 ml malt extract broth (Merck, BioLAB) and incubated at 28 ^{o}C for 15 days in the dark without agitation. The fungal mat was separated from the culture filtrate by filtering through sterilised cheesecloth. The mycelia were blotted on Whatman no.1 filter paper to remove excess moisture, weighed and blended for 20 s in 200 ml sterile water in a Waring blender. The resulting suspension was added to nutrient solution at the rate of 7 g/l (Meera *et al*., 1994; Utkhede *et al*., 2002).

Inocula of the rhizobacterial isolates were prepared from 48-hour-old cultures on STD1 nutrient agar (Merck, BioLAB). Bacterial cells were gathered by means of swabs, suspended in Ringers' solution (Merck) and vortexed for two minutes. The final cell concentration was adjusted to 2x10^{6} CFU/ml using a Petroff-Hauser counting chamber. The inocula were applied to the nutrient solution of the relevant treatments in the re-circulating hydroponic system at the rate of 100 ml/100 l of solution in each reservoir. The commercial biocontrol products Biostart™ and Bactolife™ were both applied at the rate of 10 ml/100 l water according to the recommendation of the manufacturers. Inocula of all biocontrol treatment were added four days prior to introduction of the pathogen inoculum. Two controls were included in each experiment namely an untreated control receiving only the standard nutrient solution and a negative control receiving only *Pythium* inoculum.

A pathogenic isolate of *Pythium* group-F originally isolated from lettuce plant roots was obtained from a previous study (Gull, 2003). Inoculum was prepared by blending three 5-day-old V8-juice agar cultures of the isolate for 15 seconds in distilled water in a Waring blender

and diluting the resulting suspension to a volume of five liters. Prior to the start of the various experiments butterhead lettuce seedlings were inoculated by suspending their roots in the above *Pythium* suspension for two days. Four of these pre-inoculated seedlings were planted at the top of each trough to serve as a source of inoculum. The remainder of each trough was planted with 12 uninoculated seedlings.

The incidence of *Pythium* in the variously treated nutrient solutions was determined at weekly intervals. Samples of 500 ml of the nutrient solution were taken at the return pipe exiting from the bottom end of each trough and baited with 30 mm diameter citrus leaf discs as described by Grim and Alexander (1973). After 24 hours exposure in the nutrient solutions, the citrus leaf discs were transferred to BNPRA selective media (Masago *et al*., 1997) as modified by Botha and Coetzer (1996). *Pythium* growth was verified under a compound microscope at 100x magnification. The number of leaf discs rendering *Pythium* growth was recorded.

Three weeks after the start of the experiment, the seedlings were removed from the gravel and their roots rinsed clean under tap water. Their roots and shoots were subsequently separated and weighed. Root and shoot mass as well as total biomass of each plant was recorded. Ten root segments, ca 10 mm long were excised from each seedling, plated on BNPRA selective medium and incubated at 25^{o}C for three to six days. The number of root pieces rendering *Pythium* growth was recorded for each seedling. The identity of the *Pythium* that was re-isolated was confirmed on water agar supplemented with 30 µg ml^{-1} β-sitosterol (Bates and Stanghellini, 1984).

In the final biocontrol experiment the most promising isolates were evaluated, the four pre-inoculated plants at the top of each trough were evaluated as a separate set to give an indication of a curative effect of the treatments (Fig. 4.9). For these seedlings from each treatment, only fresh leaf weight, root weight and total biomass were recorded.

4.2.2. Growth-promotion experiment

The isolates and commercial biocontrol products that were evaluated in four greenhouse experiments for possible growth-promoting ability are listed in Table 4.2. These included one fungus isolate, nine rhizobacterial isolates and one biocontrol commercial product. Each rhizosphere fungus, bacterial isolate and commercial biocontrol product was first screened in greenhouses in three separate experiments. The most promising isolates from each experiment

were subsequently evaluated in a final experiment. The procedures for application of the inoculum of rhizobacterial and fungal isolates were the same as described for the biological control experiments.

Three weeks after the start of the experiment, the seedlings were removed from the gravel and their roots rinsed clean under tap water. Their roots and shoots were subsequently separated and weighed. Root and shoot mass as well as total biomass of each plant was recorded.

4.2.3. Identification of rhizobacteria

The rhizobacterial isolates that showed growth promotion and biocontrol ability were characterised by macroscopic (margin, elevation and configuration of the colony) and microscopic (cell shapes and arrangements) characteristics. They were Gram stained (Richard, 1994; Schaad *et al*., 2001) and inoculated in Hugh-Leifson's medium (Hugh and Leifson, 1953; Krieg and Holt, 1984; Richard, 1994; Schaad *et al*., 2001) to test for their ability to oxidize or ferment glucose. Oxidase activity was determined by means of cytochrome oxidase test. API 50 CH and API 20 E (Biomérieux, France) were used for identification of the rhizobacteria (Table 4.1).

4.2.4. Statistical analysis

All data were subjected to analysis of variance (ANOVA). When a significant ($P < 0.05$) F-test was obtained from the treatment, separation was accomplished by the least significance difference ($LSD_{0.05}$ and $LSD_{0.01}$) or Critical difference ($CD_{0.05}$ and $CD_{0.01}$) test.

4.3.RESULTS

4.3.1. Growth promotion

The results of growth promotion experiments 1, 2 and 3 are presented in Table 4.2. Significant differences ($P < 0.01$) occurred between treatments in experiment 1 in terms of plant height, fresh leaf weight and total biomass per plant, but not in terms of fresh root weight. In comparison with the untreated control, treatment with isolate 24B resulted in a significant increase in plant height (10.19 %) and fresh leaf weight (42.10 %).

Similarly in experiment 2, treatment with bacterial isolates showed significant effects ($P < 0.01$) in terms of increases in fresh leaf weight. In comparison with the untreated control,

treatment with bacterial isolates BSB and 57B caused increases in fresh leaf weight of 15.87% and 10.47 % respectively. However, no significant differences were recorded in terms of plant height and fresh root weight.

Out of the four bacterial isolates tested in experiment 3, no significant differences were recorded in leaf and root weight, whereas significant differences in plant height did occur. In comparison with the untreated control increase in plant height ranged from 7.31 to 10.18 %.

The results of the final evaluation experiment in which the best performing isolates from the previous experiments were tested are presented in Table 4.3. All the bacterial isolates namely BSB, 24B and 87B resulted in a significant increase in fresh leaf weight of 28, 10 and 24 % respectively over the untreated control. Treatment with BSB also increased root weight by 24.31 % although this was not statistically significant.

4.3.2. Biocontrol

Results of the biocontrol screening experiments 1 and 2 are presented in Table 4.4. Inoculation with *Pythium* caused only a small decrease of 3.27 g in total biomass compared to the uninoculated control in experiment 1. For this reason the increases of 48.21, 27.96 and 34.57 % in fresh leaf weight and 13.87, 17.51 and 7.57 % in fresh root weight caused by isolates 91B, 43B and Biostart respectively can be ascribed primarily to a growth promoting effect rather than *Pythium* control. However, in the second experiment *Pythium* inoculation resulted in a 10.43 and 15.36 % reduction in fresh leaf and root weight respectively. In this instance only the Bactolife treatment resulted in *Pythium* control by increasing the biomass by 7.5 % in comparison with the *Pythium* infected control. In contrast to this, isolates 51B and 121B caused a further reduction in fresh leaf and root weight compared to the *Pythium* infested control.

Data on the incidence of *Pythium* in the nutrient solutions is presented in Table 4.5 and 4.8. The incidence of *Pythium* declined sharply over time in all experiments even in the *Pythium* only treatment where no biocontrol agents were added. However, in the screening experiments, some of the biocontrol agents such as 91B, 43B, 121B and Bactolife accelerated the decline of *Pythium* over the 3-week period reducing *Pythium* incidence with 60, 97, 100 and 77 % respectively compared to the treatment with *Pythium* alone where no biocontrol

agent was added. Isolates BSB, 87B and BSB + 91B reduced the *Pythium* incidence in the nutrient solution with 100 % in the final evaluation experiment.

The greatest effect was exhibited by BSB, which effectively eradicated *Pythium* from the nutrient solution over the three-week period. This effect is also reflected in the 65.8 % reduction in *Pythium* root infection with the BSB treatment (Fig. 4.4). At one week after inoculation, the bacterial isolates 91B (in the screening experiment), BSB, 87B and BSB + 91B (in the final evaluation experiment) significantly reduced *Pythium* incidence in the nutrient solution by 23.3, 100, 81.2 and 93.8 % respectively compared to the *Pythium* only treatment. However, in the first screening experiments, the only isolates capable of significantly reducing *Pythium* root infection were 91B and 43B (Fig. 4.1 and 4.4).

Results of the final evaluation of the most promising biocontrol isolates are presented in Table 4.7 and 4.8. The effect of *Pythium* inoculation on growth of butterhead lettuce in the re-circulating hydroponic system in the greenhouse was relatively small, reducing total biomass per plant by an average of 3.8 g (4.4 %), 17.89 g (17.95 %) and 9.8 g (11.8 %) in experiments 1, 2 and 3 respectively. With the exception of isolate 91B, none of the various bacterial treatments had a significant effect in terms of growth promotion of lettuce plants. In contrast to this, in the pre-inoculated lettuce plants (Table 4.7) *Pythium* caused dramatic reductions in total biomass of the plants averaging 20.4 g (72.5 %), 36.62 g (84.5 %) and 33.9 g (88.46 %) in experiments 1, 2 and 3 respectively (Table 4.7 and Figs. 4.6 and 4.8). In this instance the BSB + 43B treatment (experiment 1) significantly reduced the effect of *Pythium* on root weight by 32.2 % (Fig. 4.7). Although Biostart^{TM} and isolate 91B individually also showed positive effects, these were not statistically significant, with the exception of isolate 91B having reduced the effect of *Pythium* on root weight significantly by 7.3 %. In terms of *Pythium* root infection, the bacterial combinations BSB + 43B and BSB + 43B + 91B + 87B and isolate BSB significantly reduced root infection by 10.1, 17.8 and 65.8 % respectively compared to the *Pythium* only treatment (Fig. 4.3 and 4.4).

4.3.3. Identification of rhizobacteria

Results of the characterization and identification of rhizobacterial isolates are summerised in Table 4.1. Most of the rhizobacteria were rod shaped except for isolate 121B and 7B that were staphylococci and coccobacilli respectively. All the rhizobacterial isolates were cytochrome oxidase negative. All the Gram-negative rhizobacteria were able to ferment glucose in Hugh-

Leifson medium. None was able to oxidize glucose in the same medium. All The Gram positive rhizobacteria were belong to *Bacillus* spp. All the rhizobacterial isolates, except 114B (*Enterobacter cloacae*) were identified as *Proteus penneri* using the API 20 E microtechnique (Table 4.1).

4.4.DISCUSSION

In the present study, several isolates of rhizobacteria, especially BSB and 87B, exhibited growth promoting effects on lettuce plants whilst one fungal isolate 9F also had a significant effect on plant growth. The growth promotion activity of rhizosphere isolates was demonstrated by increasing plant height, fresh leaf weight and total biomass of lettuce plants in the absence of the pathogen thereby qualifying these isolates as growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1991). Plant growth promoting rhizobacteria strains have been shown to increase yields in many other crops, including peanut (Turner and Backman, 1989), wheat (de Freitas and Germida, 1989), cotton (Backman *et al*., 1994) and container-grown plants (Harris, 1994). Over the last two decades, there have been an increasing number of reports of promotion of plant growth following treatments of seeds, roots, cuttings, soil or artificial growing media with bacteria and fungi (Whipps, 1997). Depending on the plant studied, growth promotion has been expressed in a variety of ways but most commonly as increase in germination, emergence, fresh or dry weight of roots or shoots, root length, yield and flowering (Whipps, 1997). Many aspect of this phenomenon have previously been reviewed in detail (Shippers *et al*., 1987; Weller, 1988; Baker, 1989; Kloepper *et al*., 1991; Campbell, 1994; Glick, 1995; Bevivino, 1998; Enebak *et al*, 1998; Harris, 1999; Raupach and Kloepper, 2000; Bloemberg and Lugtenberg, 2001; Peix *et al*, 2001).

Pertaining to biocontrol, rhizobacterial isolates 43B and 91B reduced *Pythium* root infection by 22 and 20 % and incidence in the nutrient solution by 97 and 60 %, thereby enhancing the growth of lettuce plants by 28 and 48 % respectively. Most previous attempts to control *Pythium* on lettuce in hydroponic systems were not as successful (Goldberg and Stanghellini, 1991). Paulitz *et al*. (1992) selected rhizosphere bacteria for the control of *Pythium aphanidermatum* on cucumber. When tested under near-commercial conditions in rockwool inoculated with *P. aphanidermatum*, isolates of *P. corrugata* (strain 13) and *Pseudomonas fluorescens* (strain 15) significantly reduced disease under high disease levels, but did not increase yields comparable to the healthy controls (Rankin and Paulitz, 1994). Moulin *et al*.

(1994) found one strain of *Pseudomonas* spp. that controlled *P. aphanidermatum* under near-commercial conditions, resulting in yields comparable to the uninoculated control. *P. fluorescens* strain WCS365 and the commercial product Mycostop (*Streptomyces griseoviridis*) reduced disease caused by *P. aphanidermatum* on cucumber by 60-50 % in an ebb and flow hydroponic system (Postma *et al*., 1995). *Bacillus subtilis* has also been shown to reduce *Phytophthora nicotianae* var. *nicotianae* (Breda de Haan) Sarej on tomato (Bochow, 1992). In the present study rhizobacterial isolates BSB, 43B and 91B reduced *Pythium* root rot and enhanced the growth of lettuce.

In the biocontrol experiments of the present study, inoculation with *Pythium* had a drastic effect on the growth of the pre-inoculated plants at the top of each trough (inoculum-source plants). In contrast to this, the effect of *Pythium* on the plants below the pre-inoculated plants in the troughs was relatively small. This can be attributed to the fact that infection in these plants was dependent on the zoospores released into the nutrient solution from the inoculum source plant at the top of each trough. However, there was an observed trend for the *Pythium* levels in the nutrient solution to decline over time. This decline can most probably be ascribed to the weekly replacement of nutrient solution, thereby removing most of the *Pythium* inoculum (zoospores) from the solution. Secondly it must be noted that the lettuce plants were growing under optimal conditions without any stress. Under these conditions *Pythium* is known to have little effect on plants as opposed to conditions that induce stress in the host plants (Favrin *et al*., 1988; Stanghellini, 1994).

Kloepper *et al.* (2000) indicated that the performance of individual PGPR strains could be enhanced by combination with other PGPR organisms. In this study, the combination of rhizobacterial isolates BSB and 43B rendered the maximum level of growth promotion and the best control of *Pythium* as reflected in yield. Different mechanism of action for different rhizobacterial strains may explain why combinations of strains provide a synergistic effect on disease suppression. These results are in agreement with studies by Pierson and Weller (1994) and Duffy and Weller (1995), both of which demonstrated that certain mixtures of fluorescent pseudomonads were significantly more suppressive of take-all than either treatment alone.

There is still a very limited number of compatible and effective mixtures of biocontrol agents available (Raupach and Kloepper, 1998). In the present study some combinations did not suppress *Pythium* infection or enhance the growth of lettuce plants. This might be due to several factors. The majority of mixtures have no additional benefit over the individual

isolates. A mixture that shows improved activity under one set of conditions or on one host may show reduced efficacy under another set of conditions or on a different host. From an economical point of view, a biocontrol product composed of a mixture of strains has a potential drawback, because producing and registering such a product will likely be more costly than a product composed of a single strain (Scisler *et al*., 1997). However, greater emphasis on developing mixtures of biocontrol agents is needed, because they may result in better plant colonisation, be better adapted to the environmental changes that occur throughout the growing season, present a larger number of pathogen-suppressive mechanisms or protect against a broader range of pathogens (Raupach and Kloepper, 1998).

The results of the present study show a number of reproducible trends. Bacterial isolate BSB performed the best of all strains tested and had a growth promoting effect in the absence of measurable disease pressure or *Pythium* inoculation (Growth promotion experiment). These strains, originally obtained from the Department of Microbiology and Plant Pathology culture collection, improved lettuce yields by 16 to 28%. This is comparable to the growth promotion seen by other growth-promoting rhizobacteria in greenhouse crops (McCullagh *et al.*, 1996; Utkhede *et al.*, 2000). Not only did isolate BSB promote the growth of lettuce plants but it also resulted in a low percentage yield reduction due to *Pythium* infection, low root infection compared to all other treatments and significantly reduced *Pythium* incidence in the nutrient solution of the re-circulating gravel bed hydroponic system. Indeed, the term plant Growth-promoting rhizobacteria (PGPR) has been coined specifically to describe bacteria, which colonize roots and have the ability to stimulate plant growth (Kloepper and Schroh, 1978). Frequently growth promotion involved application of known biocontrol agents. However, growth promotion was observed in nutrient solution lacking the pathogen. It is then thought to be due to a direct effect on the plant (Wipps, 1997). Gibberellins have been detected in some cultures of *B. subtilis*, but not auxins (Broadbent *et al*., 1971).

Although the precise mode of action of the potential biocontrol PGPR used here is beyond the scope of this work, plant growth promotion may be at least partially responsible for the action BSB, 43B, 91B and 87B isolates. All of the isolates promoted growth of lettuce plants in at least one of the experiments. Numerous speculative explanations of mechanisms involved in increased growth responses have been advanced namely inhibition and alteration of normal root microflora, growth-stimulating substances (hormones, growth factors), nutrient availability or stimulation of nutrient uptake and decreasing substances inhibitory to plant

growth (Baker, 1989; Glick, 1994; Bevivino, 1998; Harris, 1999). Van Peer *et al*. (1989) also reported an increased growth response of cucumber from *Pseudomonas* spp. in hydroponic culture. The isolates in the present study may reduce disease losses by increasing plant vigor and reducing the susceptibility of the plant to stress and subsequent damage from *Pythium* (Rankin and Paulitz, 1994). Competition for nutrients in root exudates and interference with zoospore chemotaxis and encystement may also be a mechanism of biocontrol by this isolate (Zhou and Paulitz, 1993). It could be due to indirect effects on the plant by directly antagonising pathogens via siderophore, antibiotic, or hydrogen cyanide production (Kloepper *et al*., 1991). PGPR have also been shown to induce resistance to root pathogen (Lui *et al*., 1995; Zhou and Paulitz, 1995). PGPR may also inhibit deleterious rhizobacteria, or directly stimulate plant growth through the production of plant hormones (Arshad and Frankenberger, 1991) or increase phosphorus uptake.

In the biocontrol experiments a low level of contamination (1.1 %) with *Pythium* was experienced. Fungus gnats (*Bradysia impatiens*) (Gardiner *et al*., 1990) and shore flies (*Scatella stagnalis*) (Goldberg and Stanghellini, 1990) may also be involved in the introduction and spread of *Pythium* in greenhouses. The potential, documented sources of pathogen introduction include the following: air, sand, soil, peat, water and insects (Stanghellini and Rasmussen, 1994). The low level of contamination in the present study is considered to have had little effect on the results.

In conclusion, several isolates of rhizosphere bacteria showed potential as lettuce inoculants for growth promotion and biocontrol of *Pythium* root rot. The use of biocontrol or PGPR bacteria can be included as a useful component in an integrated disease management strategy for lettuce grown in soilless system. Before commercialization of these isolates can take place, the mode of action and toxicity of the antagonist as well as commercial trials to evaluate the formulated product under commercial conditions should be determined. Additional aspects that should be studied to optimise efficiency include formulation and delivery systems.

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Table 4.1. Characteristics and identification of rhizobacteria isolated from Nylsvley Nature Reserve

Isolate code	**Cell shapes**	**Gram staining**	**Hugh-Leifson's test**	**Cytochromeoxidase test**	**Micro-techniques**	**Identity**
121B	Staphylococcus	_	Fermentative	_	API 20 E	*Proteus penneri*
76B	Coccobacillus	_	Fermentative	_	API 20 E	*P. penneri*
24B	Rod	+	NA	_	API 50 CH	*Bacillus stearotermophilus*
57B	Rod	+	NA	_	API 50 CH	*B. cereus*
114B	Rod	_	Fermentative	_	API 20 E	*Enterobacter cloacae*
107B	Rod	+	NA	_	API 50 CH	*B. pumilus*
43B	Rod	+	NA	_	API 50 CH	*B. pumilus*
91B	Rod	_	Fermentative	_	API 20 E	*P. penneri*
87B	Rod	_	Fermentative	_	API 20 E	*P. penneri*
106B	Rod	+	NA	_	API 50 CH	*B. mycoides*
51B	Rod	+	NA	_	API 50 CH	*B. cereus*
BSB	Rod	+	NA	_	API 50 CH	*B. subtilis*

NA = not applicable.

Table 4.2. Effect of different rhizosphere microflora and commercial products on growth enhancement of lettuce plants in a re-circulating gravel bed hydroponic system in the greenhouse (growth promotion screening experiment)

Experiments	Treatments	Plant height (mm)[a]	Fresh leaf weight per plant (g)[b]	Fresh root weight per plant (g)[b]	Total biomass per plant (g)[b]	Increase in plant height (%)[c]	Increase in fresh leaf height (%)[c]	Increase in fresh root weight (%)[c]
1	Control	227.25	61.03	3.32	64.35	0.00	0.00	0.00
	107B	221.00	65.78	3.84	69.62	-2.67	7.79	15.74
	9F	239.17	73.50	3.91	77.41	5.29	20.44	18.16
	24B	250.33	86.77	3.61	90.39	10.19	42.10	9.31
	S.E.[1]	2.26	2.27	0.28	2.44	1.63	3.15	9.53
	CD (0.05)[2]	7.37	7.42	0.91	7.95	5.34	10.29	31.09
	CD (0.01)[3]	10.72	10.79	1.33	11.57	7.77	14.98	45.23
2	BSB	235.09	132.76	10.57	143.34	-4.72	15.87	9.30
	57B	246.75	126.50	11.17	137.68	-0.01	10.47	14.17
	114B	245.66	120.09	11.05	131.14	-0.43	4.80	12.97
	Control	246.75	114.61	9.86	124.47	0.00	0.00	0.00
	Bactolife A+B	242.50	11.04	9.48	120.52	-1.71	-3.01	-2.59
	S.E.[1]	3.79	2.75	0.63	3.16	1.46	1.56	7.50
	CD (0.05)[2]	11.69	8.48	1.94	9.76	4.52	4.83	23.13
	CD (0.01)[3]	16.40	11.90	2.73	13.69	6.35	6.78	32.43
3	51B	250.50	100.31	6.98	107.29	7.31	-2.17	-15.13
	76B	257.08	104.18	7.85	112.03	10.18	1.56	-5.15
	Control	233.41	102.58	8.50	11.08	0.00	0.00	0.00
	106B	251.33	102.81	9.49	112.30	7.70	0.30	13.71
	87B	251.33	106.95	9.18	116.13	7.65	4.19	9.83
	S.E.[1]	3.54	2.43	0.62	2.65	1.42	2.11	8.84
	CD (0.05)[2]	10.90	7.50	1.93	8.18	4.39	6.50	27.24
	CD (0.01)[3]	15.29	10.52	2.70	11.47	6.15	9.11	38.19

[a] Each value is the mean of three replicate troughs with five representative plants in each, evaluated three weeks after planting.
[b] Each value is the mean of three replicate trough with 12 plant in each, evaluated three weeks after planting.
[c] Each value representing percents increment compared to non-inoculated control.
[1] Standard error of the treatment
[2] Critical difference at $P \leq 0.05$.
[3] Critical difference at $P \leq 0.01$.

Table 4.3. Effect of different rhizobacteria on growth of lettuce plants in re-circulating gravel bed hydroponic system in the greenhouse (final evaluation for growth promotion)

Treatments	**Fresh leaf weight per plant (g)** [a]	**Fresh root weight per plant (g)** [a]	**Total biomass per plant (g)** [a]	**Increase in fresh leaf weight (%)** [b]	**Increase in fresh root weight (%)** [b]
BSB	70.43	1.83	72.23	29.82	24.31
24B	60.16	1.46	61.66	10.43	3.71
87B	67.10	1.40	68.43	24.45	-6.34
Control	54.60	1.46	56.00	0.00	0.00
S.E.[1]	3.04	0.12	3.05	8.62	8.96
CD (0.05)[2]	9.92	0.41	9.96	28.11	29.23
CD (0.01)[3]	14.44	0.59	14.49	40.89	42.54

[a] Each value is the mean of three replicates with 12 plants in each trough, evaluated three weeks after planting.
[b] Each value represents percentage increment compared to uninoculated lettuce plant.
[1] Standard error of the treatment.
[2] Critical difference at $P \leq 0.05$.
[3] Critical difference at $P \leq 0.01$.

Table 4.4. Effect of bacterial isolates and commercial biocontrol products on growth parameters of *Pythium* inoculated lettuce plants in a re-circulating gravel bed hydroponic system in a greenhouse (Biocontrol screening experiments)

Experiments	Treatments	Fresh leaf weight per plant (g)[a]	Fresh root weight per plant (g)[a]	Total biomass per plant (g)[a]	Reduction in fresh leaf weight (%)[b]	Reduction in fresh root weight (%)[b]
1	91B	123.03	7.71	130.74 (15.1)[c]	-13.83	-48.21
	43B	127.01	6.63	133.64 (16.9)	-17.51	-27.96
	Biostart	115.94	6.99	122.92 (9.7)	-7.14	-34.57
	Pythium alone	105.48	5.51	110.99 (0.0)	3.28	-6.11
	Uninfected control	109.07	5.19	114.26 (2.8)	0.00	0.00
	S.E.[1]	5.80	0.39	5.94	6.43	6.67
	CD (0.05)[2]	18.30	1.23	18.73	19.82	20.57
	CD (0.01)[3]	26.03	1.74	26.64	27.78	28.84
2	51B	102.44	5.40	107.84 (-7.0)	17.07	4.68
	121B	101.06	4.93	106 (-8.8)	18.25	8.95
	Bactolife	119.43	5.43	124.86 (7.5)	3.33	0.92
	Pythium alone	110.67	4.72	115.39 (0.0)	10.43	15.36
	Uninfected control	123.54	5.64	129.18 (10.6)	0.00	0.00
	S.E.[1]	2.60	0.52	2.64	1.23	12.13
	CD (0.05)[2]	8.20	1.66	8.34	3.80	37.38
	CD (0.01)[3]	11.66	2.36	11.87	5.34	52.42

[a] Each value is the mean of three replicates with 12 plants in each trough, evaluated three weeks after planting.
[b] Each value represents the percentage reduction compared to the uninfected control.
[c] Each value in brackets represents percentage increase in total biomass in comparison with *Pythium* inoculated control.
[1] Standard error of the treatment.
[2] Critical difference at $P \leq 0.05$.
[3] Critical difference at $P \leq 0.01$.

Table 4.5. Effect of rhizobacterial isolates and a commercial biocontrol product on *Pythium* incidence in the nutrient solution of a re-circulating gravel bed hydroponic system in the greenhouse (biocontrol screening experiments).

Experiments	Treatments	*Pythium* incidence in the nutrient solution (%)		
		1st week after inoculation [a]	2nd week after inoculation [a]	At harvest (three weeks) [a]
1	91B	76.66 (23.3) [b]	60.00	13.33 (60.0)
	43B	96.66 (3.3)	63.33	1.00 (97.0)
	Biostart	100 (0.0)	100.00	23.33 (30.0)
	Pythium alone	100 (0.0)	90.00	33.33 (0.0)
	Uninfected control	0 (100.0)	0.00	0.00 (100.0)
	S.E.[1]	4.21	9.77	14.38
	CD (0.05)[2]	13.29	30.80	45.31
	CD (0.01)[3]	18.90	43.80	64.46
2	51B	100.00 (0.0)	93.33	33.33 (-150.0)
	121B	100.00 (0.0)	6.66	0.00 (100.0)
	Bactolife	83.33 (16.6)	76.66	3.00 (77.49)
	Pythium alone	100.00 (0.0)	73.33	13.33 (0.0)
	Uninfected control	0.00 (100.0)	0.00	0.00 (100.0)
	S.E.[1]	3.94	6.49	6.32
	CD (0.05)[2]	12.15	20.02	19.48
	CD (0.01)[3]	17.04	28.07	27.32

[a] Each value is the mean of three replicate Petri dishes with ten citrus leaf discs in each, evaluated two days after plating V8-juice agar.
[b] Each value in bracket represents percentage reduction of *Pythium* incidence in the nutrient solution in comparison with the untreated, *Pythium* inoculated control.
[1] Standard error of the treatments
[2] Critical difference at $P \leq 0.05$.
[3] Critical differences at $P \leq 0.01$.

Table 4.6. Effect of bacterial isolates and commercial biocontrol products on growth parameters of *Pythium* inoculated lettuce plants in a re-circulating gravel bed hydroponic system in the greenhouse (biocontrol final evaluation experiment)

Experiment	Treatments	Fresh leaf weight per plant (g)[a]	Fresh root weight per plant (g)[a]	Total biomass per plant (g)[a]	Reduction in fresh leaf weight (%)[b]	Reduction in fresh root weight (%)[b]
1	Uninfected control	86.83	1.20	88.06 (0.0)*	0.00	0.00
	BSB + 43B	89.73	1.36	91.10 (-3.5)	-3.27	-12.88
	Pythium alone	83.03	1.26	84.26 (4.3)	4.39	-3.32
	BSB + 43B + 91B + 87B	82.50	1.60	84.10 (4.5)	4.79	-34.33
	S.E.[1]	3.13	0.15	3.27	2.23	12.30
	CD (0.05)[2]	10.21	0.50	10.67	7.28	40.12
	CD (0.01)[3]	14.86	0.73	15.53	10.59	58.37
2	Uninfected control	95.83	3.70	99.53 (0.0)	0.00	0.00
	Biostart	79.40	2.46	81.90 (17.7)	17.11	31.96
	Pythium alone	77.13	2.40	79.53 (20.1)	19.50	34.69
	43B	78.06	2.66	80.73 (18.9)	18.54	27.25
	BSB	81.80	2.56	84.40 (15.2)	14.61	28.40
	S.E.[1]	2.82	0.21	2.76	2.88	7.11
	CD (0.05)[2]	8.90	0.68	8.70	9.10	22.42
	CD (0.01)[3]	12.66	0.96	12.38	12.95	31.89
3	*Pythium* alone	71.00	2.03	73.03 (11.9)	8.07	20.34
	87B	74.36	2.40	76.76 (7.4)	3.76	6.13
	Uninfected control	80.16	2.66	82.86 (0.0)	0.00	0.00
	91B	85.16	2.43	87.60 (-5.7)	-10.10	5.20
	BSB + 91B	66.76	2.66	69.43 (16.2)	14.00	-4.21
	S.E.[1]	3.97	0.14	4.07	7.02	5.78
	CD (0.05)[2]	12.97	0.46	12.82	22.11	18.22
	CD (0.01)[3]	17.81	0.66	18.24	31.46	25.92

[a] Each value is the mean of three replicates with 12 plants in each trough, evaluated three weeks after planting.
[b] Each value represent percentage reduction compared to none inoculated lettuce plant.
* Each value in brackets represents percentage reduction in total biomass compared to uninfected control.
[1] Standard error of the treatments
[2] Critical difference at $P \leq 0.05$.
[3] Critical differences at $P \leq 0.01$.

Table 4.7. Effect of bacterial isolates and commercial biocontrol products on growth parameters of *Pythium-pre*-inoculated [a] lettuce plants in re-circulating gravel bed hydroponic system in the greenhouse (biocontrol final evaluation experiments)

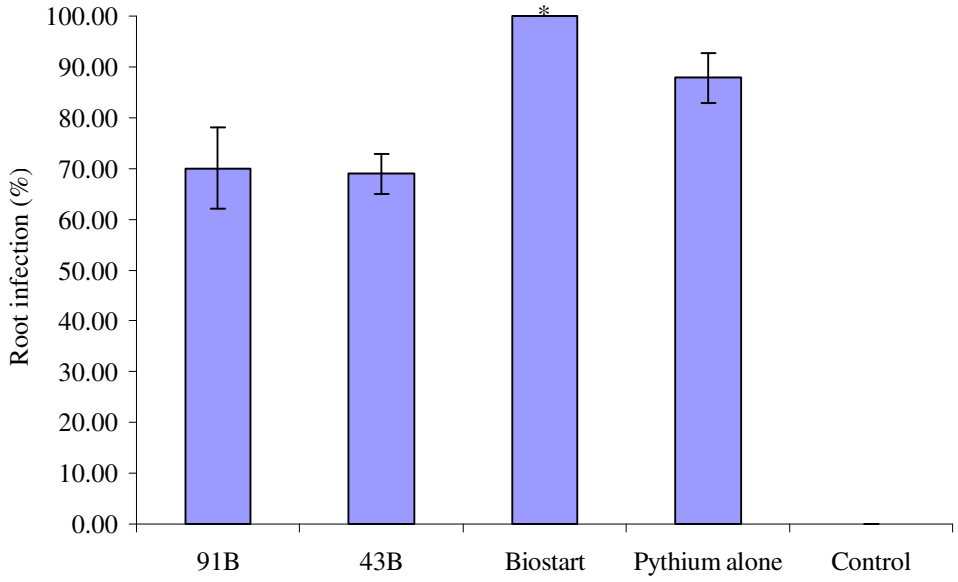
Experiments	Treatments	Fresh leaf weight per plant (g) [b]	Fresh root weight per plant (g) [b]	Total biomass per plant (g) [b]	Reduction in fresh leaf weight (%) [c]	Reduction in fresh root weight (%) [c]
1	Uninfected control	25.36	2.80	28.13 (0.0) [d]	0.00	0.00
	BSB + 43B	8.46	1.20	9.63 (65.8)	68.32	58.29
	Pythium alone	6.16	0.40	6.56 (76.7)	76.06	86.05
	BSB + 43B + 91B + 87B	6.80	0.23	7.03 (75.0)	69.80	90.59
	S.E.[1]	1.50	0.18	1.50	5.91	3.59
	CD (0.05)[2]	4.91	0.60	4.92	19.30	11.73
	CD (0.01)[3]	7.14	0.88	7.15	28.08	17.07
2	Uninfected control	37.03	6.30	43.30 (0.0)	0.00	0.00
	Biostart	5.50	1.76	7.23 (83.3)	85.00	70.86
	Pythium alone	4.03	1.16	5.16 (88.1)	89.05	80.11
	43B	4.40	0.83	5.20 (88.0)	87.84	87.89
	BSB	7.93	1.20	9.13 (78.9)	77.58	80.63
	S.E.[1]	1.27	0.48	1.37	4.00	4.30
	CD (0.05)[2]	4.03	1.51	4.34	12.60	13.56
	CD (0.01)[3]	5.73	2.15	6.17	17.92	19.30
3	*Pythium* alone	2.90	0.53	3.43 (91.1)	91.56	86.95
	87B	3.86	0.70	4.56 (88.2)	89.32	82.46
	Uninfected control	34.56	4.03	38.60 (0.0)	0.00	0.00
	91B	3.10	0.80	3.86 (90.0)	91.37	80.58
	BSB + 91B	5.36	0.60	5.96 (84.6)	86.70	86.88
	S.E.[1]	1.68	0.08	1.65	1.75	1.32
	CD (0.05)[2]	5.31	0.25	5.20	5.53	4.18
	CD (0.01)[3]	7.56	0.35	7.39	7.87	5.95

[a] Four plants at the top of the trough that were pre-inoculated prior to transplanting serving as a source of inoculum, evaluated three weeks after planting.
[b] Each value is the mean of three replicates with 12 plants in each trough, evaluated three weeks after planting.
[c] Each value represent percentage reduction compared to non inoculated lettuce plants.
[d] Each value in brackets represent percentage reduction in total biomass compared to uninfected control.
[1] Standard error of the treatments
[2] Critical difference at $P \leq 0.05$.
[3] Critical differences at $P \leq 0.01$.

Table 4.8. Effect of rhizobacterial isolates and a commercial biocontrol product on *Pythium* incidence in the nutrient solution of a re-circulating gravel bed hydroponic system in the greenhouse (biocontrol final evaluation experiments)

Experiments	Treatments	*Pythium* incidence in the nutrient solution (%)		
		1st week after inoculation [a]	2nd week after inoculation [a]	At harvest (three weeks)
1	Uninfected control	0.00 (100.0) [b]	0.00	0.00 (0.0)
	BSB + 43B	93.33 (6.7)	100.00	0.00 (0.0)
	Pythium alone	100.00 (0.0)	76.66	0.00 (0.0)
	BSB+43B+91B+87B	100.00 (0.0)	83.33	3.33 (-100.0)
	S.E.[1]	1.66	6.87	1.66
	CD (0.05)[2]	5.43	22.41	5.43
	CD (0.01)[3]	7.90	32.60	7.90
2	Uninfected control	0.00 (100.0)	0.00	0.00 (100.0)
	Biostart	86.66 (3.7)	40.00	3.33 (0.0)
	Pythium alone	90.00 (0.0)	10.00	3.33 (0.0)
	43B	3.33 (96.3)	0.00	33.33 (-900.0)
	BSB	0.00 (100.0)	0.00	0.00 (100.0)
	S.E.[1]	6.66	3.65	2.58
	CD (0.05)[2]	20.54	11.25	7.95
	CD (0.01)[3]	28.80	15.77	11.15
3	*Pythium* alone	53.33 (0.0)	0.00	20.00 (0.0)
	87B	10.00 (81.2)	0.00	0.00 (100.0)
	Uninfected control	0.00 (100.0)	0.00	0.00 (100.0)
	91B	23.33 (23.33)	0.00	16.66 (16.7)
	BSB + 91B	3.33 (93.8)	0.00	0.00 (100.0)
	S.E.[1]	5.16	-	6.49
	CD (0.05)[2]	15.91	-	20.02
	CD (0.01)[3]	22.31	-	28.07

[a] Each value is the mean of three replicate Petri dishes with ten citrus leaf discs in each, evaluated two days after plating on V8-juice agar.
[b] Each value represents percentage reduction of *Pythium* incidence in the nutrient solution in comparison with the *Pythium* inoculated control.
[1] Standard error of the treatments
[2] Critical difference at $P \leq 0.05$.
[3] Critical difference at $P \leq 0.01$.



* All replicates were 100 % infected.

Figure 4.1. Effect of rhizosphere bacterial isolates and a commercial biocontrol product on *Pythium* root infection of butterhead lettuce in a re-circulating gravel bed hydroponic system in the greenhouse (Biocontrol screening experiment 1).

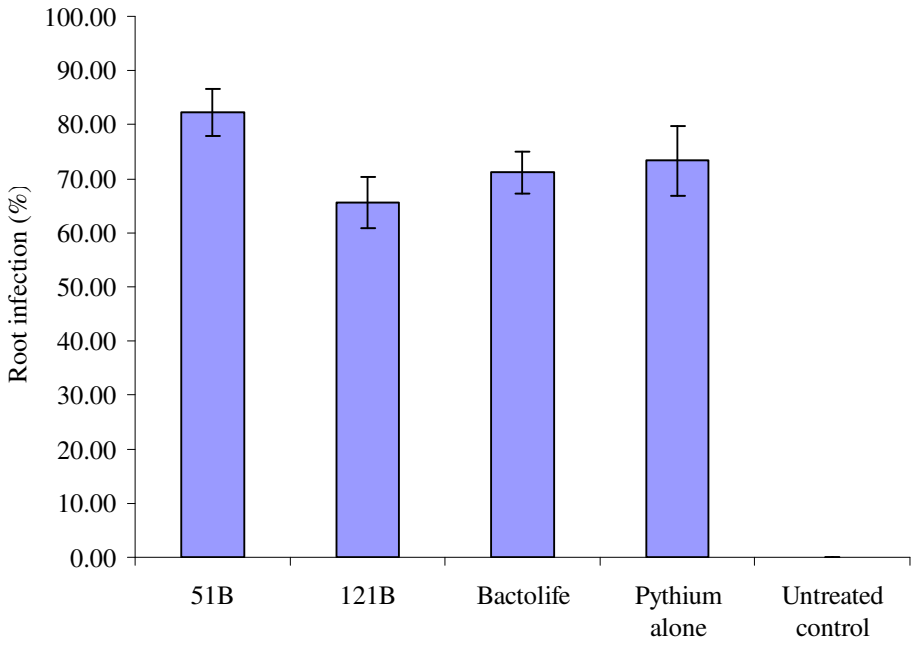
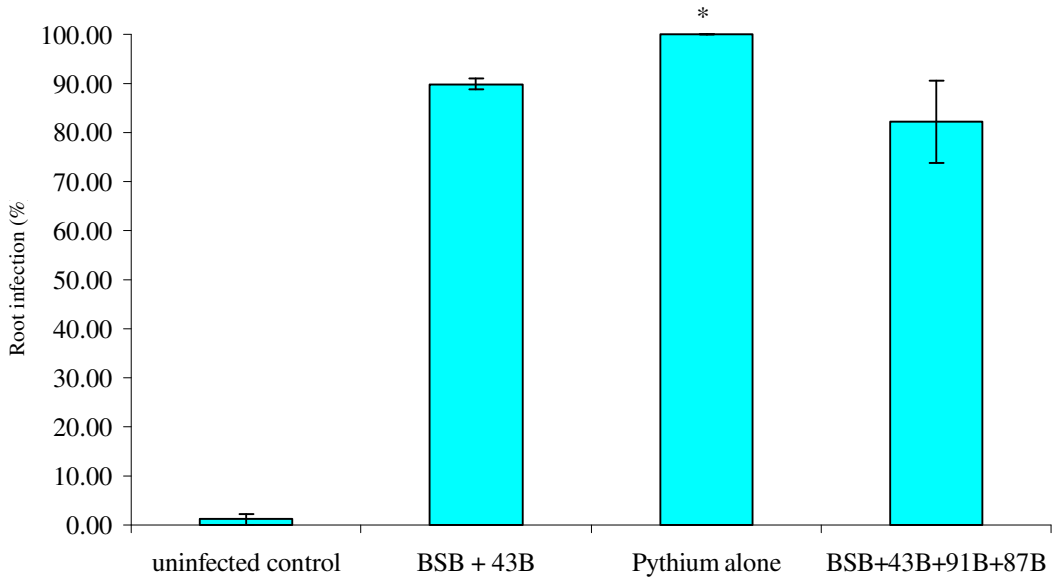


Figure 4.2. Effect of rhizosphere bacterial isolates and a commercial biocontrol product on *Pythium* root infection of butterhead lettuce in a recirculating gravel bed hydroponic system in the greenhouse (Biocontrol screening experiment 2).



* All replicates were 100 % infected.

Figure 4.3. Effect of rhizobacterial isolates combinations on *Pythium* root infection of butterhead lettuce in a recirculating gravel bed hydroponic system in the greenhouse. (Biocontrol final evaluation experiment 1).

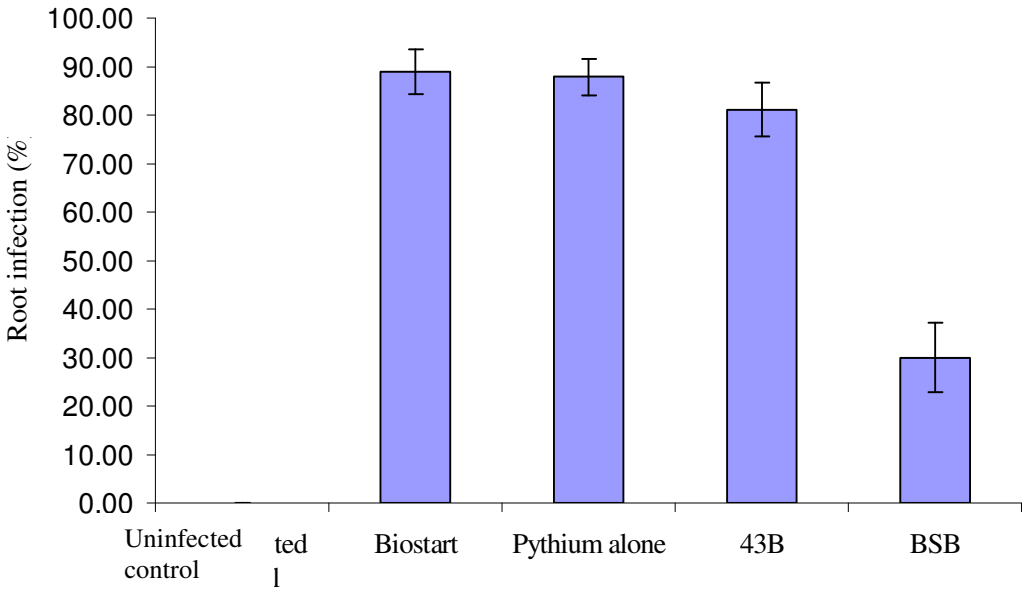


Figure 4.4. Effect of rhizobacterial isolates and a commercial biocontrol product on root infection of butterhead lettuce in a recirculating gravel bed hydroponic system in the greenhouse, (Biocontrol final evaluation experiment 2).

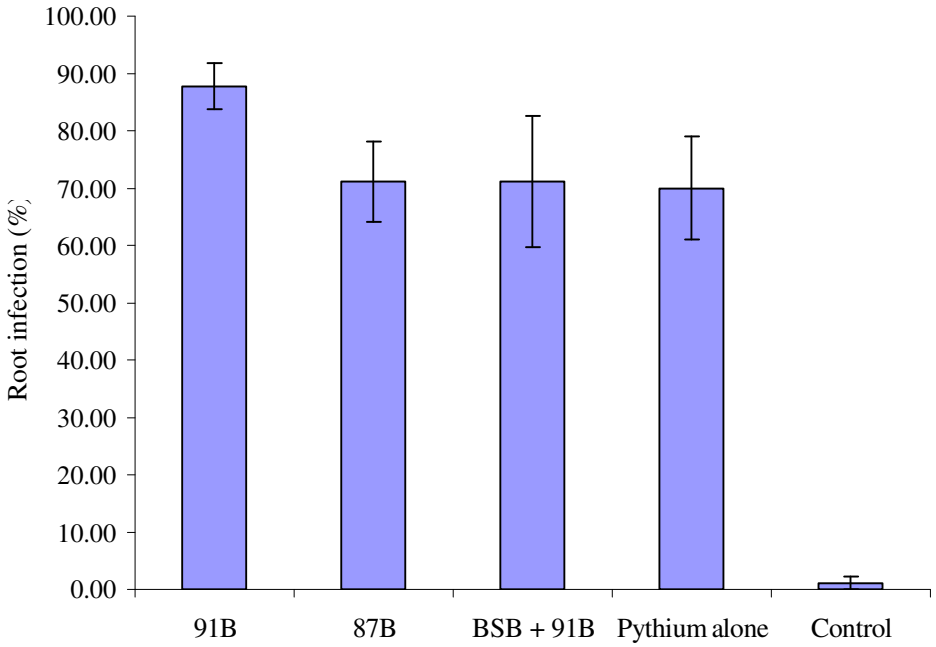


Figure 4.5. Effect of rhizobacterial isolates and a commercial biocontrol product on root infection of butterhead lettuce in a recirculating gravel bed hydropoinc system in the greenhouse(Biocontrol final evaluation experiment 3).

CHAPTER 5

GENERAL DISCUSSION

The ultimate goal of this study was to screen rhizosphere microbial isolates for plant growth promoting activities and for disease control of *Pythium* group-F in re-circulating gravel bed hydroponic systems. Among the genus *Pythium*, *Pythium* group-F was previously found to be the most dominant pathogen consistently isolated from commercial re-circulating gravel bed hydroponic systems (Niederwieser, 2001; Gull, 2003). Although it significantly reduces the growth of various crops including lettuce, it does not kill the plant and can thus be classified as a successful pathogen (Gull, 2003). In general, *Pythium* spp. are important pathogens in hydroponic systems and difficult to control particularly in re-circulating gravel bed systems (Stanghellini and Rasmussen, 1994; Niederwieser, 2001; Gull, 2003). Hydroponic systems offer a unique environment for biocontrol since various parameters can be managed to favour the antagonist (Paulitz, 1997). Ample scope, therefore, exists for development of biological control in such systems, particularly when the biocontrol agent was originally isolated from aquatic environments or has adapted to such systems (Linderman *et al*., 1983).

The present study constitutes the first attempt to isolate rhizosphere microflora mainly from partially aquatic environments at the edge of the Nyl flood plain at Nylsvley Nature Reserve, Limpopo province, South Africa. Isolation of the rhizosphere microflora from partially aquatic environments assume that the potential antagonists would be ecologically adapted to periods of flooding and might be able to survive and express biocontrol activity if applied in a similar environment such as a re-circulating hydroponic system. Large collections of antagonistic bacteria and fungi have previously been isolated from similar environments and where effectively applied as biocontrol or plant growth promotion agents (Kloepper *et al*., 1988; Renwick *et al*., 1991, Swadling and Jeffries, 1996). In the present study, more than 150 rhizobacterial and 49 fungal isolates were collected from the rhizosphere grasses such as *Oryza longistaminata* A. Chev. & Roehr., *Panicum schinizii* Hack., *Setaria sphacelata* (Schumach.) Moss, *Leersia* spp., *Sporobolus* spp., *Acrocera* spp. and sedges (*Cyprus fastigiatus*, *Schoenoplectus* spp. and *Eleocharis* spp.), as well as flowering plants (*Brassica* spp.) grown at open grasslands and underneath acacia trees from the edge of Nylsvley flood plain and dry savanna grassland of the Nylsvley Nature Reserve. The isolation of the microflora from the rhizosphere of these grasses and sedges was done since this region is

known to provide a niche of intense microbial activity where beneficial free-living bacteria (Benizri *et al*., 2001; Bloemberg and Lugtenberg, 2001) and fungi (Meera *et al*., 1994) exist. The present study is in agreement with Meera *et al*. (1995) and Shivanna *et al*. (1994), who successfully isolated fungi from the zoysia grass rhizosphere, which were capable of inducing growth promotion activity in a variety of crops. In addition, some of these isolates also acted as biocontrol agents when tested further against soilborne diseases of several crop plants (Hyakumachi, 1992). Similarly this study found potential rhizosphere microorganism from several grasses that subsequently proved to be potential antagonists and growth promotion agents.

Among the total rhizosphere microflora that were isolated from the Nylsvley Nature Reserve, bacteria were found to be the dominant microorganisms isolated in the area. Most of the bacteria were Gram negative, rod shaped and had oxidative metabolism. Roveria and Brisbane (1967) found that bacteria isolated from the rhizosphere of wheat and clover were also predominantly Gram negative. According to Glick (1995) bacteria are by far the most common type of rhizosphere microorganisms compared to the more commonly found fungi, actinomycetes, protozoa and algae. This is mainly due to the fact that bacteria grow faster and have the ability to utilize a wide range of substances as either carbon or nitrogen sources making them more ideally suited for such environments (Glick, 1995; Bown and Rovira, 1999).

In vitro screening for antibiosis is frequently used to select prospective antagonists (Fravel, 1988). The results presented in Chapter 2 indicate that general broad-spectrum antagonism against *Pythium irregulare*, *Penicillium digitatum*, two isolates of *Colletotrichum gloeosporioides*, *Fusarium solani* and *Geotrichum candidum* exist when rhizosphere isolates were screened *in vitro*, albeit to differing degree. Previously, Broadbent *et al*. (1971) and Spadaro *et al*. (2002) also used a broad spectrum of common pathogens for initial evaluation in order to determine general antagonism. The results from the initial screening showed that rhizosphere samples collected at Nylsvley Nature Reserve harboured some potential useful microorganisms that can be used for further screening in biocontrol systems. Even though *in vitro* antibiosis may not be related to biocontrol under field conditions (Fravel, 1988), there are several reports dealing with a strong correlation between *in vitro* and *in vivo* assays. For instance, production of the antibiotic chetomin by *Chaetomium globosum in vitro* was

positively correlated with antagonism of *Venturia inequalis* on apple seedlings in a growth chamber (Cullen and Andrews, 1984).

Results obtained in Chapter 3 indicate that some of the rhizosphere microflora screened reduced *Pythium* root rot infection and promoted the growth of lettuce seedlings. Most of the bacterial isolates and Bactolife™ (a commercial biocontrol product included for comparative purposes) evaluated in growth promotion experiments enhanced the growth of lettuce seedlings more effectively than the untreated control. In contrast, Romeiro *et al*. (2000) found that amongst the 26 rhizobacterial isolates evaluated in his study with tomato plants, two rhizobacteria resulted in better growth promotion compared to the control plants, although not statistically significant. Conditions that favour disease development also favours the management of disease by means of biological control agents. In subsequent screenings, 13 rhizobacteria were found to be most effective with respect to growth promotion and biocontrol of *Pythium* root rot of lettuce plants in this study.

Most biocontrol studies identified rhizobacterial *Pseudomonas* and *Bacillus* spp. as potential antagonist (Paulitz, 1997). In the present study, however, *Bacillus* spp. were found to be the most dominant and effective potential antagonists. Of these, *B. stearotermophilus* (24B), *B. subtilis* (BSB) and *B. cereus* (57B) were found to promote growth of lettuce plants in re-circulating gravel bed hydroponic systems (Chapter 4). Amongst these isolates, *B. subtilis* was most effective and consistently enhanced the fresh leaf and root weight by 29.82 and 24.3% compared to the untreated control. Similar growth promotion due to the use of *B. subtilis* has been reported on peanut, carrot and oats (Turner and Backman, 1991). The authors indicated that peanut yields (ranged from –3.5 % to 37 %) increased due to *B. subtilis* applications when compared to the untreated control. Yield increase up to 40 % in oats and 48 % in carrots were also reported with *B. subtilis* applications.

Besides *Proteus penneri* (isolate 91B and 121B), *Bacillus* spp. also acted as biocontrol agents when tested further against *Pythium* root rot in re-circulating gravel bed hydroponic systems. As indicated in chapter 4, *B. subtilis* (BSB) and *B. pumilus* (43B) suppressed *Pythium* root infection thereby enhancing the leaf weight of lettuce. This finding is in agreement with Bochow (1992) who found that *B. subtilis* partially controlled *Fusarium oxysporum* (Schltdl. em. W.C. Snydere H.N. Hansen) f.sp. *radicis-lycopersici* (Sacc.) W.C. Snydere H.N. Hansen and *Phytophthora* disease on tomato in hydroponic systems. The strains of *B. subtilis* and *B. pumilus* used in the current study have potential value in controlling root rot and promoting

growth of lettuce plants. Similar species were previously reported as having potential as biocontrol agents in other kinds of hydroponic systems (Rankin and Paulitz, 1994; McCullagh *et al*., 1996; Ongena *et al*., 1999; Utkhede *et al*., 1999). Some isolates in the present study, especially *B. subtilis* appear to cause a growth response independent of pathogen presence and could be classified as a typical plant growth promoting rhizobacteria (PGPR) (Kloepper *et al*., 1991). From previous studies, PGPR can be used as effective inoculants for biofertilisation, phytostimulation and biocontrol. Antagonistic isolates used in the current study (viz., BSB, 43B, 91B and 87B) promoted growth of lettuce plants in at least one of the experiments (growth promotion and / or biocontrol experiments). This indicating that growth promotion may be responsible for suppressing root rot of lettuce and reducing the incidence of *Pythium* group-F in the re-circulating gravel bed system.

In the present study a combination of different groups of bacteria were evaluated since mixture of introduced biocontrol agents could more closely mimic the natural situation and might broaden the spectrum of biocontrol activity and enhance efficacy and reliability of control (Duffy and Weller, 1995). However, only a combination of rhizobacteria BSB and 43B resulted in a synergistic effect as reflected by growth enhancement of lettuce plants whilst suppressing root rot caused by *Pythium* group-F. It was evident from this study that most combinations of bacterial isolates did not show growth promotion and biocontrol activities (Chapter 4). Incompatibility of co- inoculants can arise because biocontrol agents may also inhibit the growth of each other as well as the target pathogen or pathogens (Raupach and Kloepper, 1998). Thus, an important prerequisite for successful development of mixtures of strains appear to be the compatibility of the co-inoculated microorganisms (Raupach and Kloepper, 1998).

Finally, more work needs to be done on the mode of action of rhizobacteria that promote the growth of lettuce plants and suppress *Pythium* root rot. Given the ease of introducing nutrients into a fertigation system, similar products containing bacteria may be developed that could be added to the injector system for rapid dispersal of biocontrol agents through a re-circulating gravel bed hydroponic systems. In conclusion, biological control using PGPRs may offer another tool for disease management in re-circulating hydroponic systems. This is particularly important when considering the current public concern over the use of chemical pesticides, the lack of chemical alternatives in hydroponic systems and the innovative technology of these systems, which makes it ideally suitable for biocontrol applications. This study

highlighted the potential of using natural microorganisms in hydroponic systems for disease control and growth promotion attributes.

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Utilisation of rhizosphere microflora in the biocontrol of root rot and growth enhancement of lettuce under hydroponic systems

by

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RESUMÉ

About 150 rhizobacteria and 49 rhizosphere fungi were isolated from the rhizosphere of grasses and sedges at Nylsvley Nature Reserve, Limpopo Province, South Africa. The rhizobacterial isolated were mostly Gram negative (72%) and rod shaped (73%). The dominant fungal genera were *Trichoderma, Aspergillus*, *Pencillium, Neosartorya* and *Fusarium*. The antagonistic activity of the above isolates were determined in a dual culture assay against a range of plant pathogens namely *Colletotrichum gloeosporioides*, *Pythium irregulare*, *Penicillium digitatum*, *Fusarium solani* and *Geotrichum candidum*. All rhizosphere fungal isolates showed positive antagonism against *G. candidum* (100%) and *F. solani* (100%). The rhizobacterial isolates showed positive antagonism against *G. candidum* (71%) and *C. gloeosporioides* (76%).

The growth promotion and biocontrol activity of the rhizosphere isolates that showed broad-spectrum antagonistic activity against the fore mentioned pathogens were further evaluated on Canadian peat substrate under greenhouse condition. Although most of the rhizosphere isolates resulted in improved fresh leaf weight in comparison with the non-inoculated control in final growth promotion experiments, no statistical difference could be found in increasing leaf weight

by one of the tested isolates. Some isolates and Bactolife^{TM} prevented root infection by *Pythium*. However, only isolate 68B showed significant prevention of root infection compared to the *Pythium* inoculated control.

The selected rhizobacteria, fungal and commercial biocontrol products that showed the most effective growth promotion and biocontrol activities were further evaluated in a re-circulating hydroponic system. Overall, isolate BSB (*Bacillus subtilis*) consistently enhanced the fresh leaf and root weight by 29.82 and 24.31% compared to the untreated control. Treatments with rhizobacteria isolate 91B and 43B significantly increased fresh leaf weight and suppressed *Pythium* root infection of lettuce. Isolate 91B and 121B significantly decreased the incidence of *Pythium* after the 1st and 2nd week of inoculation respectively. The combination of rhizobacteria BSB and 43B showed a synergistic effect as reflected in increased fresh leaf weight and total biomass per plant whilst suppressing root rot caused by *Pythium* group-F.

Appendix 1. Effect of different rhizosphere fungal isolates on mycelia growth of *Pythium irregulare*

Fungal isolate code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
1	*Penicillium* sp.	61.48	60.00	NI*	NI	0.39	0.40	NI	NI
2	*Penicillium* sp.	45.90	76.80	TI**	TI	0.54	0.23	TI	TI
3	Sterile	46.72	48.00	50.00	TI	0.53	0.52	0.50	TI
4	*Aspergillus* sp.	46.72	64.00	75.38	98.46	0.53	0.36	0.25	0.02
5	*Aspergillus* sp.	43.44	54.40	56.92	61.54	0.57	0.46	0.43	0.38
6	*Aspergillus* sp.	46.72	72.80	TI	TI	0.53	0.27	TI	TI
7	*Penicillium* sp	50.82	50.40	TI	TI	0.49	0.50	TI	TI
8	*Penicillium* sp.	46.72	69.60	TI	TI	0.53	0.30	TI	TI
9	*Aspergillus* sp.	60.66	81.60	TI	TI	0.39	0.18	TI	TI
10	*Aspergillus* sp	46.72	53.60	51.54	58.46	0.53	0.46	0.48	0.42
11	*Aspergillus* sp.	49.18	82.40	TI	TI	0.51	0.18	TI	TI
12	*Trichoderma harzianum*	61.48	59.20	TI	TI	0.39	0.41	TI	TI
13	*T. harzianum*	63.11	62.40	NI	NI	0.37	0.38	NI	NI
14	*Neosartorya fischeri*	47.54	67.20	TI	TI	0.52	0.33	TI	TI
15	*Aspergillus* sp.	67.21	60.80	67.69	65.38	0.33	0.39	0.32	0.35
16	*Penicillium* sp.	60.66	64.00	TI	TI	0.39	0.36	TI	TI
17	*Penicillium* sp.	62.30	62.40	NI	NI	0.38	0.38	NI	NI
18	*N. fischeri*	59.02	84.00	TI	TI	0.41	0.16	TI	TI
19	*T. harzianum*	47.54	80.00	TI	TI	0.52	0.20	TI	TI
20	*T. hamatum*	50.82	59.20	53.85	53.08	0.49	0.41	0.46	0.47
21	*Fusarium* sp.	49.18	84.80	TI	TI	0.51	0.15	TI	TI
22	*Fusarium* sp.	46.72	84.80	TI	TI	0.53	0.15	TI	TI
23	*Trichderma* sp.	62.30	64.80	TI	TI	0.38	0.35	TI	TI
24	*Aspergillus* sp.	60.66	68.00	TI	TI	0.39	0.32	TI	TI
25	*Aspergillus* sp.	50.82	60.00	63.08	66.15	0.49	0.40	0.37	0.34
26	*Fusarium* sp.	63.93	95.20	TI	TI	0.36	0.05	TI	TI
27	*Penicillium* sp.	50.82	53.60	54.62	53.85	0.49	0.46	0.45	0.46
28	*N. fischeri*	51.64	61.60	TI	TI	0.48	0.38	TI	TI
29	*Trichoderma* sp.	45.08	45.60	56.15	TI	0.55	0.54	0.44	TI
30	*T. harzianum*	61.48	61.60	NI	NI	0.39	0.38	NI	NI
31	*Aspergillus* sp.	46.72	60.80	TI	TI	0.53	0.39	TI	TI
32	*Aspergillus* sp.	58.20	76.00	TI	TI	0.42	0.24	TI	TI
33	*Aspergillus* sp.	50.82	72.80	TI	TI	0.49	0.27	TI	TI
34	*N. fischeri*	59.02	53.60	TI	TI	0.41	0.46	TI	TI
35	*T. harzianum*	42.62	44.80	60.00	47.69	0.57	0.55	0.40	0.52
36	Sterile	62.30	64.00	NI	NI	0.38	0.36	NI	NI
37	*T. harzianum*	67.21	69.60	NI	NI	0.33	0.30	NI	NI
38	*Penicillium* sp.	49.18	51.20	93.85	TI	0.51	0.49	0.06	TI
39	*N. fischeri*	54.10	60.00	50.00	TI	0.46	0.40	0.50	TI
40	*T. harzianum*	51.64	60.00	49.23	53.85	0.48	0.40	0.51	0.46
41	*T. harzianum*	59.02	59.20	NI	NI	0.41	0.41	NI	NI
42	*T. harzianum*	63.93	66.40	NI	NI	0.36	0.34	NI	NI
43	*T. harzianum*	63.11	64.00	NI	NI	0.37	0.36	NI	NI
44	*Penicillium* sp.	59.02	69.60	TI	TI	0.41	0.30	TI	TI
45	*N. fischeri*	63.11	58.40	TI	TI	0.37	0.42	TI	TI

Appendix 1 cont…

Fungal isolate code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
46	*N. fischeri*	48.36	68.00	TI	TI	0.52	0.32	TI	TI
47	*N. fischeri*	42.62	44.80	46.15	46.92	0.57	0.55	0.54	0.53
48	Sterile	40.98	47.20	66.92	76.15	0.59	0.53	0.33	0.24
49	Sterile	58.20	60.00	NI	NI	0.42	0.40	NI	NI
Control (*Pythium irregulare)*		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
Mean		54.03	64.15	59.69	61.96	0.46	0.36	0.40	0.38
Range		40.98 to 67.21	44.80 to 95.20	46.15 to 93.85	46.92 to 98.46	0.33 to 0.59	0.05 to 0.55	0.06 to 0.54	0.02 to 0.53
CD (0.05)		6.04	10.11	13.48	13.48	_	_	_	_
CD (0.01)		8.06	13.49	18.38	18.38	_	_	_	_
Coefficient of variation (CV)		5.68	8.01	10.91	10.91	_	_	_	_

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)
* Growth overlap or no inhibition zone is observed
** Total inhibition of mycelium growth of the pathogen by the tested isolate

Appendixes 2. Effect of different rhizosphere fungal isolates on mycelia growth of *Penicillium digitatum*

Fungal isolates code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
1	*Penicillium* sp.	44.44	86.67	90.00	NI*	0.56	0.13	0.10	NI
2	*Penicillium* sp.	40.74	74.44	69.00	75.38	0.59	0.26	0.31	0.25
3	Sterile	40.74	74.44	76.00	75.38	0.59	0.26	0.24	0.25
4	*Aspergillus* sp.	3.70	68.89	80.00	NI	0.96	0.31	0.20	NI
5	*Aspergillus* sp.	29.63	73.33	78.00	NI	0.70	0.27	0.22	NI
6	*Aspergillus* sp.	14.81	73.33	78.00	NI	0.85	0.27	0.22	NI
7	*Penicillium* sp	33.33	64.44	69.00	73.08	0.67	0.36	0.31	0.27
8	*Penicillium* sp.	22.22	72.22	82.00	NI	0.78	0.28	0.18	NI
9	*Aspergillus* sp.	29.63	72.22	74.00	77.69	0.70	0.28	0.26	0.22
10	*Aspergillus* sp	25.93	74.44	80.00	NI	0.74	0.26	0.20	NI
11	*Aspergillus* sp.	22.22	73.33	86.00	NI	0.78	0.27	0.14	NI
12	*Trichoderma harzianum*	29.63	76.67	86.00	NI	0.70	0.23	0.14	NI
13	*T. harzianum*	44.44	78.89	80.00	NI	0.56	0.21	0.20	NI
14	*Neosartorya fischeri*	25.93	74.44	81.00	NI	0.74	0.26	0.19	NI
15	*Aspergillus* sp.	22.22	76.67	83.00	NI	0.78	0.23	0.17	NI
16	*Penicillium* sp.	29.63	78.89	TI**	NI	0.70	0.21	TI	NI
17	*Penicillium* sp.	37.04	80.00	83.00	NI	0.63	0.20	0.17	NI
18	*N. fischeri*	18.52	67.78	72.00	77.69	0.81	0.32	0.28	0.22
19	*T. harzianum*	33.33	57.78	67.00	85.38	0.67	0.42	0.33	0.15
20	*Trichoderma hamatum*	33.33	58.89	63.00	71.54	0.67	0.41	0.37	0.28
21	*Fusarium* sp.	29.63	76.67	84.00	NI	0.70	0.23	0.16	NI
22	*Fusarium* sp.	29.63	64.44	85.00	NI	0.70	0.36	0.15	NI
23	*Trichoderma* sp.	37.04	74.44	72.00	77.69	0.63	0.26	0.28	0.22
24	*Aspergillus* sp.	33.33	56.67	67.00	71.54	0.67	0.43	0.33	0.28
25	*Aspergillus* sp.	29.63	72.22	78.00	NI	0.70	0.28	0.22	NI
26	*Fusarium* sp.	18.52	65.56	71.00	NI	0.81	0.34	0.29	NI
27	*Penicillium* sp.	33.33	TI	NI	NI	0.67	TI	NI	NI
28	*N. fischeri*	33.33	76.67	81.00	NI	0.67	0.23	0.19	NI
29	*Trichoderma* sp.	40.74	53.33	57.00	66.92	0.59	0.47	0.43	0.33
30	*T. harzianum*	40.74	83.33	80.00	NI	0.59	0.17	0.20	NI
31	*Aspergillus* sp.	25.93	77.78	84.00	NI	0.74	0.22	0.16	NI
32	*Aspergillus* sp.	25.93	76.67	83.00	NI	0.74	0.23	0.17	NI
33	*Aspergillus* sp.	-11.11	64.44	79.00	NI	1.11	0.36	0.21	NI
34	*N. fischeri*	22.22	70.00	79.00	NI	0.78	0.30	0.21	NI
35	*T. harzianum*	22.22	42.22	46.00	63.85	0.78	0.58	0.54	0.36
36	Sterile	37.04	82.22	84.00	NI	0.63	0.18	0.16	NI
37	*T. harzianum*	44.44	83.33	87.00	NI	0.56	0.17	0.13	NI
38	*Penicillium* sp.	25.93	76.67	80.00	NI	0.74	0.23	0.20	NI
39	*N. fischeri*	33.33	62.22	68.00	NI	0.67	0.38	0.32	NI
40	*T. harzianum*	29.63	78.89	80.00	NI	0.70	0.21	0.20	NI
41	*T. harzianum*	40.74	83.33	83.00	NI	0.59	0.17	0.17	NI
42	*T. harzianum*	48.15	83.33	85.00	NI	0.52	0.17	0.15	NI
43	*T. harzianum*	44.44	82.22	85.00	NI	0.56	0.18	0.15	NI
44	*Penicillium* sp.	33.33	77.78	82.00	NI	0.67	0.22	0.18	NI
45	*N. fischeri*	33.33	75.56	82.00	NI	0.67	0.24	0.18	NI

Appendixes 2 cont…

Fungal isolate code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
46	*N. fischeri*	29.63	75.56	81.00	NI	0.70	0.24	0.19	NI
47	*N. fischeri*	33.33	77.78	83.00	NI	0.67	0.22	0.17	NI
48	Sterile	25.93	51.11	56.00	63.08	0.74	0.49	0.44	0.37
49	Sterile	40.74	80.00	73.00	NI	0.59	0.20	0.27	NI
Control (*P. digitatum*)		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
Mean		30.46	72.55	77.28	73.27	0.70	0.27	0.23	0.27
Range		-11.11 to 48.15	42.22 to 86.67	46.00 to 90.00	63.08 to 85.38	0.52 to 1.11	0.13 to 0.58	0.10 to 0.54	0.15 to 0.37
CD (0.05)		32.68	13.46	16.11	3.96	—	—	—	—
CD (0.01)		43.59	17.96	21.5	5.52	—	—	—	—
CV %		56.28	9.48	10.73	2.71	—	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)
* Growth overlap or no inhibition zone is observed
** Total inhibition of mycelium growth of the pathogen by the tested isolate

Appendix 3. Effect of different rhizosphere fungal isolates on mycelial growth of *Geotrichum candidum*

Fungal isolates code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
1	*Penicillium* sp.	-16.67	13.51	30.95	36.84	1.17	0.86	0.69	0.63
2	*Penicillium* sp.	38.89	54.05	64.29	70.18	0.61	0.46	0.36	0.30
3	Sterile	50.00	32.43	40.48	49.12	0.50	0.68	0.60	0.51
4	*Aspergillus* sp.	0.00	21.62	38.10	49.12	1.00	0.78	0.62	0.51
5	*Aspergillus* sp.	0.00	27.03	38.10	52.63	1.00	0.73	0.62	0.47
6	*Aspergillus* sp.	-11.11	21.62	38.10	50.88	1.11	0.78	0.62	0.49
7	*Penicillium* sp	-11.11	16.22	28.57	47.37	1.11	0.84	0.71	0.53
8	*Penicillium* sp.	-27.78	21.62	35.71	54.39	1.28	0.78	0.64	0.46
9	*Aspergillus* sp.	-16.67	27.03	38.10	54.39	1.17	0.73	0.62	0.46
10	*Aspergillus* sp	-22.22	13.51	33.33	47.37	1.22	0.86	0.67	0.53
11	*Aspergillus* sp.	5.56	24.32	35.71	50.88	0.94	0.76	0.64	0.49
12	*Trichoderma harzianum*	-27.78	18.92	30.95	47.37	1.28	0.81	0.69	0.53
13	*T. harzianum*	38.89	64.86	45.24	54.39	0.61	0.35	0.55	0.46
14	*Neosartorya fischeri*	-11.11	18.92	28.57	45.61	1.11	0.81	0.71	0.54
15	*Aspergillus* sp.	-16.67	13.51	28.57	47.37	1.17	0.86	0.71	0.53
16	*Penicillium* sp.	-5.56	24.32	40.48	52.63	1.06	0.76	0.60	0.47
17	*Penicillium* sp.	38.89	67.57	42.86	52.63	0.61	0.32	0.57	0.47
18	*N. fischeri*	16.67	21.62	26.19	57.89	0.83	0.78	0.74	0.42
19	*T. harzianum*	16.67	45.95	47.62	56.14	0.83	0.54	0.52	0.44
20	*Trichoderma hamatum*	-16.67	21.62	38.10	45.61	1.17	0.78	0.62	0.54
21	*Fusarium* sp.	-11.11	18.92	33.33	50.88	1.11	0.81	0.67	0.49
22	*Fusarium* sp.	-16.67	13.51	30.95	47.37	1.17	0.86	0.69	0.53
23	*Trichoderma* sp.	-22.22	8.11	23.81	43.86	1.22	0.92	0.76	0.56
24	*Aspergillus* sp.	-16.67	0.00	23.81	47.37	1.17	1.00	0.76	0.53
25	*Aspergillus* sp.	-22.22	13.51	28.57	47.37	1.22	0.86	0.71	0.53
26	*Fusarium* sp.	-5.56	21.62	30.95	54.39	1.06	0.78	0.69	0.46
27	*Penicillium* sp.	-22.22	13.51	28.57	42.11	1.22	0.86	0.71	0.58
28	*N. fischeri*	-11.11	13.51	50.00	45.61	1.11	0.86	0.50	0.54
29	*Trichoderma* sp.	5.56	24.32	54.76	50.88	0.94	0.76	0.45	0.49
30	*T. harzianum*	50.00	21.62	45.24	49.12	0.50	0.78	0.55	0.51
31	*Aspergillus* sp.	0.00	29.73	35.71	54.39	1.00	0.70	0.64	0.46
32	*Aspergillus* sp.	-5.56	24.32	30.95	50.88	1.06	0.76	0.69	0.49
33	*Aspergillus* sp.	5.56	29.73	35.71	49.12	0.94	0.70	0.64	0.51
34	*N. fischeri*	-11.11	21.62	26.19	54.39	1.11	0.78	0.74	0.46
35	*T. harzianum*	16.67	16.22	28.57	54.39	0.83	0.84	0.71	0.46
36	Sterile	44.44	32.43	40.48	52.63	0.56	0.68	0.60	0.47
37	*T. harzianum*	27.78	18.92	28.57	42.11	0.72	0.81	0.71	0.58
38	*Penicillium* sp.	5.56	24.32	30.95	50.88	0.94	0.76	0.69	0.49
39	*N. fischeri*	5.56	13.51	23.81	43.86	0.94	0.86	0.76	0.56
40	*T. harzianum*	-27.78	-5.41	-7.14	17.54	1.28	1.05	1.07	0.82
41	*T. harzianum*	50.00	18.92	40.48	33.33	0.50	0.81	0.60	0.67
42	*T. harzianum*	66.67	24.32	47.62	47.37	0.33	0.76	0.52	0.53
43	*T. harzianum*	5.56	18.92	30.95	43.86	0.94	0.81	0.69	0.56
44	*Penicillium* sp.	-5.56	21.62	33.33	50.88	1.06	0.78	0.67	0.49
45	*N. fischeri*	-5.56	18.92	33.33	47.37	1.06	0.81	0.67	0.53

Appendix 3 cont…

Fungal isolate code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
46	*N. fischeri*	5.56	10.81	9.52	19.30	0.94	0.89	0.90	0.81
47	*N. fischeri*	5.56	27.03	35.71	54.39	0.94	0.73	0.64	0.46
48	Sterile	5.56	8.11	14.29	19.30	0.94	0.92	0.86	0.81
49	Sterile	-72.22	-27.03	38.10	15.79	1.72	1.27	0.62	0.84
Control (*G. candidum*)		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
Mean		1.36	21.35	33.82	47.01	0.99	0.79	0.66	0.53
Range		-72.22 to 66.67	-27.03 to 67.57	-7.14 to 64.29	15.79 to 70.18	0.33 to 1.72	0.32 to 1.27	0.36 to 1.07	0.30 to 0.84
CD (0.05)		52.64	31.87	23.68	18.8	—	—	—	—
CD (0.01)		70.23	42.51	31.59	25.08	—	—	—	—
CV		8734.5	77.65	36.23	20.46	—	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)

Appendix 4. Effect of different rhizosphere fungal isolates on mycelial growth of *Colletotrichum gloeosporioides* (503) isolate

Fungal isolates code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
1	*Penicillium* sp.	60.00	73.33	73.53	NI*	0.40	0.27	0.26	NI
2	*Penicillium* sp.	16.00	42.22	64.71	86.67	0.84	0.58	0.35	0.13
3	Sterile	32.00	26.67	38.24	65.00	0.68	0.73	0.62	0.35
4	*Aspergillus* sp.	64.00	55.56	67.65	75.00	0.36	0.44	0.32	0.25
5	*Aspergillus* sp.	40.00	40.00	60.29	74.17	0.60	0.60	0.40	0.26
6	*Aspergillus* sp.	48.00	44.44	63.24	77.50	0.52	0.56	0.37	0.23
7	*Penicillium* sp	40.00	53.33	67.65	80.00	0.60	0.47	0.32	0.20
8	*Penicillium* sp.	20.00	46.67	58.82	75.00	0.80	0.53	0.41	0.25
9	*Aspergillus* sp.	48.00	51.11	67.65	85.00	0.52	0.49	0.32	0.15
10	*Aspergillus* sp	28.00	42.22	60.29	75.83	0.72	0.58	0.40	0.24
11	*Aspergillus* sp.	44.00	48.89	67.65	80.83	0.56	0.51	0.32	0.19
12	*Trichoderma harzianum*	64.00	80.00	85.29	NI	0.36	0.20	0.15	NI
13	*T. harzianum*	64.00	77.78	85.29	NI	0.36	0.22	0.15	NI
14	*Neosartorya fischeri*	40.00	42.22	60.29	75.00	0.60	0.58	0.40	0.25
15	*Aspergillus* sp.	64.00	37.78	45.59	70.83	0.36	0.62	0.54	0.29
16	*Penicillium* sp.	68.00	51.11	70.59	NI	0.32	0.49	0.29	NI
17	*Penicillium* sp.	76.00	80.00	85.29	NI	0.24	0.20	0.15	NI
18	*N. fischeri*	20.00	44.44	66.18	84.17	0.80	0.56	0.34	0.16
19	*T. harzianum*	52.00	48.89	66.18	80.83	0.48	0.51	0.34	0.19
20	*Trichoderma hamatum*	40.00	31.11	45.59	66.67	0.60	0.69	0.54	0.33
21	*Fusarium* sp.	16.00	35.56	54.41	73.33	0.84	0.64	0.46	0.27
22	*Fusarium* sp.	48.00	51.11	58.82	75.00	0.52	0.49	0.41	0.25
23	*Trichoderma* sp.	32.00	35.56	55.88	70.83	0.68	0.64	0.44	0.29
24	*Aspergillus* sp.	48.00	51.11	52.94	69.17	0.52	0.49	0.47	0.31
25	*Aspergillus* sp.	52.00	48.89	61.76	75.83	0.48	0.51	0.38	0.24
26	*Fusarium* sp.	28.00	37.78	60.29	76.67	0.72	0.62	0.40	0.23
27	*Penicillium* sp.	16.00	44.44	66.18	NI	0.84	0.56	0.34	NI
28	*N. fischeri*	92.00	48.89	52.94	70.00	0.08	0.51	0.47	0.30
29	*Trichoderma* sp.	16.00	15.56	35.29	63.33	0.84	0.84	0.65	0.37
30	*T. harzianum*	52.00	75.56	85.29	NI	0.48	0.24	0.15	NI
31	*Aspergillus* sp.	32.00	40.00	61.76	79.17	0.68	0.60	0.38	0.21
32	*Aspergillus* sp.	20.00	42.22	63.24	78.33	0.80	0.58	0.37	0.22
33	*Aspergillus* sp.	20.00	40.00	67.65	79.17	0.80	0.60	0.32	0.21
34	*N. fischeri*	20.00	46.67	66.18	80.83	0.80	0.53	0.34	0.19
35	*T. harzianum*	20.00	15.56	41.18	66.67	0.80	0.84	0.59	0.33
36	Sterile	48.00	75.56	80.88	NI	0.52	0.24	0.19	NI
37	*T. harzianum*	60.00	68.89	85.29	NI	0.40	0.31	0.15	NI
38	*Penicillium* sp.	24.00	42.22	64.71	NI	0.76	0.58	0.35	NI
39	*N. fischeri*	24.00	20.00	41.18	68.33	0.76	0.80	0.59	0.32
40	*T. harzianum*	12.00	22.22	42.65	60.83	0.88	0.78	0.57	0.39
41	*T. harzianum*	52.00	71.11	85.29	NI	0.48	0.29	0.15	NI
42	*T. harzianum*	60.00	77.78	85.29	NI	0.40	0.22	0.15	NI
43	*T. harzianum*	56.00	73.33	86.76	NI	0.44	0.27	0.13	NI
44	*Penicillium* sp.	56.00	46.67	61.76	66.67	0.44	0.53	0.38	0.33

Appendix 4 cont…

Fungal isolate code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
45	*N. fischeri*	16.00	46.67	64.71	81.67	0.84	0.53	0.35	0.18
46	*N. fischeri*	24.00	48.89	64.71	81.67	0.76	0.51	0.35	0.18
47	*N. fischeri*	28.00	46.67	64.71	81.67	0.72	0.53	0.35	0.18
48	Sterile	36.00	22.22	30.88	58.33	0.64	0.78	0.69	0.42
49	Sterile	52.00	73.33	85.29	NI	0.48	0.27	0.15	NI
Control (*Colletotricum* 503)		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
	Mean	40.57	48.62	63.84	74.57	0.59	0.51	0.36	0.25
	Range	12.00 to 92.00	15.56 to 80.00	30.88 to 86.76	58.33 to 86.67	0.08 to 0.88	0.20 to 0.84	0.13 to 0.69	0.13 to 0.42
	CD (0.05)	16.89	16.72	17.17	5.54	—	—	—	—
	CD (0.01)	22.54	22.31	22.91	7.44	—	—	—	—
	CV	21.2	17.55	13.76	3.76	—	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)
* Growth overlap or no inhibition zone is observed

Appendix 5. Effect of different rhizosphere fungal isolates on mycelial growth of *Colletotrichum gloeosporioides* (500) isolate

Fungal isolates code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
1	*Penicillium* sp.	63.33	77.59	84.88	NI**	0.37	0.22	0.15	NI
2	*Penicillium* sp.	26.67	62.07	TI	TI*	0.73	0.38	TI	TI
3	Sterile	-6.67	29.31	53.49	74.17	1.07	0.71	0.47	0.26
4	*Aspergillus* sp.	10.00	51.72	65.12	76.67	0.90	0.48	0.35	0.23
5	*Aspergillus* sp.	13.33	53.45	67.44	NI	0.87	0.47	0.33	NI
6	*Aspergillus* sp.	10.00	50.00	65.12	75.00	0.90	0.50	0.35	0.25
7	*Penicillium* sp	13.33	37.93	53.49	66.67	0.87	0.62	0.47	0.33
8	*Penicillium* sp.	10.00	44.83	65.12	75.00	0.90	0.55	0.35	0.25
9	*Aspergillus* sp.	16.67	58.62	68.60	TI	0.83	0.41	0.31	TI
10	*Aspergillus* sp	16.67	53.45	69.77	78.33	0.83	0.47	0.30	0.22
11	*Aspergillus* sp.	16.67	56.90	67.44	76.67	0.83	0.43	0.33	0.23
12	*Trichoderma harzianum*	20.00	56.90	69.77	NI	0.80	0.43	0.30	NI
13	*T. harzianum*	60.00	79.31	88.37	NI	0.40	0.21	0.12	NI
14	*Neosartorya fischeri*	23.33	56.90	69.77	80.00	0.77	0.43	0.30	0.20
15	*Aspergillus* sp.	10.00	48.28	63.95	74.17	0.90	0.52	0.36	0.26
16	*Penicillium* sp.	33.33	48.28	62.79	75.00	0.67	0.52	0.37	0.25
17	*Penicillium* sp.	63.33	82.76	83.72	NI	0.37	0.17	0.16	NI
18	*N. fischeri*	16.67	43.10	56.98	70.00	0.83	0.57	0.43	0.30
19	*T. harzianum*	66.67	81.03	87.21	NI	0.33	0.19	0.13	NI
20	*Trichoderma hamatum*	-13.33	29.31	53.49	67.50	1.13	0.71	0.47	0.33
21	*Fusarium* sp.	20.00	50.00	67.44	75.83	0.80	0.50	0.33	0.24
22	*Fusarium* sp.	6.67	44.83	65.12	74.17	0.93	0.55	0.35	0.26
23	*Trichoderma* sp.	3.33	48.28	61.63	71.67	0.97	0.52	0.38	0.28
24	*Aspergillus* sp.	23.33	46.55	59.30	75.00	0.77	0.53	0.41	0.25
25	*Aspergillus* sp.	13.33	44.83	65.12	75.00	0.87	0.55	0.35	0.25
26	*Fusarium* sp.	16.67	46.55	63.95	71.67	0.83	0.53	0.36	0.28
27	*Penicillium* sp.	-13.33	24.14	55.81	66.67	1.13	0.76	0.44	0.33
28	*N. fischeri*	16.67	53.45	68.60	80.83	0.83	0.47	0.31	0.19
29	*Trichoderma* sp.	3.33	18.97	50.00	62.50	0.97	0.81	0.50	0.38
30	*T. harzianum*	63.33	81.03	GOL	NI	0.37	0.19	GOL	NI
31	*Aspergillus* sp.	20.00	53.45	68.60	77.50	0.80	0.47	0.31	0.23
32	*Aspergillus* sp.	16.67	43.10	62.79	70.00	0.83	0.57	0.37	0.30
33	*Aspergillus* sp.	20.00	48.28	TI	76.67	0.80	0.52	TI	0.23
34	*N. fischeri*	10.00	46.55	62.79	75.00	0.90	0.53	0.37	0.25
35	*T. harzianum*	0.00	31.03	53.49	68.33	1.00	0.69	0.47	0.32
36	Sterile	63.33	81.03	87.21	NI	0.37	0.19	0.13	NI
37	*T. harzianum*	63.33	82.76	86.05	NI	0.37	0.17	0.14	NI
38	*Penicillium* sp.	36.67	56.90	72.09	NI	0.63	0.43	0.28	NI
39	*N. fischeri*	0.00	31.03	51.16	66.67	1.00	0.69	0.49	0.33
40	*T. harzianum*	20.00	31.03	39.53	59.17	0.80	0.69	0.60	0.41
41	*T. harzianum*	63.33	82.76	88.37	NI	0.37	0.17	0.12	NI
42	*T. harzianum*	66.67	82.76	TI	NI	0.33	0.17	TI	NI
43	*T. harzianum*	66.67	79.31	88.37	NI	0.33	0.21	0.12	NI
44	*Penicillium* sp.	16.67	53.45	67.44	76.67	0.83	0.47	0.33	0.23

Appendix 5 cont…

Fungal isolate code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
45	*N. fischeri*	20.00	43.10	66.28	75.00	0.80	0.57	0.34	0.25
46	*N. fischeri*	26.67	56.90	74.42	76.67	0.73	0.43	0.26	0.23
47	*N. fischeri*	23.33	50.00	69.77	76.67	0.77	0.50	0.30	0.23
48	Sterile	-3.33	15.52	37.21	55.83	1.03	0.84	0.63	0.44
49	Sterile	66.67	79.31	88.37	NI	0.33	0.21	0.12	NI
Control (*Coletotricum*-500)		0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
	Mean	24.90	53.24	67.05	72.63	0.75	0.47	0.33	0.27
	Range	-13.33 to 66.67	15.52 to 82.76	37.21 to 88.37	55.83 to 80.83	0.33 to 1.13	0.17 to 0.84	0.12 to 0.63	0.19 to 0.44
	CD (0.05)	14.79	13.83	7.34	3.83	—	—	—	—
	CD (0.01)	19.73	18.45	9.79	5.14	—	—	—	—
	CV	30.17	13.21	5.52	2.67	—	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)

* Growth overlap or no inhibition zone is observed

** Total inhibition of mycelium growth of the pathogen by rhizosphere isolates

Appendix 6. Effect of different rhizosphere fungal isolates on mycelial growth of *Fusarium solani*

Fungal isolates code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
1	*Penicillium* sp.	15.38	36.96	54.55	68.37	0.85	0.63	0.45	0.32
2	*Penicillium* sp.	57.69	73.91	TI*	TI	0.42	0.26	TI	TI
3	Sterile	3.85	36.96	43.94	51.02	0.96	0.63	0.56	0.49
4	*Aspergillus* sp.	7.69	36.96	54.55	65.31	0.92	0.63	0.45	0.35
5	*Aspergillus* sp.	19.23	43.48	60.61	66.33	0.81	0.57	0.39	0.34
6	*Aspergillus* sp.	15.38	34.78	54.55	67.35	0.85	0.65	0.45	0.33
7	*Penicillium* sp	15.38	28.26	39.39	58.16	0.85	0.72	0.61	0.42
8	*Penicillium* sp.	3.85	30.43	50.00	66.33	0.96	0.70	0.50	0.34
9	*Aspergillus* sp.	11.54	34.78	62.12	67.35	0.88	0.65	0.38	0.33
10	*Aspergillus* sp	19.23	39.13	56.06	71.43	0.81	0.61	0.44	0.29
11	*Aspergillus* sp.	7.69	36.96	56.06	70.41	0.92	0.63	0.44	0.30
12	*Trichoderma harzianum*	7.69	36.96	56.06	69.39	0.92	0.63	0.44	0.31
13	*T. harzianum*	50.00	71.74	84.85	TI	0.50	0.28	0.15	TI
14	*Neosartorya fischeri*	7.69	15.22	25.76	48.98	0.92	0.85	0.74	0.51
15	*Aspergillus* sp.	3.85	32.61	54.55	67.35	0.96	0.67	0.45	0.33
16	*Penicillium* sp.	30.77	45.65	66.67	77.55	0.69	0.54	0.33	0.22
17	*Penicillium* sp.	50.00	73.91	78.79	TI	0.50	0.26	0.21	TI
18	*N. fischeri*	7.69	39.13	57.58	71.43	0.92	0.61	0.42	0.29
19	*T. harzianum*	53.85	76.09	80.30	TI	0.46	0.24	0.20	TI
20	*Trichoderma hamatum*	11.54	26.09	46.97	63.27	0.88	0.74	0.53	0.37
21	*Fusarium* sp.	11.54	36.96	56.06	68.37	0.88	0.63	0.44	0.32
22	*Fusarium* sp.	19.23	41.30	56.06	68.37	0.81	0.59	0.44	0.32
23	*Trichoderma* sp.	15.38	43.48	62.12	69.39	0.85	0.57	0.38	0.31
24	*Aspergillus* sp.	7.69	28.26	42.42	65.31	0.92	0.72	0.58	0.35
25	*Aspergillus* sp.	19.23	41.30	57.58	67.35	0.81	0.59	0.42	0.33
26	*Fusarium* sp.	0.00	34.78	50.00	66.33	1.00	0.65	0.50	0.34
27	*Penicillium* sp.	38.46	63.04	68.18	77.55	0.62	0.37	0.32	0.22
28	*N. fischeri*	11.54	41.30	60.61	68.37	0.88	0.59	0.39	0.32
29	*Trichoderma* sp.	0.00	10.87	27.27	48.98	1.00	0.89	0.73	0.51
30	*T. harzianum*	42.31	71.74	81.82	TI	0.58	0.28	0.18	TI
31	*Aspergillus* sp.	19.23	39.13	56.06	69.39	0.81	0.61	0.44	0.31
32	*Aspergillus* sp.	11.54	34.78	54.55	69.39	0.88	0.65	0.45	0.31
33	*Aspergillus* sp.	11.54	36.96	53.03	68.37	0.88	0.63	0.47	0.32
34	*N. fischeri*	11.54	34.78	54.55	67.35	0.88	0.65	0.45	0.33
35	*T. harzianum*	0.00	15.22	34.85	54.08	1.00	0.85	0.65	0.46
36	Sterile	53.85	73.91	83.33	TI	0.46	0.26	0.17	TI
37	*T. harzianum*	61.54	78.26	84.85	TI	0.38	0.22	0.15	TI
38	*Penicillium* sp.	0.00	34.78	54.55	68.37	1.00	0.65	0.45	0.32
39	*N. fischeri*	15.38	30.43	51.52	69.39	0.85	0.70	0.48	0.31
40	*T. harzianum*	15.38	30.43	43.94	67.35	0.85	0.70	0.56	0.33
41	*T. harzianum*	61.54	76.09	75.76	TI	0.38	0.24	0.24	TI
42	*T. harzianum*	65.38	73.91	TI	TI	0.35	0.26	TI	TI
43	*T. harzianum*	53.85	73.91	81.82	TI	0.46	0.26	0.18	TI
44	*Penicillium* sp.	7.69	36.96	54.55	63.27	0.92	0.63	0.45	0.37

Appendix 6 cont…

Fungal isolates code	Identity	Growth inhibition (%)[1]				Relative growth ratio[1]			
		After 5 days	After 7 days	After 10 days	After 14 days	After 5 days	After 7 days	After 10 days	After 14 days
45	*N. fischeri*	3.85	34.78	54.55	68.37	0.96	0.65	0.45	0.32
46	*N. fischeri*	15.38	36.96	57.58	68.37	0.85	0.63	0.42	0.32
47	*N. fischeri*	3.85	36.96	53.03	65.31	0.96	0.63	0.47	0.35
48	Sterile	0.00	10.87	24.24	47.96	1.00	0.89	0.76	0.52
49	Sterile	50.00	73.91	74.24	86.73	0.50	0.26	0.26	0.13
	Control (*F. solani*)	0.00	0.00	0.00	0.00	1.00	1.00	1.00	1.00
	Mean	20.96	43.39	57.29	66.25	0.79	0.57	0.43	0.34
	Range	0.00 to 65.38	10.87 to 78.26	24.24 to 84.85	47.96 to 86.73	0.35 to 1.00	0.22 to 0.89	0.15 to 0.76	0.13 to 0.52
	CD (0.05)	17.95	8.1	6.72	3.32	—	—	—	—
	CD (0.01)	23.95	10.8	8.96	4.44	—	—	—	—
	CV	43.51	9.48	5.96	2.54	—	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)
* Total inhibition of mycelium growth of the pathogen by the test isolate

Appendix 7. Effect of different rhizosphere bacterial isolates on the mycelium growth of *Fusarium solani*

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
1	1	6.47	0.00	0.00	0.94	1.00	1.00
2	2	17.76	0.00	0.00	0.82	1.00	1.00
3	3	6.47	0.00	0.00	0.94	1.00	1.00
4	4	14.53	0.00	0.00	0.85	1.00	1.00
5	5	3.24	0.00	0.00	0.97	1.00	1.00
6	6	12.11	0.00	0.00	0.88	1.00	1.00
7	7	-1.60	0.00	0.00	1.02	1.00	1.00
8	8	37.11	0.00	0.00	0.63	1.00	1.00
9	9	39.53	55.00	0.00	0.60	0.45	1.00
10	10	38.72	0.00	0.00	0.61	1.00	1.00
11	11	16.14	0.00	0.00	0.84	1.00	1.00
12	12	20.98	0.00	0.00	0.79	1.00	1.00
13	13	54.04	0.00	0.00	0.46	1.00	1.00
14	14	0.02	0.00	0.00	1.00	1.00	1.00
15	15	8.08	0.00	0.00	0.92	1.00	1.00
16	16	-0.79	0.00	0.00	1.01	1.00	1.00
17	17	17.76	0.00	0.00	0.82	1.00	1.00
18	18	0.02	0.00	0.00	1.00	1.00	1.00
19	19	58.07	0.00	0.00	0.42	1.00	1.00
20	20	53.23	0.00	0.00	0.47	1.00	1.00
21	21	70.97	TIP*	TIP	0.29	TIP	TIP
22	22	4.05	0.00	0.00	0.96	1.00	1.00
23	23	0.02	0.00	0.00	1.00	1.00	1.00
24	24	41.94	26.11	23.33	0.58	0.74	0.77
25	25	10.50	0.00	0.00	0.90	1.00	1.00
26	26	39.53	0.00	0.00	0.60	1.00	1.00
27	27	-3.21	0.00	0.00	1.03	1.00	1.00
28	28	4.05	0.00	0.00	0.96	1.00	1.00
29	29	2.44	0.00	0.00	0.98	1.00	1.00
30	30	3.24	0.00	0.00	0.97	1.00	1.00
31	31	TIP	TIP	TIP	TIP	TIP	TIP
32	32	33.08	2.22	28.89	0.67	0.98	0.71
33	33	52.43	0.00	0.00	0.48	1.00	1.00
34	34	-3.21	0.00	0.00	1.03	1.00	1.00
35	35	52.43	0.00	0.00	0.48	1.00	1.00
36	36	4.05	0.00	0.00	0.96	1.00	1.00
37	37	0.82	0.00	0.00	0.99	1.00	1.00
38	38	26.62	0.00	0.00	0.73	1.00	1.00
39	39	8.89	0.00	0.00	0.91	1.00	1.00
40	40	8.08	0.00	0.00	0.92	1.00	1.00
41	41	16.95	6.67	0.00	0.83	0.93	1.00
42	42	14.53	10.00	0.00	0.85	0.90	1.00
43	43	12.92	4.44	0.00	0.87	0.96	1.00
44	44	19.37	7.22	0.00	0.81	0.93	1.00
45	45	27.43	5.00	0.00	0.73	0.95	1.00

Appendix 7 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
46	46	16.95	20.00	0.00	0.83	0.80	1.00
47	47	19.37	5.56	0.00	0.81	0.94	1.00
48	48	23.40	4.44	0.00	0.77	0.96	1.00
49	49	22.59	12.78	0.00	0.77	0.87	1.00
50	50	41.94	56.11	55.00	0.58	0.44	0.45
51	51	12.92	20.00	0.00	0.87	0.80	1.00
52	52	10.50	5.56	0.00	0.90	0.94	1.00
53	53	7.27	0.00	0.00	0.93	1.00	1.00
54	54	TIP	TIP	TIP	TIP	TIP	TIP
55	56	23.40	0.56	0.00	0.77	0.99	1.00
56	57	17.76	17.22	0.00	0.82	0.83	1.00
57	58	25.82	5.56	0.00	0.74	0.94	1.00
58	59	10.50	6.67	0.00	0.90	0.93	1.00
59	60	25.01	5.56	0.00	0.75	0.94	1.00
60	61	16.14	3.33	0.00	0.84	0.97	1.00
61	62	19.37	8.89	0.00	0.81	0.91	1.00
62	63	14.53	9.44	0.00	0.85	0.91	1.00
63	64	16.95	7.78	0.00	0.83	0.92	1.00
64	65	19.37	11.67	10.00	0.81	0.88	0.90
65	66	20.98	16.11	0.00	0.79	0.84	1.00
66	67	17.76	7.78	0.00	0.82	0.92	1.00
67	68	20.17	13.33	0.00	0.80	0.87	1.00
68	69	11.30	4.44	0.00	0.89	0.96	1.00
69	70	14.53	10.00	0.00	0.85	0.90	1.00
70	71	25.82	0.56	0.00	0.74	0.99	1.00
71	72	33.88	0.56	0.00	0.66	0.99	1.00
72	75	33.08	3.33	0.00	0.67	0.97	1.00
73	76	42.75	2.22	0.00	0.57	0.98	1.00
74	77	27.43	3.89	0.00	0.73	0.96	1.00
75	78	27.43	0.56	0.00	0.73	0.99	1.00
76	79	19.37	10.00	0.00	0.81	0.90	1.00
77	80	12.92	0.56	0.00	0.87	0.99	1.00
78	81	37.11	3.33	0.00	0.63	0.97	1.00
79	82	15.34	6.11	0.00	0.85	0.94	1.00
80	83	28.24	13.33	10.56	0.72	0.87	0.89
81	84	25.01	1.67	0.00	0.75	0.98	1.00
82	85	20.17	2.22	0.00	0.80	0.98	1.00
83	86	17.76	1.67	0.00	0.82	0.98	1.00
84	87	14.53	16.67	0.00	0.85	0.83	1.00
85	88	35.49	-23.89	0.00	0.65	1.24	1.00
86	89	18.56	14.44	0.00	0.81	0.86	1.00
87	90	12.92	4.44	0.00	0.87	0.96	1.00
88	91	19.37	7.78	10.56	0.81	0.92	0.89
89	92	23.40	1.11	0.00	0.77	0.99	1.00
90	93	14.53	6.67	0.00	0.85	0.93	1.00

Appendix 7 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
91	94	13.72	9.44	3.89	0.86	0.91	0.96
92	95	35.49	0.56	0.00	0.65	0.99	1.00
93	96	16.14	3.33	0.00	0.84	0.97	1.00
94	96	33.08	3.89	0.00	0.67	0.96	1.00
95	97	14.53	27.78	0.00	0.85	0.72	1.00
96	99	19.37	25.56	22.22	0.81	0.74	0.78
97	100	25.01	13.89	12.78	0.75	0.86	0.87
98	101	6.47	3.89	0.00	0.94	0.96	1.00
99	102	22.59	6.11	0.00	0.77	0.94	1.00
100	103	13.72	17.78	0.00	0.86	0.82	1.00
101	104	19.37	0.56	0.00	0.81	0.99	1.00
102	105	11.30	2.22	0.00	0.89	0.98	1.00
103	106	12.92	32.22	30.56	0.87	0.68	0.69
104	107	33.08	25.56	20.00	0.67	0.74	0.80
105	108	20.17	4.44	0.00	0.80	0.96	1.00
106	109	12.11	5.56	0.00	0.88	0.94	1.00
107	110	16.95	6.67	0.00	0.83	0.93	1.00
108	111	19.37	6.67	0.00	0.81	0.93	1.00
109	112	23.40	1.11	0.00	0.77	0.99	1.00
110	113	15.34	11.11	0.00	0.85	0.89	1.00
111	114	48.40	12.78	0.00	0.52	0.87	1.00
112	115	10.50	5.56	0.00	0.90	0.94	1.00
113	116	11.30	6.67	0.00	0.89	0.93	1.00
114	117	21.79	7.22	0.00	0.78	0.93	1.00
115	118	18.56	3.33	0.00	0.81	0.97	1.00
116	119	29.04	0.56	0.00	0.71	0.99	1.00
117	120	32.27	1.11	0.00	0.68	0.99	1.00
118	121	42.75	22.78	20.00	0.57	0.77	0.80
119	122	14.53	5.00	0.00	0.85	0.95	1.00
120	123	20.17	20.00	0.00	0.80	0.80	1.00
121	124	28.24	16.67	0.00	0.72	0.83	1.00
122	125	36.30	43.89	41.11	0.64	0.56	0.59
123	126	11.30	21.11	0.00	0.89	0.79	1.00
124	127	20.98	3.33	0.00	0.79	0.97	1.00
125	128	44.36	1.11	0.00	0.56	0.99	1.00
126	129	35.49	8.33	0.00	0.65	0.92	1.00
127	130	31.46	15.00	0.00	0.69	0.85	1.00
128	131	36.30	1.67	0.00	0.64	0.98	1.00
129	132	39.53	0.56	0.00	0.60	0.99	1.00
130	134	33.88	22.78	0.00	0.66	0.77	1.00
131	135	27.43	32.78	28.89	0.73	0.67	0.71
132	136	42.75	7.78	0.00	0.57	0.92	1.00
133	137	16.14	11.11	0.00	0.84	0.89	1.00
134	139	20.98	23.33	0.00	0.79	0.77	1.00
135	140	15.34	3.33	0.00	0.85	0.97	1.00
136	141	15.34	3.89	0.00	0.85	0.96	1.00

Appendix 7 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
137	142	16.95	15.00	0.00	0.83	0.85	1.00
138	143	19.37	14.44	0.00	0.81	0.86	1.00
139	144	10.50	5.56	0.00	0.90	0.94	1.00
140	145	10.50	2.78	0.00	0.90	0.97	1.00
141	146	14.53	16.67	0.00	0.85	0.83	1.00
142	147	22.59	17.22	0.00	0.77	0.83	1.00
Control (*F. solani*)		0.00	0.00	0.00	1.00	1.00	1.00
Mean		21.19	7.40	2.29	0.79	0.93	0.98
Range		-3.21 to 70.97	-23.89 to 56.11	0.00 to 55.00	0.29 to 1.03	0.44 to 1.24	0.45 to 1.00
CD (0.05)		6.55	7.61	2.51	—	—	—
CD (0.01)		8.61	10.013	3.31	—	—	—
CV %		19.62	65.99	53.92	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)
* The rhizobacterial isolate totally inhibited by the pathogen

Appendix 8. Effect of different rhizosphere bacterial isolates on the mycelial growth of *Geotrichum candidum*

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
1	1	14.27	25.00	25.00	0.86	0.75	0.75
2	2	24.36	33.89	33.33	0.76	0.66	0.67
3	3	11.75	18.89	18.33	0.88	0.81	0.82
4	4	29.40	46.67	44.44	0.71	0.53	0.56
5	5	9.23	17.78	15.56	0.91	0.82	0.84
6	6	28.56	50.56	50.00	0.71	0.49	0.50
7	7	42.01	57.78	57.22	0.58	0.42	0.43
8	8	TI*	TI	TI	TI	TI	TI
9	9	TI	TI	TI	TI	TI	TI
10	10	TI	TI	TI	TI	TI	TI
11	11	17.63	37.78	35.56	0.82	0.62	0.64
12	12	22.68	41.11	40.00	0.77	0.59	0.60
13	13	TI	TI	TI	TI	TI	TI
14	14	5.03	21.67	20.00	0.95	0.78	0.80
15	15	14.27	26.11	27.22	0.86	0.74	0.73
16	16	24.36	42.22	52.22	0.76	0.58	0.48
17	17	27.72	50.00	51.11	0.72	0.50	0.49
18	18	6.71	20.00	0.00	0.93	0.80	1.00
19	19	3.35	15.56	0.00	0.97	0.84	1.00
20	20	17.63	37.22	35.56	0.82	0.63	0.64
21	21	TI	TI	TI	TI	TI	TI
22	22	74.79	TI	TI	0.25	TI	TI
23	23	74.79	TI	TI	0.25	TI	TI
24	24	74.79	TI	TI	0.25	TI	TI
25	25	54.61	65.00	66.11	0.45	0.35	0.34
26	26	TI	TI	TI	TI	TI	TI
27	27	15.95	20.00	0.00	0.84	0.80	1.00
28	28	14.27	20.56	0.00	0.86	0.79	1.00
29	29	18.47	22.22	0.00	0.82	0.78	1.00
30	30	16.79	22.22	0.00	0.83	0.78	1.00
31	31	TI	TI	TI	TI	TI	TI
32	32	TI	TI	TI	TI	TI	TI
33	33	0.68	10.00	0.00	0.99	0.90	1.00
34	34	2.04	14.44	0.00	0.98	0.86	1.00
35	35	TI	TI	TI	TI	TI	TI
36	36	8.84	20.00	0.00	0.91	0.80	1.00
37	37	8.39	25.00	0.00	0.92	0.75	1.00
38	38	45.37	51.11	51.67	0.55	0.49	0.48
39	39	12.59	23.33	0.00	0.87	0.77	1.00
40	40	10.91	26.67	22.22	0.89	0.73	0.78
41	41	23.81	28.89	7.78	0.76	0.71	0.92
42	42	26.53	36.67	15.56	0.73	0.63	0.84
43	43	30.61	40.00	23.33	0.69	0.60	0.77
44	44	25.17	24.44	4.44	0.75	0.76	0.96
45	45	30.61	37.78	16.67	0.69	0.62	0.83
46	46	14.29	25.56	8.89	0.86	0.74	0.91
47	47	6.12	16.67	0.00	0.94	0.83	1.00
48	48	8.84	17.78	0.00	0.91	0.82	1.00
49	49	7.48	17.78	2.22	0.93	0.82	0.98
50	50	22.45	26.67	11.11	0.78	0.73	0.89
51	51	TI	TI	TI	TI	TI	TI
52	52	4.76	10.00	0.00	0.95	0.90	1.00

Appendix 8 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
53	53	25.17	30.00	11.11	0.75	0.70	0.89
54	56	22.45	22.22	2.22	0.78	0.78	0.98
55	57	15.65	24.44	7.78	0.84	0.76	0.92
56	58	21.09	31.11	14.44	0.79	0.69	0.86
57	59	8.84	20.00	1.11	0.91	0.80	0.99
58	60	0.68	14.44	0.00	0.99	0.86	1.00
59	61	-0.68	12.22	0.00	1.01	0.88	1.00
60	62	4.76	13.33	0.00	0.95	0.87	1.00
61	63	8.84	14.44	0.00	0.91	0.86	1.00
62	64	8.84	21.11	8.89	0.91	0.79	0.91
63	65	19.73	28.89	8.89	0.80	0.71	0.91
64	66	10.20	23.33	3.33	0.90	0.77	0.97
65	67	27.89	30.00	15.56	0.72	0.70	0.84
66	68	29.25	38.89	23.33	0.71	0.61	0.77
67	69	27.89	36.67	13.33	0.72	0.63	0.87
68	70	12.93	23.33	6.67	0.87	0.77	0.93
69	71	22.45	33.33	16.67	0.78	0.67	0.83
70	72	25.17	33.33	17.78	0.75	0.67	0.82
71	75	23.81	30.00	18.89	0.76	0.70	0.81
72	76	26.53	35.56	15.56	0.73	0.64	0.84
73	77	22.45	31.11	13.33	0.78	0.69	0.87
74	78	19.73	30.00	16.67	0.80	0.70	0.83
75	79	7.48	23.33	2.22	0.93	0.77	0.98
76	80	30.61	34.44	14.44	0.69	0.66	0.86
77	81	22.45	23.33	3.33	0.78	0.77	0.97
78	82	21.09	28.89	10.00	0.79	0.71	0.90
79	83	21.09	31.11	11.11	0.79	0.69	0.89
80	84	19.73	25.56	5.56	0.80	0.74	0.94
81	85	25.17	36.67	14.44	0.75	0.63	0.86
82	86	3.40	10.00	0.00	0.97	0.90	1.00
83	87	TI	TI	TI	TI	TI	TI
84	88	22.45	25.56	12.22	0.78	0.74	0.88
85	89	27.89	34.44	14.44	0.72	0.66	0.86
86	90	3.40	17.78	1.11	0.97	0.82	0.99
87	91	TI	TI	TI	TI	TI	TI
88	92	29.25	36.67	16.67	0.71	0.63	0.83
89	93	2.04	13.33	0.00	0.98	0.87	1.00
90	94	6.12	15.56	0.00	0.94	0.84	1.00
91	95	23.81	32.22	10.00	0.76	0.68	0.90
92	96	8.84	17.78	0.00	0.91	0.82	1.00
93	97	23.81	33.33	15.56	0.76	0.67	0.84
94	98	7.48	17.78	1.11	0.93	0.82	0.99
95	99	23.81	32.22	13.33	0.76	0.68	0.87
96	100	19.73	28.89	7.78	0.80	0.71	0.92
97	101	6.12	17.78	0.00	0.94	0.82	1.00
98	102	10.20	21.11	3.33	0.90	0.79	0.97
99	103	10.20	20.00	1.11	0.90	0.80	0.99
100	104	2.04	14.44	0.00	0.98	0.86	1.00
101	105	7.48	20.00	0.00	0.93	0.80	1.00
102	106	22.45	25.56	12.22	0.78	0.74	0.88
103	107	25.56	33.33	23.33	0.74	0.67	0.77
104	108	19.73	28.89	11.11	0.80	0.71	0.89
105	109	6.12	18.89	0.00	0.94	0.81	1.00
106	110	6.12	20.00	0.00	0.94	0.80	1.00
107	111	3.40	14.44	0.00	0.97	0.86	1.00

Appendix 8 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
108	112	22.45	33.33	16.67	0.78	0.67	0.83
109	113	6.12	16.67	0.00	0.94	0.83	1.00
110	114	31.97	37.78	16.67	0.68	0.62	0.83
111	115	7.48	16.67	0.00	0.93	0.83	1.00
112	116	6.12	16.67	0.00	0.94	0.83	1.00
113	117	8.84	21.11	0.00	0.91	0.79	1.00
114	118	6.12	18.89	0.00	0.94	0.81	1.00
115	119	26.53	32.22	11.11	0.73	0.68	0.89
116	120	23.81	33.33	12.22	0.76	0.67	0.88
117	121	37.41	45.56	36.67	0.63	0.54	0.63
118	122	2.04	15.56	0.00	0.98	0.84	1.00
119	123	4.76	17.78	0.00	0.95	0.82	1.00
120	124	21.09	26.67	8.89	0.79	0.73	0.91
121	125	25.17	33.33	17.78	0.75	0.67	0.82
122	126	17.01	26.67	8.89	0.83	0.73	0.91
123	127	23.81	28.89	16.67	0.76	0.71	0.83
124	128	6.12	17.78	1.11	0.94	0.82	0.99
125	129	22.45	28.89	10.00	0.78	0.71	0.90
126	130	22.45	28.89	8.89	0.78	0.71	0.91
127	131	25.17	30.00	14.44	0.75	0.70	0.86
128	132	22.45	30.00	11.11	0.78	0.70	0.89
129	134	23.81	35.56	14.44	0.76	0.64	0.86
130	135	23.81	35.56	6.67	0.76	0.64	0.93
131	136	26.53	32.22	14.44	0.73	0.68	0.86
132	137	6.12	17.78	0.00	0.94	0.82	1.00
133	139	7.48	21.11	0.00	0.93	0.79	1.00
134	140	0.68	11.11	0.00	0.99	0.89	1.00
135	141	2.04	12.22	0.00	0.98	0.88	1.00
136	142	7.48	21.11	1.11	0.93	0.79	0.99
137	143	8.84	16.67	1.11	0.91	0.83	0.99
138	144	2.04	16.67	0.00	0.98	0.83	1.00
139	145	2.04	13.33	0.00	0.98	0.87	1.00
140	146	21.09	31.11	10.00	0.79	0.69	0.90
141	147	29.25	35.56	15.56	0.71	0.64	0.84
Control (*G. candidum*)		0.00	0.00	0.00	1.00	1.00	1.00
Mean		17.96	26.45	11.19	0.82	0.74	0.89
Range		-0.68 to 74.79	10.00 to 65.00	0.00 to 66.11	0.25 to 1.01	0.35 to 0.90	0.34 to 1.00
CD (0.05)		5.08	6.38	5.91	—	—	—
CD (0.01)		6.67	8.38	7.77	—	—	—
CV %		17.89	15.23	33.60	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)

* Total inhibition of mycelium growth of the pathogen by isolate

Appendix 9: Effect of different rhizosphere bacterial isolates on the mycelial growth of *Colletotrichum gloeosporioides*

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
1	1	19.54	31.37	25.98	0.80	0.69	0.74
2	2	48.28	59.48	55.85	0.52	0.41	0.44
3	3	14.94	30.72	27.28	0.85	0.69	0.73
4	4	34.48	60.78	57.80	0.66	0.39	0.42
5	5	13.79	35.95	29.23	0.86	0.64	0.71
6	6	18.39	52.94	50.01	0.82	0.47	0.50
7	7	14.94	29.41	27.28	0.85	0.71	0.73
8	8	TI*	TI	TI	TI	TI	TI
9	9	TI	TI	TI	TI	TI	TI
10	10	TI	TI	TI	TI	TI	TI
11	11	47.13	54.25	49.36	0.53	0.46	0.51
12	12	57.47	67.32	61.69	0.43	0.33	0.38
13	13	TI	TI	TI	TI	TI	TI
14	14	9.20	27.45	22.74	0.91	0.73	0.77
15	15	17.24	31.37	29.88	0.83	0.69	0.70
16	16	8.05	21.57	20.79	0.92	0.78	0.79
17	17	17.24	43.14	48.71	0.83	0.57	0.51
18	18	10.34	13.07	16.24	0.90	0.87	0.84
19	19	13.79	13.07	14.30	0.86	0.87	0.86
20	20	22.99	41.83	42.22	0.77	0.58	0.58
21	21	TI	TI	TI	TI	TI	TI
22	22	57.47	61.44	57.15	0.43	0.39	0.43
23	23	-1.15	18.95	20.79	1.01	0.81	0.79
24	24	16.09	33.33	31.83	0.84	0.67	0.68
25	25	47.13	67.32	70.13	0.53	0.33	0.30
26	26	TI	TI	TI	TI	TI	TI
27	27	18.39	42.48	40.27	0.82	0.58	0.60
28	28	22.99	41.18	43.51	0.77	0.59	0.56
29	29	24.14	42.48	42.86	0.76	0.58	0.57
30	30	26.44	42.48	43.51	0.74	0.58	0.56
31	31	TI	TI	TI	TI	TI	TI
32	32	TI	TI	TI	TI	TI	TI
33	33	TI	TI	TI	TI	TI	TI
34	34	TI	TI	TI	TI	TI	TI
35	35	TI	TI	TI	TI	TI	TI
36	36	TI	TI	TI	TI	TI	TI
37	37	34.48	43.79	44.16	0.66	0.56	0.56
38	38	63.22	60.13	64.94	0.37	0.40	0.35
39	39	36.78	42.48	38.32	0.63	0.58	0.62
40	40	25.29	37.91	38.97	0.75	0.62	0.61
41	41	12.00	22.72	1.11	0.88	0.77	0.99
42	42	16.00	38.63	2.22	0.84	0.61	0.98
43	43	46.67	51.13	38.89	0.53	0.49	0.61

Appendix 9 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
44	44	54.67	59.09	46.67	0.45	0.41	0.53
45	45	52.00	56.81	53.33	0.48	0.43	0.47
46	46	32.00	17.04	0.00	0.68	0.83	1.00
47	47	0.00	9.08	0.00	1.00	0.91	1.00
48	48	6.67	7.94	0.00	0.93	0.92	1.00
49	49	12.00	15.90	1.11	0.88	0.84	0.99
50	50	45.33	49.99	44.44	0.55	0.50	0.56
51	51	17.33	19.31	0.00	0.83	0.81	1.00
52	52	17.33	18.17	5.56	0.83	0.82	0.94
53	53	41.33	23.85	15.56	0.59	0.76	0.84
54	54	53.33	57.95	55.56	0.47	0.42	0.44
55	56	45.33	51.13	41.11	0.55	0.49	0.59
56	57	33.33	36.36	30.00	0.67	0.64	0.70
57	58	44.00	48.86	36.67	0.56	0.51	0.63
58	59	2.67	6.81	1.11	0.97	0.93	0.99
59	60	25.33	23.85	17.78	0.75	0.76	0.82
60	61	2.67	10.22	1.11	0.97	0.90	0.99
61	62	45.33	29.54	34.44	0.55	0.70	0.66
62	63	26.67	27.26	20.00	0.73	0.73	0.80
63	64	54.67	54.54	45.56	0.45	0.45	0.54
64	65	30.67	38.63	23.33	0.69	0.61	0.77
65	66	25.33	27.26	0.00	0.75	0.73	1.00
66	67	52.00	53.40	42.22	0.48	0.47	0.58
67	68	1.33	13.63	0.00	0.99	0.86	1.00
68	69	1.33	4.53	0.00	0.99	0.95	1.00
69	70	4.00	19.31	0.00	0.96	0.81	1.00
70	71	56.00	54.54	56.67	0.44	0.45	0.43
71	72	48.00	52.27	50.00	0.52	0.48	0.50
72	75	49.33	53.40	47.78	0.51	0.47	0.52
73	76	53.33	54.54	45.56	0.47	0.45	0.54
74	77	49.33	45.45	31.11	0.51	0.55	0.69
75	78	44.00	48.86	41.11	0.56	0.51	0.59
76	79	36.00	36.36	15.56	0.64	0.64	0.84
77	80	40.00	43.18	27.78	0.60	0.57	0.72
78	81	49.33	52.27	46.67	0.51	0.48	0.53
79	82	38.67	35.22	10.00	0.61	0.65	0.90
80	83	50.67	43.18	53.33	0.49	0.57	0.47
81	84	28.00	34.08	13.33	0.72	0.66	0.87
82	85	57.33	60.22	54.44	0.43	0.40	0.46
83	86	6.67	17.04	1.11	0.93	0.83	0.99
84	87	33.33	30.67	16.67	0.67	0.69	0.83
85	88	44.00	47.72	35.56	0.56	0.52	0.64
86	89	56.00	55.68	52.22	0.44	0.44	0.48
87	90	9.33	17.04	5.56	0.91	0.83	0.94
88	91	2.67	9.08	1.11	0.97	0.91	0.99
89	92	50.67	54.54	51.11	0.49	0.45	0.49

Appendix 9 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
90	93	13.33	17.04	0.00	0.87	0.83	1.00
91	94	0.00	13.63	0.00	1.00	0.86	1.00
92	95	50.67	48.86	42.22	0.49	0.51	0.58
93	96	46.67	51.13	44.44	0.53	0.49	0.56
94	97	20.00	23.85	3.33	0.80	0.76	0.97
95	98	12.00	12.49	3.33	0.88	0.88	0.97
96	99	46.67	47.72	41.11	0.53	0.52	0.59
97	100	9.33	19.31	2.22	0.91	0.81	0.98
98	101	24.00	27.26	16.67	0.76	0.73	0.83
99	102	26.67	27.26	0.00	0.73	0.73	1.00
100	103	20.00	18.17	0.00	0.80	0.82	1.00
101	104	9.33	13.63	0.00	0.91	0.86	1.00
102	105	2.67	5.67	1.11	0.97	0.94	0.99
103	106	25.33	31.81	13.33	0.75	0.68	0.87
104	107	40.00	51.13	50.00	0.60	0.49	0.50
105	108	-76.00	53.40	42.22	1.76	0.47	0.58
106	109	9.33	14.76	2.22	0.91	0.85	0.98
107	110	16.00	13.63	0.00	0.84	0.86	1.00
108	111	13.33	26.13	2.22	0.87	0.74	0.98
109	112	46.67	52.27	45.56	0.53	0.48	0.54
110	113	50.67	53.40	48.89	0.49	0.47	0.51
111	114	20.00	32.95	6.67	0.80	0.67	0.93
112	115	40.00	44.31	38.89	0.60	0.56	0.61
113	116	8.00	15.90	0.00	0.92	0.84	1.00
114	117	20.00	19.31	0.00	0.80	0.81	1.00
115	118	18.67	13.63	1.11	0.81	0.86	0.99
116	119	41.33	45.45	42.22	0.59	0.55	0.58
117	120	53.33	56.81	53.33	0.47	0.43	0.47
118	121	50.67	52.27	48.89	0.49	0.48	0.51
119	122	9.33	15.90	0.00	0.91	0.84	1.00
120	123	21.33	22.72	0.00	0.79	0.77	1.00
121	124	20.00	24.99	0.00	0.80	0.75	1.00
122	125	18.67	23.85	6.67	0.81	0.76	0.93
123	126	24.00	27.26	25.56	0.76	0.73	0.74
124	127	44.00	49.99	35.56	0.56	0.50	0.64
125	128	53.33	57.95	50.00	0.47	0.42	0.50
126	129	49.33	53.40	48.89	0.51	0.47	0.51
127	130	50.67	55.68	46.67	0.49	0.44	0.53
128	131	44.00	39.77	33.33	0.56	0.60	0.67
129	132	52.00	57.95	57.78	0.48	0.42	0.42
130	134	48.00	53.40	47.78	0.52	0.47	0.52
131	135	45.33	46.58	35.56	0.55	0.53	0.64
132	136	48.00	54.54	51.11	0.52	0.45	0.49
133	137	53.33	59.09	54.44	0.47	0.41	0.46
134	139	38.67	38.63	32.22	0.61	0.61	0.68
135	140	14.67	17.04	0.00	0.85	0.83	1.00

Appendix 9 cont…

No.	Bacterial isolates code	Growth inhibition (%)[1]			Relative growth ratio[1]		
		After 5 days	After 10 days	After 15 days	After 5 days	After 10 days	After 15 days
136	141	30.67	37.49	15.56	0.69	0.63	0.84
137	142	9.33	19.31	1.11	0.91	0.81	0.99
138	143	14.67	31.81	5.56	0.85	0.68	0.94
139	144	1.33	4.53	0.00	0.99	0.95	1.00
140	145	4.00	7.94	0.00	0.96	0.92	1.00
141	146	45.33	45.45	41.11	0.55	0.55	0.59
142	147	20.00	24.99	0.00	0.80	0.75	1.00
Control (*C. gloeosporioides*)		0.00	0.00	0.00	1.00	1.00	1.00
	Mean	28.94	35.84	26.69	0.71	0.64	0.73
	Range	-76.00 to 63.22	4.53 to 67.32	0.00 to 70.13	0.37 to 1.76	0.33 to 0.95	0.30 to 1.00
	CD (0.05)	31.82	10.8	8.4	—	—	—
	CD (0.01)	41.83	14.2	11.05	—	—	—
	CV %	69.47	19.06	20.01	—	—	—

[1] Each value represents a mean of three Petri dish replicates evaluated after the listed dates at room temperature (25°C)
* Total inhibition of pathogen mycelium growth by isolate

Appendix 10. Bacterial and fungal isolates and commercial biocontrol products included in the present study

Isolate code	Identity	Type of experiment			
		Biocontrol		Growth promotion	
		Screening	Final evaluation	Screening	Final evaluation
9F	*Aspergillus* sp.			x	
107B	*Bacillus pumilus*			x	
24B	*B. stearotermophilus*			x	x
BSB	*B. subtilis*		x	x	x
57B	*B. cereus*			x	
114B	*Enterobacter cloacae*			x	
51B	*B. cereus*	x		x	
76B	*Proteus penneri*			x	
106B	*B. mycoides*			x	
87B	*P. penneri*		x	x	x
91B	*P. penneri*	x	x		
43B	*B. pumilus*	x	x		
121B	*P. penneri*	x			
BSB+43B	*B. subtilis* and *B. pumilus*		x		
BSB+91B	*B. subtilis* and *P. penneri*		x		
BSB+43B+ 91B+87B	*B. subtilis*, *B. pumilus*, *P. penneri* and *P. penneri*		x		
	Biostart™	x	x		
	Bactolife A+B™	x		x	