Mechanisms of Phyllosphere Biological Control Of *Leptosphaeria Maculans*, The Blackleg Pathogen of Canola, Using Antagonistic Bacteria

BY

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A Thesis
Submitted to the Faculty of Graduate Studies in
Partial fulfillment of the Requirements
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GENERAL ABSTRACT

Blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces & De Not (anamorph *Phoma lingam* (Tode:Fr./Desm.)), is an economically important disease of canola (*Brassica napus* L.) in Canada and worldwide. In W. Canada, a shift in the pathogen population to more virulent races (PG3, PGT and PG4) has increased the risk of a blackleg epidemic, which is a huge threat for the canola industry. Biological control using non-pathogenic, antagonistic bacteria has been successfully used in other cropping systems for the control of plant diseases. This study identified that bacteria originating from the stubble and leaf tissues (endophytes) had the highest antifungal activity, against *L. maculans*, in plate and plant assays, respectively. Bacteria with the highest disease suppression in cotyledon assays were also effective at the 3-4 leaf stage. In field assays, bacteria applied at the cotyledon leaf stage suppressed the disease. PCR screening for antibiotic biosynthetic genes identified *Pseudomonas chlororaphis* (DF190, DF202, DF210) and *P. aurantiaca* (DF200) isolates harboring biosynthetic genes of phenazine and pyrrolnitrin. Similarly, PCR-screening also detected the presence of biosynthetic genes of: iturin A and bacillomycin D in *Bacillus cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6; surfactin in strains DFE4 and DFE16; zwittermicin A and the self-resistance protein (ZmaR) in *B. cereus* isolates DFE4, DFE8 and DFE13. The GC-MS analysis of the methanol-broth extract of strain DF190 revealed the presence of phenazine, 2-hydroxyphenazine and 2-acetamidophenol. MALDI-TOF-MS analysis of the cell surface extracts of strains DFE4 and DFE16 detected the production of antifungal lipopeptide antibiotics iturin A, bacillomycin D and surfactin, confirming the PCR detection of the biosynthetic genes of these antibiotics. The production of 2-acetamidophenol by a *P.*

chlororaphis isolate, and lipopeptide antibiotics iturin A, bacillomycin D and surfactin by a *B. cereus* isolate has not been reported before. Antifungal antibiotics-producing bacteria were also tested for elicitation of ISR in blackleg control. Bacterial cells and broth extracts, when inoculated away from the pathogen, failed to elicit ISR towards the suppression of blackleg lesions on cotyledons. However, the bacteria and broth extracts, when inoculated locally with the pathogen, significantly suppressed the pycnidiospores and lesion development. The absence of localized host antifungal defense enzyme activity further indicated direct antifungal activity of the bacteria and metabolites present in their broth extracts. A *gacS* mutant of strain PA23 lost its secondary metabolite production and biocontrol activity, which was restored with the complementation of the *gacS* gene. Interestingly, a phenazine non-producing mutant of strain PA23, producing same levels of pyrrolnitrin as the wild type, exhibited same or better levels of blackleg disease suppression. This suggests the potential role of pyrrolnitrin in antibiosis mediated by strain PA23, along with extracellular lytic enzyme activity for the biocontrol of *L. maculans* in canola. Overall, this study has demonstrated the potential of bacterial biocontrol agents to mediate phyllopshere control of *L. maculans*, which could be incorporated in an integrated disease management system

FOREWORD

This thesis follows the manuscript style outlined by the Department of Plant Science, University of Manitoba. Manuscripts follow the style recommended by the Canadian Journal of Plant Pathology. The thesis is presented as five manuscripts, each containing an introduction, materials and methods, results and discussion. A general review of literature precedes the manuscripts, and a general discussion follows the manuscripts.

1.0 INTRODUCTION

Canola (*Brassica napus* L) is grown worldwide, with Canada being the biggest single producer, with a 10 year average at 11.3 million acres harvested. Blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces & De Not (anamorph *Phoma lingam* (Tode:Fr./Desm.)), is an economically important disease of canola worldwide. The disease is endemic in countries where canola is grown, except China. Disease management strategies include stubble management, crop rotation, chemical control and the use of resistant cultivars. Stubble-borne inoculum was previously controlled by burning and deep burial, the latter enhancing the decomposition of stubble. Burning is not practiced anymore due to environmental concerns and prevention of depletion of soil organic matter, and burial with tillage is also not in use due to the shift in agronomic practices towards zero-tillage and soil conservation. This leads to enhanced risk of inoculum build-up. A 3-4 year crop rotation, involving growing of non-canola crops has been recommended to control the disease. The trend towards shortening the length of the rotation increases the risk of inoculum build-up, as the 2-3 year old stubble is the most potent producer of the inoculum. Combined with the stubble remaining on the surface, and the hot summer conditions which favor the longevity of the stubble, this could enhance blackleg threat. In western Canada, the use of cultivars, which are bred for resistance to the predominant pathogen race PG2, had helped to achieve moderate to good resistance control of the disease. During the summer of 2002, there was a breakdown in the resistance of the PG2-resistant cultivar Q2, which was followed by a report of the occurrence of the more virulent race PG3 in 2003. Subsequently, the most virulent PG4, which is predominant in Australia, was reported in 2005. Also, a new

group, PGT, present only in Canada has been reported since 2005. This shift in the pathogen population to more virulent races (PG3, PGT and PG4), for which cultivar resistance has not been achieved yet in Canada, is an emerging threat for the canola industry. This can lead to enhanced fungicide use, which is the case in Australia and Europe. Therefore, the disease has to be managed using an integrated approach that incorporates several strategies against the pathogen. Biological control using antagonistic bacteria has been successfully used in other cropping systems for the control of plant diseases. Bacteria utilize several mechanisms, such as competition, antibiosis (volatile and non-volatile) and induction of systemic resistance in the host plant, to control fungal pathogens. The role of antibiosis in biological control of plant diseases has been well established in many cropping systems. However, there are no reports on antagonistic bacteria-mediated biological control of blackleg, at the phyllosphere, in canola. Our study focused on the identification of potential antibiotic-producing bacteria for the biological control of blackleg, which could be incorporated in the integrated management of the disease, with reduced dependency on fungicides.

2.0 LITERATURE REVIEW

2.1 The Host

2.1.1 History

Canola (*Brassica napus* L.), also known as oilseed rape, is produced worldwide, with China, Canada, Australia and the European Union leading the acreage. In Canada, the 10-year production average is 11.3 million acres, making it the largest single producer of canola (Canola Council of Canada, 2006). The cultivation of *Brassica* dates back several thousand years, when rapeseed, the precursor of canola, was grown in China around 5000 BC (Yan 1990). Ancient literature from India suggests that *Brassica campestris* variety "Yellow Sarson" was grown around the same time as in China (Singh 1958). *Brassica rapa*, one of the diploid ancestors of *B. napus,* has the widest distribution among the Brassicas and had its center of origin the Himalayan regions of India (Downey and Robbelen 1989).

In Canada, the edible oilseed rape was first grown only in 1942 (Special Crops 1944). However, the high erucic acid content (C22:1) (40%) in these oilseeds only enabled them to be used as lubricants for steam engines (Henkel and Cie 1967). The shortage to meet supply demands led to the import of *B. rapa* from Poland in 1936 and *B. napus* in 1943 from the United States, whose original accession was from Argentina. Hence, *B. rapa* is known as Polish rape and *B. napus* as Argentine rapeseed. Extensive research efforts were focused at reducing the erucic acid content in the oil. In the early 1960s, rapeseed varieties low in erucic acid, but still high in the glucosinolate levels were grown (Greer 1950). Glucosinolates, due to their toxicity to animals, were not preferred

at high levels and hence their reduction was essential. In 1968, Dr. Keith Downey from the Agriculture Canada Research Station, developed the first variety containing low erucic acid. But, it was Dr. Baldur Stefansson at the University of Manitoba, who in 1974 derived canola from rapeseed and a closely-related forage (Redekop 2002). The variety "Tower", developed by Dr. Stefansson, was the first "double low" variety and the term 'canola' was coined to describe the combined oil and meal quality. Western Canadian Oilseed Crusher's Association (now the Canadian Oilseed Processors Association) registered the canola trademark, which became the property of the Canola Council of Canada in 1980, and was amended in 1986 by the Trademarks Branch of Consumer and Corporate Affairs. A rapeseed variety, to qualify as canola, has to fit the following definition, "an oil must contain less than 2% of C22:1, and the solid component of the seed must contain less than 30 micromoles of any one or mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3-butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air dry, oil free solid" (Adolphe et al 2002).

2.1.2 Economic Importance

Canola is currently more valuable than peanut, cottonseed and sunflower, as a source of vegetable oil (Sovero 1993). It is the third most important source of vegetable oil worldwide. The steady increase in its value has resulted in a steady increase in its production. In Canada, the rapeseed acreage increased from 10,000 to 2 million hectares during the period from 1948 to 1970 (Ohlson 1972). A similar trend was seen in the world rapeseed production, with an annual rate of increase of 4.3% (Ohlson 1972). Canada is the largest consumer of canola oil per capita, and canola oil represents 70% of

vegetable oil consumed by Canadians (Canola Council of Canada 2007). Canola acreage in the United States has also seen a steady increase (Bunting 1986), and the canola industry is a key contributor to the Australian economy (Jones et al. 2001). In Manitoba, canola has been a key player in the province's economy, with an annual farm cash receipt of $150 million in 1978 (Rogalsky et al 1980) increasing to $596.8 million in 1998. Manitoba's contribution to Canadian-grown canola receipts was 29.6% in 2002, up from 21.1% in 2001 (Manitoba Agriculture Food and Rural Initiative 2006).

2.1.3 Production Practices

Canola is a cool season crop that requires low night temperatures to recover from extreme heat or dry weather. In Canada, the crop is best adapted to the Parkland and transition zones of the western Prairie Provinces (Canola Council of Canada 2006). Soil conditions also play a vital role for good seed germination. In western Canada, black and gray soil zones have the maximum canola production. Loamy soil, which does not crust or hamper seedling emergence, is best suited for canola production. Canola must be seeded into a well aerated, well-structured seedbed, with little stubble and soil temperature >5°C. Weeds, both annuals and perennials, found in all canola-growing areas of the world are controlled by pre-seeding tillage, the use of pre-plant herbicides or foliar applied herbicides, or both, when herbicide resistant GM cultivars are grown (Orson 1995).

2.1.4 Diseases of Canola

Canola is affected by many fungal diseases such as: blackleg (*Leptosphaeria maculans* (Desmaz.) Ces & De Not.); sclerotinia stem rot (*Sclerotinia sclerotiorum* (Lib.) de Bary); alternaria leaf and pod spot or black spot (*Alternaria brassica* (Berk.)

Sacc., *A. brassicola* (Schwein.) Wiltshire and *A. raphani* Groves & Skolko); light leaf spot (*Pyrenopezziza brassicae* Sutton & Rawlinson); white leaf spot (*Mycosphaerella capsellae* (Ellis & Everh.)Deighton); white rust (*Albugo candida* (Pers.) Kunze); downy mildew (*Peronospora parasitica* (Pers.:Fr) Fr.); clubroot (*Plasmidiophora brassicae* Woronin.); and soil-borne seedling diseases (*Fusarium spp, Pythium* spp and *Rhizoctonia solani* Kuhn) (Rimmer and Buchwald 1995).

2.2 The Blackleg Pathogen

2.2.1 Biology

Leptosphaeria maculans (Desm.) Ces & De Not, the causal agent of blackleg disease of canola, is a pseudothecial loculoascomycete. The characteristic feature of this group is that the ascus is bitunicate; it has two separable walls. The outer wall does not stretch readily, but tears off laterally or at its apex to allow the stretching of the thin inner wall. The asci are contained in a single loculed, flask-like structure called pseudothecium. Pseudothecia are the sexual fruiting bodies. According to Ainsworth's (1973) system of classification, *L. maculans* belongs to: Sub-Division- Ascomycotina; Class- Loculoascomycetes; Order-Pleosporales; Family- *Leptosphaeriaceae*; Genus- *Leptosphaeria*; Species- *maculans*. The black shining pseudothecia are conical and flattened at the base. The bitunicate asci elongate within a group of preformed branching pseudoparaphyses, which on close observation would reveal both ascending and descending growth. The ostiole of the pseudothecium is formed by the lysis of the thin layer of cells at the apex of the fruiting body (Webster 1993). The ascospores are released from the pseudothecia and are windborne to cause the primary infection. The ascospores

are discharged successfully in about 5 seconds and the asci tend to appear shorter after the release of each ascospore (Hodgetts 1917).

The anamorph, *Phoma lingam* (Tode:Fr./Desm.) is characterized by thin-walled, slightly smaller globose pycnidia. Pycnidia are asexual fruiting bodies and they produce numerous rod-shaped pycnidiospores. The pycnidiospores are held together in the form of ooze at the apex of the pycnidia and are released through splashing, when a rain droplet falls on the pycnidia.

2.2.2 Pathogenecity Groups (PG) of *L. maculans*

The morphological similarity of several distinct species that are associated with *L. maculans* makes its taxonomy and nomenclature a little confusing (Howlett et al 2001). The ability of the strains to cause stem cankers and produce the phytotoxin, sirodesmin PL, has been used as a criteria to classify them into two groups. Strains that are classified under the "A" group are capable of causing stem canker on *B. napus* and also produce the phytotoxin. These isolates are considered aggressive (highly virulent or virulent). On the other hand, strains that are incapable of causing the stem canker on canola are classified under the "B" group. These isolates are considered non-aggressive (weakly virulent or avirulent). There are some isolates that have been isolated from cruciferous weeds, such as *Thlaspi arvense*, *Sysimbrium spp.*, *Descurainia spp.*, *Lepidium spp.*, and *Erysimum spp.*, (Williams and Fitt 1999), which although classified as *L. maculans,* are genetically dissimilar to both A and B groups (Balesdent et al 1998; Purwantara et al 2000). Somda et al (1997) observed crossing barriers between the A and B group isolates, when pseudothecial production was absent between crosses among the groups. This has led to the recent renaming of the B pathotype group as *Leptosphaeria biglobosa* sp. nov

(Shoemaker and Brun 2001). The presence of a distinct beak on the ascomata that is greatly enlarged at the apex, distinguishes this species from the A pathotype isolates. Bipolar heterothallism is seen in both isolates. Sequence related amplified polymorphism data also suggested that the B group strains were genetically distinct from the A group strains (Chen 2006).

Isolates of pathotype group A have been subdivided into pathogenicity groups PG2, PG3 and PG4 based on their differential reaction on cotyledons of *B. napus* cultivars 'Westar', 'Glacier' and 'Quinta', and the degree of genetic similarity among these isolates is high (Koch et al 1991). PGT, a new, aggressive pathotype was detected in the North American populations of *L. maculans* (Chen and Fernando 2005, Rimmer 2006) Contrastingly, three genetically distinct subgroups have been recognized in the B pathotype group, namely NA1 (*L. biglobosa*), NA2 and NA3 (of which only one isolate has been reported) (Koch et al 1991). The distinction between these pathotype groups and subgroups is supported by a variety of isozyme and protein studies (Balesdent et al 1992; Gall et al 1995; Somda et al 1996), analysis of internal transcribed spacers (Balesdent et al 1998) and amplified fragment length polymorphism (AFLP) (Purwantara et al 2000). The AFLP analysis, the most sensitive of all, did not yield any common bands between these subgroups (Purwantara et al 2000).

2.2.3 Life cycle and mode of infection

Ascospores are the primary and main source of infection of canola. They are produced from pseudothecia in the infected stubble. The release of the ascospores during a rain event usually coincides with the sowing period. Seedling infection is achieved by penetration of the cotyledons or young leaves through stomata or natural openings. The

initial infection of the tissue is biotrophic, but most of the hyphal front becomes necrotrophic. The necrotrophic infection leads to the production of the asexual fruiting bodies in the dead tissue (Hammond et al 1985; Hammond and Lewis 1987). During a rain event, the pycnidiospores are spread by splashing and thus are thought to infect other leaves and neighbouring plants. Hence, pycnidiospores are believed to be secondary sources of infection. However, pycnidiospores have been occasionally shown to be sources of primary inoculum (Hall 1992; Williams 1992). The continuous release of the ascospores, throughout the growing season, causes leaf and stem lesions. The colonization of the intercellular spaces follows the initial infection, which leads to the colonization of the xylem or the spaces between the xylem parenchyma and the cortex in the petiole. This intercellular growth is systemic, biotrophic and visually symptomless (Hammond et al 1985). The fungus finally invades and kills the cells of the stem cortex, resulting in a blackened canker, which completely girdles the base of the stem. Thus, the disease is named "blackleg".

2.2.4 Pathogenicity factors

2.2.4.1 Phytotoxins

Understanding the fungal-host interaction is a key to understanding the disease and employing successful control measures. Phytotoxins, produced by the invading pathogen, play an important role in the establishment of disease in a host by working as a pathogenicity factor. Understanding the chemistry, toxic effect and mode of action of these compounds sheds light on the pathogen-host interaction. Of the various phytotoxins produced by *L. maculans*, the first major compound to be reported was sirodesmin PL (Figure 2.1 A), which is obtained from a non-toxic precursor phomamide. The molecule

is a dioxopiperazine, containing an epipolysulfide bridge and its corresponding diacetyl derivative (Férézou et al 1980). HPLC analysis revealed that all pathotype A group isolates produce a range of sirodesmins, including sirodesmin PL, diacetylsirodesmin PL, sirodesmin J, sirodesmin K, sirodesmin H and phomalirazine, and in most cases sirodesmin PL was the major constituent of the phytotoxic extracts (Pedras et al 1990; Pedras 1998a, b; Pedras and Biesenthal 2000). Protoplast (*B. carinata, B. juncea, B. napus* and *Sinapsis alba*) bioassays determined the phytotoxic effects of sirodesmin PL, at concentration as low as $\geq$ 1 μM (Sjödin et al 1988; Pedras and Séguin-Swartz 1992; Pedras 1998a). Microspores of *B. juncea* cv 'Cutlass' and *B. napus* cv 'Westar', had highly reduced viability when exposed to 5 or 10 μM of sirodesmin PL, diacetylsirodesmin PL and phomalirazine (Pedras 1998a). UV-generated sirodesmin PL mutants produced smaller stem lesions than the wild type, but infected the cotyledons equally well, thus establishing their role as a pathogenecity factor during stem cankering (Sock and Hoppe, 1999). Older cultures of germinated spores of pathotype group A produce a novel host selective toxin, phomalide (Figure 2.1 B), which is characterized by its short production period and distinct structural unrelatedness to sirodesmins (Pedras and Biesenthal 1998). Lesions caused by phomalide on *B. napus* leaves resembles that caused by blackleg, but shows low toxicity on *B. juncea* (Pedras and Biesenthal 1998). This high selectivity of phomalide and its role in plant disease resistance could be of help in breeding programs inclined towards blackleg resistance in *Brassica.* The abundant presence of sirodesmin PL in the group A and its absence in the group B, has led to a classification of the group A as tox^+ and group B as tox^- (Williams and Fitt 1999).

However, this is based on incomplete information, as there are other phytotoxins produced by isolates of both groups.

2.2.4.2 Detoxification of host defense compounds

Phytoalexins are an important group of host defense compounds produced by the plant in response to pathogen invasion. Detoxification of phytoalexins seems to be a key pathogenicity factor for *L. maculans* (Howlett et al 2001). Phytoalexins from the Crucifereae have an indole or indole related ring and at least one sulfur atom as common structural features. Accumulation of phytoalexins, following a hypersensitive response to *L. maculans* infection, is observed 1 or 2 days post-infection (Rouxel et al 1989, 1991). Detoxification of the phytoalexin brassinin, occurs via different pathways in different isolates in the pathotype subgroups (Pedras and Taylor 1993; Pedras 1998b). Leroy, a pathotype A isolate, detoxifies brassinin to indole-3-carboxylic acid via α-dithiocarbamate S-oxide and indole 3-carbaldehyde, whereas Unity, an NA2 isolate of the pathotype B group, mostly the aldehyde through 3-methylacetylamine and indole 3-methylamine (Figure 2.1 C). The detoxification of brassinin, a biogenetic precursor of several other phytoalexins, seems to be a key step in the infection process, as it makes the plant more susceptible to further fungal colonization (Pedras 1993). But at the same time, brassinin is capable of affecting the production of sirodesmins, but not phomalide (Pedras et al 1998a, b). Again, *L. maculans* seem to outwit the host by producing phomalide, which is not affected by brassinin.

The other phytoalexin, cyclobrassinin, is detoxified by different isolates of the two pathotype groups in different pathways (Pedras and Okanga 1999; Pedras et al 2000).

BJ125, an A group isolate, detoxifies cyclobrassinin by transformation to dioxybrassinin, whereas Unity, a pathotype B isolate, detoxifies cyclobrassinin, by transforming it into the phytoalexin, brassilexin (Figure 2.1 D). Both dioxybrassinin and brassilexin are non-toxic. Thus, a 'mimicking' of the host pathway is seen in *L. maculans*, which has coevolved with the host defense system. The only phytoalexin that has not been metabolized by isolates of the A group or Unity is spirobrassinin (Pedras, 1998a, b).

2.3 The Disease

Blackleg occurs as an economically important disease in most of the rapeseed-producing areas of the world, except China. In Canada, since the detection of the non-aggressive isolate in the 1960s (Vanterpool 1961, Vanterpool 1963), both the aggressive and non-aggressive isolates have been identified in western Canada, on the prairies (McGee and Petrie 1978, Petrie 1979, Petrie et al 1985, Fernando and Chen, 2003; Chen and Fernando 2005), and Ontario (Gugel and Petrie 1992, Rempel and Hall 1993). In Europe, the presence of isolates belonging to both pathotypes A and B has been reported in Denmark (Jensen 1994), Great Britain (Brown et al 1976; Fitt et al 1997), Germany (Kuswinanti et al 1995), France (Somda and Brun 1995) and Hungary (Szlavik et al., 2006). In Australia, the disease occurred in devastating proportions in the early 1970s (Bokor et al 1975), and the presence of both pathotypes has been consistently reported (Plummer et al 1994, Salisbury et al 1995). The occurrence of the disease in the United States occurred much later, as compared to the other canola growing regions of the world (Lamey 1995; Bradley et al 2005). Brazil is the only country in South America to report

blackleg disease on canola (Fernando et al., 2003). The only report from China is isolating the B-group from a few fields in 1999 (West et al 2000).

Yield losses due to blackleg have been considerably high. In Ontario, up to 25% yield loss has been reported in some fields (Gugel and Petrie 1992) and in western Canada, yield losses up to 50% have been reported (Petrie et al 1985). High yield losses have also been reported in other canola growing regions of the world as well: Germany- as high as 50% (Gugel and Petrie 1992); Logan County in Kentucky- 75-90% (Lamey and Hershman 1993); UK- up to 93% (Sansford 1995). In Australia, the entire rapeseed industry was wiped out due to a devastating epidemic of the disease (Bokor et al 1975). However, in the oilseed rape growing areas of India (*B. juncea* and *B. rapa*) and China (*B. napus*), the occurrence of the disease is very rare.

2.4 Disease Control

2.4.1 Cultural Control

Crop rotation, which involves the rotation of non hosts of the pathogen, is considered the most important of the cultural control methods. Disease control is achieved through deprivation of the pathogen of its susceptible host, thus reducing the pathogen inoculum. Severity of blackleg can be reduced by a crop rotation of at least 4 years between canola crops (West et al 2001). Blackleg severity was reduced when canola was rotated with wheat, and wheat and flax, under both conventional and zero till systems (Guo et al 2005). Even though the fungus survives in the stubble for more than 5 years, the most serious infections are caused by 2 or 3 year old stubble (Kharbanda and Tewari 1996). But, Marcroft et al (2004) did not observe any difference in blackleg

severity between canola crops sown into 18-month-old canola stubble (short rotation) and crops sown into paddocks that had no history of canola for at least the previous 3 years (long rotation). In Alberta, under the Alberta Agriculture Pest Act, *L. maculans* was declared a pest and sowing or transportation of infected seed was prohibited (West et al 2001). Also, farmers could not grow oilseed rape in a field in which the disease was found. This legislation considerably reduced the rate of spread of blackleg in Alberta.

The production of *L. maculans* ascospores from the canola stubble in adjacent fields could reduce the effect of crop rotation in disease control (Kharbanda and Tewari 1996). Isolation of the canola crop from previous year's canola stubble is considered an important disease management strategy. In W. Australia a distance of 5 – 8 km (Bokor et al 1975) and in W. Canada, a distance of 2 km (Petrie 1978) was recommended for isolating a canola crop from the previous year's stubble. Marcroft et al (2004) observed that blackleg severity was highest where canola crops had been sown adjacent to 6-month-old canola stubble, with the level of blackleg severity decreasing markedly in the first 100 m, and declining further up to 500 m. They recommend that canola crops should be sown at distances greater than 100 m and preferably 500 m from last season's canola stubble.

Altering the date of seeding is also one of the methods of disease control, where the crop escapes the high amount of inoculum or the conditions conducive that favor the development of a disease. Early sowing is practiced in France (LePage and Penaud 1995), whereas delayed sowing is practiced in Australia (McGee 1977) for the seedlings to escape the period when most ascospores are present. In Canada, the release of

ascospores throughout the growing season renders the date of sowing to have no effect on the severity of blackleg (Kharbanda and Tewari 1996).

Minimum or zero tillage aims at conservation of soil moisture, prevention of soil erosion and reduction of input costs; whereas conventional tillage hastens the decomposition of crop residue and shortens the time pathogens survive on infected host material (Kharbanda and Tewari 1996). From a disease control perspective, minimum or zero tillage increases the disease potential by retaining the infected stubble at the surface, as compared to conventional tillage which ensures the burial and prevention of release of windborne pathogen spores (Tekauz and Howard 1988). It is suggested that blackleg, a pathogen spread by windborne ascospores released from surface stubble, can be controlled by burying the stubble in the fall and directly seeding the non host in the spring (Kolte 1985). Guo et al (2005) found that tillage, along with a simple rotation, reduced blackleg incidence and severity, as compared to zero-till which had significantly higher disease levels, with or without rotation. Also, tillage influenced the dynamics of spore release, as seen in the lower release of ascospores and pycndiospores in conventional till plots as compared to zero till plots, irrespective of the crop rotation system followed (Guo et al 2006). In Australia, raking, burning or burying is recommended for the destruction of the infected residues along with other methods of residue management that enhance their breakdown (Barbetti and Khangura 1999, Barbetti and Khangura 2000). Deep ploughing of the stubble is followed in Europe, whereas no tillage is popular in Canada (West et al 2001). Fall tillage is considered one of the best methods of stubble burial and reduction of blackleg severity, along with a good 3 year crop rotation with barley, field peas and wheat (Turkington et al 2000).

2.4.2 Chemical Control

The economics of the crop and the epidemiology of the disease determine the use of fungicides for disease control, in the form of fungicide seed treatments, soil treatments or foliar applications (West et al 2001). Different fungicide treatments and combinations are used in different parts of the world for the control of blackleg in canola. Seedborne infection plays an important role in the introduction of the pathogen to new areas (Rimmer and Buchwaldt 1995). In Canada, carbathin, iprodione and thiram are currently registered as seed treatments for the prevention of seedborne infection (West et al 2001). In Canada, foliar spray of propiconazole is sometimes used for the control of blackleg, but with less success (Kharbanda et al 1999). Unlike in Europe, where the greater yields obtained can justify the use of these expensive fungicides, it is uneconomical to use them in Canada, where the yield is generally low due to shorter day lengths and/or early harvesting caused by hot weather (West et al 2001). Also, in Canada a disease forecasting system has not been in place which would facilitate making fungicide spray decisions during seasons of high disease risk and protect the crop from yield loss. Also, agronomic traits such as cultivar susceptibility, soil type, growth stage, and plant vigor have to be considered in making a meaningful and economically feasible decision.

2.4.3 Host Resistance

The ability of a given host plant to resist the growth and colonization of a pathogen is defined as host plant resistance. Breeding for both seedling and adult plant resistance to both A and B group isolates of *L. maculans* is an important step in the management of blackleg in canola. Adult plant resistance prevents the development of

crown cankers and is thought to have a different genetic basis to seedling resistance, which prevents initial spread in the leaf lamina or spread down the petiole to the hypocotyls or stem (Rimmer and van den Berg 1992). In Canada, Australia and Europe, a major objective of canola breeding is the development of resistant cultivars. Breeding for host resistance to blackleg aims at achieving "field resistance", which encompasses genetic resistance, disease escape and disease tolerance (West et al 2001). Field resistance is influenced not only by host and pathogen factors but also by cultural and environmental factors. In Canada, up until the 1980's, *B. napus* (Argentine) cultivars were susceptible to blackleg, but by the year 2000 nearly 20 resistant or moderately resistant cultivars were developed (Keri 2000). The *B. rapa* (Polish) cultivars still remain susceptible to the disease (Canola Council of Canada 2006). Avirulence genes in *L. maculans* and their corresponding race-specific resistance (R) genes in canola have been established, thus supporting the gene-for-gene relationship in this system (Yu et al 2005). The race-specific resistance gene, *Rlm1* of 'Quinta', is matched by the avirulence gene, AvrRlm1, in the pathogen (Yu et al 2005), and similarly *Rlm2* of 'Glacier' is matched by AvrRlm2 (Ansan-Melayah et al 1998). In addition, six avirulence genes have been identified (Balesdent et al 2001) and their corresponding resistant genes mapped (Delourme et al 2004). This genetic dissection has suggested that a single gene (or a cluster) has been used widely in breeding for resistance to blackleg in canola / oilseed rape cultivars (Yu et al 2005).

2.4.4 Systemic Acquired Resistance

Systemic acquired resistance (SAR) is a plant defense response, in which the plant is in a state of enhanced defense capacity that is often induced after a local

infection. SAR protects the plants from a wide range of pathogens, which includes bacteria, fungi and viruses (Friedrich et al 1996; Sticher et al 1997). The induction of SAR requires the accumulation of salicylic acid (SA) and the expression of pathogenesis-related genes (PR), which encode small, secreted or vacuole-targeted (occur in vacuoles) proteins that are antimicrobial in nature (Gaffney et al 1993; Malamy et al 1990; Metraux et al 1990; Van loon and Van Strien 1999; Ward et al 1991). SAR is induced by abiotic and biotic factors. Exogenous application of salicylic acid, or one of its functional analogs, 2,6-isochloronicotinic acid or benzathadiazole *S*-methyl ester, can induce expression of PR genes and resistance in plants without pathogen inoculation (Friedrich et al 1996; Metraux et al 1991).

SAR has been observed earlier in the *Brassica*-blackleg system. Inoculation of an avirulent strain of *L. maculans* on the leaves of resistant cultivar of Indian mustard led to the rapid necrosis of the guard cells (Chen et al 1996). Similarly, hypersensitive response and accumulation of phytoalexins was observed in *Brassica* spp. following inoculation with an avirulent strain of *L. maculans* (Pedras et al 2002). Weakly virulent isolates of *L. biglobosa* (previously classified as *L. maculans*) have also been shown to induce SAR in canola. Mahuku et al (1996) demonstrated that co-inoculation of the weakly virulent and the highly virulent strains of *L. maculans* on the highly susceptible cultivar 'Westar', led to a reduction in lesion size on the leaves, when compared to the inoculation of the virulent strain alone. Chen and Fernando (2006) also observed a significant reduction in stem infection under field conditions on plants treated with the weakly virulent strain prior to inoculation of the virulent strain, as compared to the virulent strain inoculated controls. They also demonstrated the activity of PR proteins, chitinase, β-1,3 glucanase,

peroxidase and phenylalanine ammonia lyase, when the plants were treated with the weakly virulent strain 24 h prior to the inoculation of the virulent PG2 strain.

2.5 Biological Control

2.5.1 Introduction

Baker and Cook, in 1974, defined biological control as "the reduction of inoculum density or disease producing activities of a 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 antagonist". Later, in 1983, it was redefined as "the reduction in the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man".

The work in biological control started as early as 1874, when W. Roberts observed antagonistic action between *Penicillium glaucum* and bacteria, thus introducing the word "antagonism" to microbiology (Baker 1986). It was W. Henry's work in 1931, which was the first successful transfer of a total antagonistic microflora to produce a suppressive soil. Addition of varying concentrations of suppressive field soil to sterilized soil led to the suppression of foot rot of wheat caused by *Helminthosporium sativum* and *Fusarium graminearum*, as compared to the disease levels in the sterile soil (Baker 1986). This experiment also established the role of soil microflora in disease suppression, when bacteria, fungi and actinomycetes isolated from the suppressive soil reduced disease levels upon introduction into sterile soil. Later in 1959, J. D. Menzies's work confirmed Henry's finding, when antagonistic microflora of a soil suppressive to potato

common scab could be transferred to a pot of nontreated conducive soil, thus making the soil that is conducive, suppressive.

2.5.2 Why use Biological Control?

Steady increase in the global population demands a steady and healthy food supply. The demand for food production requires further intensification of agricultural practices, which in turn may increase the pressure on crop plants. To have a healthy cropping system to meet the demands of increased food production, yield losses resulting from plant diseases need to be curtailed. Practices involving improving the genetic resistance of the host plant, management of the plant and its environment, and use of synthetic pesticides play a key role in the control of plant diseases (Strange 1993). Intensification of agricultural practices would result in increases in use of synthetic pesticides such as fungicides, which is of great concern. Use of synthetic chemicals is under extreme pressure due to their hazardous effects on the environment, and concern over non-target effects (Felton and Dahlman 1984; Elmholt 1991) and; development of resistance in pathogen populations (Ishii 2006). Especially, the use of site – specific systemic fungicides has brought about numerous cases of fungicide resistance (Schumann 1991). Also, there have been concerns over pesticide residues in food, which could lead to serious health problems (Picó et al 2006).

Biological control, which is a natural phenomenon, involving the use of microorganisms for disease control, seems to be a good alternative to chemical control. First and foremost, the highly diverse microbial community in the environment provides an endless source for this purpose (Emmert and Handelsman 1999). Various studies have shown that increasing the population of a particular microbial strain in the vicinity of a

host plant can control the disease without altering the rest of the microbial community or causing any adverse effects on other organisms in the ecosystem (Gilbert et al 1993, Osburn et al 1995, Ravnskov et al 2002). In addition to this inundative approach, management and manipulation of natural communities of antagonistic microbes through crop rotation and organic amendments have proven to be highly effective forms of biological control (Hoitink and Boehm 1999, Kurle et al 2001, Coventry et al 2005). It is also believed that the complexity in the interaction between the organisms in the ecosystem, the multiple mechanisms of disease suppression by a single microorganism, and the capability of the antagonist to adapt itself to the environment in which it is used, could make biological control a more durable system than synthetic chemicals (Cook 1993, Benbrook et al 1996, Weller 2007)

There are also a few limitations to biological control. Success with biocontrol agents is often unpredictable and too variable for large scale use (Emmert and Handelsman 1999). Two major reasons that contribute to this are a lack of understanding of the biocontrol system and difficulties in formulation. For a better understanding of the biocontrol system, more insight is needed into the interactions of the antagonist with the pathogen, the plant, the microbial community and the environment. Recent advances in microbiological and molecular techniques could be of great help in understanding the diversity, physiology and interactions of the antagonists with its above mentioned partners (Nelson 2004). A number of biocontrol agents have been successful in research, but have not advanced due to problems in scaling up production and providing a stable and inexpensive formulation (Cook 1993). A more thorough understanding of ecology can help us figure out which problems to work on, how to approach them, when and

where to apply the biocontrol agent, and predict situations in which control would not be expected to work (Fravel 2005). A table containing details of commercially available biocontrol products, the biocontrol agent, the target organism, the crop. and the manufacturer is presented in Fravel (2005)

2.5.3 Bacterial antagonists

Cook and Baker (1983) defined an antagonist as "biological agents with the potential to interfere in the life processes of plant pathogens". Antagonists can include fungi, bacteria, viruses, protozoa, nematodes, viroids and seed plants (Cook and Baker 1983). Bacteria have proven to be excellent antagonists owing to their multiple mechanisms of disease control. Earlier work on biocontrol of plant diseases was focused more on the root colonizing rhizobacteria, with special emphasis on their ability to produce secondary metabolites, such as siderophores. Siderophores efficiently sequester iron and deprive the pathogen of this vital element, which is essential for metabolic functioning and the process of pathogenesis (Kloepper 1980). Numerous studies have established the role of other mechanisms such as, antifungal antibiotics, enzymes and volatiles produced by bacteria, and their ability to induce systemic resistance toward plant disease control (Weller et al 1988, Whipps 1997, Garbeva et al 2004). Other than their direct role in pathogen control, bacteria also produce metabolites that enhance plant growth (root growth) or trigger the induction of systemic resistance, which acts like the process of immunization and thus prevents plant disease (Van Loon et al 1998). With the above mentioned multiple mechanisms of disease control, bacteria serve as potential antagonists.

2.5.4 Mechanisms of Biological Control

2.5.4.1 Competition for Nutrients

Microbes that exist in a community tend to be in a constant level of interaction. These interactions between the microbial populations, according to Atlas and Bartha (1993), can be classified into one of different categories: neutralism (lack of interaction); commensalism (one benefactor); synergism (both benefit); asmensalism (one organism produces a substance inhibitory to the other) or competition (both populations are adversely affected). According to Singh and Faull (1988), competition occurs when two or more organisms demand the same nutrient source in excess of the immediate supply. From a pathological point of view, a pathogen requires energy to infect a plant. Facultative pathogens derive food from soil organic matter, from latent infections or dead host tissue, or from their own storage organs such as sclerotia, rhizomorphs etc. (Baker 1987). The mycelia remain intact and transfer these food sources to the infection site. Infection of woody structures requires more energy than leafy structures.

Competition for space or nutrients in the rhizosphere is a common mechanism by which bacteria control pathogens. A good example of a bacterial antagonist controlling a bacterial pathogen is the use of non-pathogenic *Streptomyces* to control scab of potato caused by *Streptomyces scabies* (Ryan and Kinkel 1997, Neeno-Eckwall and Schottel 1999). Bacteria competing for nutrients bring out effective control of fungal pathogens. A few examples are: 1) Competition for fatty acids by *Enterobacter cloacae* led to the suppression of *Pythium ultimum* sporangium germination and damping off (van-Dijk and Nelson 2000); 2) Competition for seed exudates and prevention of zoospore attraction by *Burkholderia cepacia* AMMDR1 resulted in *Pythium aphanidermatum* control in peas

and sweet corn (Heungens and Parke 2000); 3) Competition for Botrytis cinerea stimulating nutrients by Pseudomonas spp., (Elad and Stewart 2004); and 4) combined application of *Pseudomonas putida* and non-pathogenic *Fusarium oxysporum* isolates in the control of Fusarium yellow of beans caused by *F. oxysporum* f.sp. *phaseoli* (Dhingra et al 2006)

In the phyllosphere, pollen, aphid honeydew, leaf guttate, fallen plant parts, or other refuse serve as food bases for both pathogens and antagonists. Bacteria, yeast and fungi are common epiphytes, which compete for nutrients needed by the pathogen and thus influence infection (Baker 1987). Biocontrol of disease on the phyllosphere, through manipulation of this biota, has lots of potential, particularly through genetic manipulation of the biocontrol agents. A good example of this is the development of the ice-minus strains of *Pseudomonas syringae* (Lindow 1987) that control the pathogenic strain through preemptive competitive exclusion. The same phenomenon is emplpoyed by the antagonistic strain *Pseudomonas syringae* strain 22d/93 for the control of bacterial blight of sybean caused by *P. syringae* pv *glycinea* (Volksch and May 2001).

2.5.4.2 Siderophores

Under iron-limiting conditions, bacteria produce a range of low molecular mass iron-chelating compounds known as siderophores. These compounds have a very high affinity for the ferric ion, which is present as ferric hydroxide that is hard to sequester. From a disease control perspective, these compounds sequester the limited supply of iron available in the rhizosphere, making it unavailable to the pathogenic fungi, thus restricting their growth and serving as a mechanism of biocontrol (O'Sullivan and

O'Gara 1992, Loper and Henkels 1999). Among the bacterial siderophores, the role of pyoverdine siderophores produced by many *Pseudomonas* species, in iron competition and pathogen control has been clearly established. The control of *Pythium* and *Fusarium* species by pyoverdine siderophores has been demonstrated with the effects of purified pyoverdine with synthetic iron chelators or through the use of pyoverdine minus mutants (Loper and Buyer 1991, Duijff et al 1993). The biological control of Fusarium wilt in flax was greater with nonpathogenic *F. oxysporum* Fo47 and *P. putida* WCS358 used in combination than with either organism applied individually (Duijff et al 1999). Enhanced competition for iron via the production of siderophores by fluorescent pseudomonads resulted in an elevated level of carbon competition between nonpathogenic and pathogenic strains of *F. oxysporum*. Also, the siderophore deficient mutant did not enhance disease control achieved by the use of the non-pathogenic *F. oxysporum* alone, thus ascertaining the role of pyoverdine siderophore in this interaction. Siderophore production also contributes to the successful colonization of the rhizophere by the Pseudomonas spp., and the suppression of root diseases (Cornelis and Matthijs 2002, De Bellis and Ercolani 2001). Other than pyoverdine siderophores, new siderophores such as thioquinolobactin, produced by P. fluorescens, is responsible for antifungal and anti-Pythium activity of the bacterium (Matthijs et al 2007) In addition to competing for iron, pyoverdine and salicylic acid have also been established in the induction of systemic resistance against pathogens (Metraux et al 1990, Leeman et al 1996).

2.5.4.3 Hyperparasitism

Among bacteria, the actinomycetes have been well established for their role in parasitizing and degrading fungal pathogens (El- Tarabily et al 1997). The bacteria derive their nutrients from the degraded fungal wall. Bacteria can bring about parasitism through simple attachment to the fungal wall, as seen in the *Enterobacter cloacae – Pythium ultimum* interaction (Nelson 1986) or through complete degradation and lysis of the fungal wall, as seen in the *Arthrobacter – Pythium debaryanum* interaction (Mitchell and Hurwitz 1965). Cell wall degrading enzymes play a critical role in the process of parasitism. The role of cellulase, produced by *Micromonospora carbonacea*, was established in the biocontrol of *Phytophthora cinnamomi* root rot of *Banksia grandis.* Similarly, chitinolytic enzymes produced by both *Bacillus cereus* and *Pantoea agglomerans* also appear to be involved in biocontrol of *Rhizoctonia solani* (Chernin et al 1995, 1997, Pleban et al 1997). Chitinolytic activity of *Bacillus circulans* GRS 243 and *Serratia marcescens* GPS 5 is responsible for the suppression of late leaf spot of groundnut, caused by *Phaeoisariopsis personata* (Kishore et al 2005). Similarly, chitinase produced by *B. cereus* 29-8 is responsible for the control of Botrytis leaf blight of lily (Huang et al 2005). Tn5 insertion mutants and subsequent complementation experiments in *Stenotrophomonas maltophila* established the role of extracellular proteases in control of *Pythium ultimum* in the rhizosphere of sugar beet (Dunne et al 1997). Even though numerous research findings have identified the cell wall degrading enzymes produced by bacterial biocontrol agents, there is little direct evidence for their presence and activity in the rhizosphere (Whipps 2001).

2.5.4.4 Antifungal volatiles

Inorganic volatiles such as ammonia, produced by *Enterobacter cloacae*, has been implemented as one of many mechanisms that bacteria employ in the biocontrol of pre-emergence damping-off caused by *Pythium* spp. (Howell et al 1988). Fungal response to bacterial volatiles appears to be species-, environment-, and age-specific (Mackie and Wheatley 1999). Allyl alcohol inhibits carpogenic germination of sclerotia of *S. sclerotiorum*, but at the same time stimulates growth and enhances sclerotial colonization by *Trichoderma* spp. (Huang et al 1997). Allyl alcohol also increases populations of beneficial bacteria such as *P. fluorescens* and *P. putida* (Domsch 1959; Altman and Lawlor 1966). The chemical nature of the organic volatiles appears to determine their antifungal activity. Aliphatic aldehydes were more effective in the post harvest control of gray mould caused by *Botrytis cineria* in strawberry, blackberry and grape (Archibold et al 1997), and aliphatic aldehydes and ketones were more effective than alcohols in the inhibition of germ tube formation of *Alternaria alternata* (Andersen et al 1994). Fernando et al (2005) for the first time isolated, characterized and established the antifungal activity of volatile organic compounds produced by *Pseudomonas spp.*, towards *S. sclerotiorum*. The antifungal volatiles that were characterized included nonanal, n- decanal, cyclohexanol, 2- ethyl, 1- hexanol, benzothiazole, and dimethyl trisulfide. These volatile compounds inhibited in vitro growth of mycelium and germination of sclerotia of *S. sclerotiorum*.

2.5.4.5 Induced Systemic Resistance

Induced systemic resistance (ISR) against pathogens develops in the plant as a result of colonization of the root by certain plant growth promoting rhizobacteria (PGPR) (Van Loon et al 1998). ISR has been demonstrated in many plant species and exhibits a broad spectrum of activity that protects against various bacterial, viral, fungal and nematode pathogens (Bostock 2005). Fluorescent *Pseudomonas* spp. have been the most successful of the PGPRs and been linked to the reduction of root diseases in natural disease-suppressive soils (Raaijmakers and Weller 1998). The role of *Bacillus* spp. in ISR has also been demonstrated recently (Kloepper et al 2004; Zhang et al 2004). The expression of ISR is genetically determined in the plant, which allows the specific recognition between the plant and the ISR inducing rhizobacterium (Pieterse et al 2002). The genetic studies by Pieterse et al (1996 and 1998) with *Arabidopsis* mutants proved that *P. fluorscens* WCS417r-mediated ISR requires responsiveness to both jasmonic acid (JA) and ethylene (ET), but functions independently of salicylic acid (SA). Other fluorescent *Pseudomonas* spp. have also been shown to induce a SA-independent ISR pathway in Arabidopsis (Van Wees et al 1997, Iavicoli et al 2003, Ryu et al 2003), tobacco (Press et al 1997, Zhang et al 2002) and tomato (Yan et al 2002).

The mode of procurement of its nutrients by a pathogen from its host seems to determine the role of JA, ET and SA in basal resistance (Pozo et al 2005). Plant pathogens are generally classified into two types: biotrophs - those that need a living host cell for its nutrient uptake, and necrotrophs – those that kill the host cell and feed on the dead tissue (Parbery 1996). SA-dependent defense responses are associated with the hypersensitive response, which restricts the growth of biotrophic pathogens by killing the

infected cell, through programmed cell death, but ineffective against necrotrophic pathogens (Govrin and Levin 2000, Thomma et al 2001). JA-dependent defense responses, which do not involve cell death, seem to offer protection against necrotrophic pathogens (McDowell and Dangl 2000). Genetic evidence, in the form of *Arabidopsis* mutants, which were reduced in their sensitivity to methyl jasmonate (MeJA) or defective in JA biosynthesis, which exhibited susceptibility to normally non-pathogenic strains of *Pythium* spp. (Staswick et al 1998, Vijayan et al 1998). Seo et al (2001) demonstrated that increased endogenous levels of MeJA, through over-expression of JA carboxy methyl transferase, resulted in higher resistance to *Botrytis cinerea*. All these studies demonstrated the role of JA-dependent defense response to restrict necrotrophs. But Ellis et al (2002) constitutively activated the JA-signaling pathway in *Arabidopsis*, which resulted in enhanced resistance to the biotrophs *Erysiphe cichoracearum*, *E. orontii* and *Oidium lycopersicum*, thus challenging the general notion that JA-dependant defense responses are predominantly effective against necrotrophic pathogens.

Rhizobacteria-mediated ISR leads to enhanced sensitivity of the induced tissue to the JA and ET, rather than increase in their production (Pozo et al 2005). Conrath et al (2002) term this phenomenon as "priming", which is the enhanced capacity of induced tissues for rapid and effective activation of cellular defense response upon challenge by pathogen infection. This has been proven with analysis of local and systemic levels of JA and ET in plants expressing ISR, where induced resistance was not associated with detectable changes in their production (Pieterse et al 2000). Priming has been well established towards disease reduction as seen in tobacco, where cells showed faster and stronger lipid peroxidation and protein phosphorylation in response to fungal elicitors

after preconditioning by MeJA treatment (Dubery et al 2000). Priming seems to be an important mechanism involved in rhizobacteria-mediated ISR. In *Arabidopsis*, micro-array analysis, after challenge inoculation of *P. fluorescens* WCS417r-induced plants with the bacterial leaf pathogen *P. syringae* pv *tomato* DC3000, revealed augmented expression pattern in a large set of genes in ISR expressing leaves, suggesting that these genes were primed to respond faster and/or more strongly upon pathogen attack (Verhagen et al 2004). These ISR-expressing leaves, prior to pathogen challenge, did not exhibit detectable changes in gene expression in response to effective colonization of the roots by WCS417r. A number of bacterial determinants seem to function as elicitors of ISR and they seem to vary between bacteria and the host. A few examples are: 2, 3-butanediol in *Bacillus amyloliquefaciens* IN937a – *Arabidopsis* (Ryu et al 2004); lipopolysaccharide (LPS), siderophore and flagellin in *P. putida* WCS358 - *Arabidopsis*, but only LPS and siderophore (no flagellin) in *P. putida* WCS358 – bean and tomato (Meziane et al 2005). A better understanding of the PGPR-ISR system could help in its utilization as a powerful tool to reduce diseases caused by pathogens that are sensitive to JA and ET dependent defenses (Van Loon and Bakker 2005). Also, ISR in combination with SAR can act as an extended protection to a broader range of pathogens, when other means of protection are absent or limited.

2.5.4.6 Antibiosis

Direct antagonism of the pathogen through antibiosis is one of the mechanisms by which disease suppressive bacteria achieve disease control. Antibiosis is mediated through the production of a chemically heterogeneous group of organic, low-molecular

mass compounds (Raaijmakers et al 2002), which at low concentrations are deleterious to the growth or metabolic activities of other microorganisms (Fravel 1988; Thomashow et al 1997). Under optimal growing conditions, such as a balanced, nutrient-rich medium, microorganisms utilize energy to synthesize cellular components and grow, not for secondary metabolism (Haas and Keel 2003). Restriction of growth, either due to limitation in nutrients or high cell density, leads to the initiation of secondary metabolism, such as antibiotic production, which helps the organism to remain competitive in its environment (Vining 1990, Haas et al 2000). Secondary metabolites are produced during growth phase called idiophase, which occurs during the transition to stationary phase, as opposed to the trophophase of maximal growth (Liao et al 1995).

Numerous studies on the role of antibiotics in biocontrol of plant pathogens have led to the isolation and characterization of numerous antibiotics from various bacterial genera. Some prominent examples are: control of *Gaeumannomyces graminis* var *tritici* in wheat by phenazine produced by *Pseudomonas chlororaphis* PCL1391 (Chin-A-Woeng et al 1998); control of multiple pathogens such as *P. aphanidermatum*, *Botryodiplodia theobromae*, and *Alternatria solani* by phenazine produced by *P. chlororaphis* PA23 (Kavitha et al 2005); control of *Rhizoctonia solani* in cotton by pyrrolnitrin produced by *P. fluorescens* BL915 (Ligon et al 2000); control of *Rhizoctonia solani* in poinsettia by pyrrolnitrin produced by *Burkholderia cepacia* (Hwang et al 2002); control of *Thielaviopsis basicola* and *Pythium ultimum* in cotton by pyoluteorin produced by *P. fluorescens* CHA0 (Maurhofer et al 1992); control of *Gaeumannomyces graminis* var *tritici* in wheat by 2-4, diacetylphloroglucinol produced by *P. fluorescens* Q8r1-96 (Raajmakers and Weller 2001); control of *Phytophthora medicaginis* in alfalfa

by zwittermicin A produced by *B. cereus* UW85 (Silo-Suh et al 1994); control of *Erwinia herbicola* in apple by pantocin A and B produced by *P. agglomerans* EH318 (Wright et al 2001); control of *P. ultimum* in sugar beets by xanthobaccins produced by *Stenotrophomonas* SB-K88 (Nakayama et al 1999).

The majority of the work has focused on the isolation and characterization of antibiotics produced by *Pseudomonas* spp. and the establishment of their role in biocontrol. Whipps (1997) attributes several reasons for this abundance of studies on the antibiotics produced by *Pseudomonas* spp., which are: i) their common inhabitation of the rhizosphere and phyllosphere environment; ii) easy isolation from these natural environments; iii) their ability to utilize a wide range of substrates; iv) their flexibility for easy culture and genetic manipulation, thus making them more suited for experimentation. In recent years, publications on antibiotics produced by bacteria other than *Pseudomonas* spp. are also on the rise.

Antibiotic mediated biocontrol is not only attributed to the diverse kinds of antibiotics produced by different bacteria, but also to the number of antibiotics produced by a single strain. For example, *B. cereus* strain UW85 (zwittermicin A and kanosamine) (Handelsman and Stabb 1996), *P. chlororaphis* strain PA23 (phenazines and pyrronitrin) (Zhang et al 2006), and *P. fluorescens* strains CHA0 and Pf5 (2,4-diacetylphloroglucinol and pyoluteorin) (Keel et al 1996, Bender et al 1999) produce multiple antibiotics, thus indicating that for at least some biocontrol agents, several antibiotics with overlapping or different degrees of activity, may account for the suppression of specific or multiple plant pathogens (Raaijmakers et al 2002). Antibiotics produced by bacterial biocontrol agents exhibit broad-spectrum activity. Few good examples are: pyrrolnitrin exhibits antifungal

activity towards plant pathogens belonging to Basidiomycetes, Deuteromycetes and Ascomycetes (Ligon et al 2000) and antibacterial activity towards pathogenic gram-positive bacteria, such as *Streptomyces* spp. (El-Banna and Winklemann 1998); 2,4-diacetylphloroglucinol exhibits antifungal, antibacterial, anthelminthic and phytotoxic properties (Keel et al 1990, Thomashow and Weller 1996).

Other than *in vitro* assays, there are several lines of evidence that have conclusively demonstrated the role of antibiotics in biocontrol of plant pathogens. They are as follows:

1. Cell-free culture filtrates or the purified antibiotics demonstrated similar levels of disease control as achieved by the antibiotic producing wild-type strain of the biocontrol bacteria (Kang et al 1998, Nakayama et al 1999).
2. Mutagenesis mediated inactivation of antibiotic production and reduction in the ability of the antagonistic bacteria to control the pathogen. This has been demonstrated in *Pseudomonas* spp. (Anjaiah et al 1998, Ching-A-Woeng et al 1998; Poritsanos 2005); *Burkholderia cepacia* (Heungens and Parke 2001); *B. cereus* (Silo-Suh et al 1994). Also, most of these studies included the complemented mutant with restored antibiotic production and biocontrol activity (Thomashow and Weller 1988).
3. Enhancement of antibiotic production in the wild-type strain, through genetic manipulation such as introduction or modification of biosynthetic genes or regulatory genes. For example, introduction of multiple copies of the regulatory *phzR* gene in *P. aureofaciens* strain 30-84 led to increasd phenazine production and inhibition of up to nine different pathogens (Fernando and Pierson 1999).

Similarly, introduction of multiple copies of the regulatory *gacA* gene into *P. fluorescens* strain BL915 resulted in a 2.5-fold increase in pyrrolnitrin production (Ligon et al 2000).

4. Heterologous expression of the antibiotic biosynthetic genes in non-producing strains and subsequent evaluation of their disease control ability. Most of the heterologous expression studies describe the use of multi-copy plasmid vectors (Raaijmakers et al 2002). But, Timms-Wilson et al (2000) introduced, into the chromosome of PCA non-producing *P. fluorescens* strain SBW25, a disabled Tn5 vector harboring the genes for phenazine-1-carboxylic acid production. They observed significantly more protection from the PCA-producing derivatives than their parental strain against *P. ultimum* on pea seedlings, and also that the single copy chromosomal insertions were less likely to affect the fitness of the heterologous strain than the plasmid-based modifications.
5. Recovery and detection of antibiotic production *in situ* complements the indirect evidence provided by genetic approaches (Raaijmakers et al 2002). But the recovery and detection of the antibiotics are hindered by the instability of the compound, irreversible binding to the soil colloid or the organic matter, or microbial decomposition (Thomashow et al 1997). Development of sensitive methods such as high performance liquid chromatography has allowed the *in situ* detection and quantification of a range of antibiotics, such as PCA, herbicolin A, pyrrolnitrin, pyoluteorin, surfactin and DAPG (reviewed by Thomashow et al 1997).

Quantitative detection methods do not determine the spatial and temporal

patterns of antibiotic production, which is crucial for the biological control of several plant pathogens (Raaijmakers et al 2002). The use of different derivatives of the green fluorescent protein acts as a powerful tool to visualize the pathogen and the biocontrol agent simultaneously. Also, it helps in determining whether the spatial colonization patterns of the pathogen coincides with the spatial colonization and antibiotic production patterns of the biocontrol agent (Bloemberg et al 2000).

2.5.4.6.1. Antifungal antibiotics and their biosynthesis

The ability of bacteria as synthetic chemists is unparalleled in nature. Bacteria produce structurally diverse compounds with a wide spectrum of activities, which have been utilized by humans for numerous purposes. Antibiotics are key among the compounds of microbial origin, providing the basis for treatment of infectious diseases of humans, animals, and plants (Emmert et al 2004). The genes responsible for the production of these antifungal metabolites have been isolated from the producing strains. The study of these genes and the enzymes that they encode gives us a significantly increased understanding of the biology and biochemistry of these interesting compounds. A few antifungal antibiotic compounds, which are relevant to this thesis, are briefly discussed below with regards to: the organisms that produce them; their mode of action; and the biosynthetic genes involved in their production.

2.5.4.6.1.1. Phenazines

Natural phenazines are produced almost exclusively by eubacteria; they often are excreted to very high levels (milligrams to grams per liter) during bacterial growth in

vitro (Mavrodi et al 2006). Fluorescent pseudomonads, are the best-studied phenazine producers, with strains of *Pseudomonas fluorescens, P.chlororaphis*, and *P. aeruginosa* known to produce these compounds. Except for *P. fluorescens,* which produces only the yellow compound phenazine-1-carboxylic acid (PCA) (Figure 2.2 A), phenazine-producing fluorescent pseudomonads typically synthesize two or more phenazines. The range of compounds synthesized by a given species is determined genetically (Mavrodi et al 2006). In addition to PCA, *P. aureofaciens* produces the orange and brick-red compounds 2-hydroxy phenazine-1-carboxylic acid and 2-hydroxyphenazine (Figure 2.2 A) (Pierson et al 1992, Delaney et al 2001), whereas *P.chlororaphis* produces PCA and the green compound phenazine-1-carboxamide (Figure 2.2 A) (Chin-A-Woeng et al 1998) (PCN). *P. aeruginosa* is the first phenazine-producing microorganism to have been reported in the literature, and its characteristic blue-green pigment pyocyanin (5-*N*methyl- 1-hydroxyphenazine) probably is the first described phenazine (Fordos 1859). *P. aeruginosa* also can produce several other phenazines including 1-hydroxyphenazine, PCA, PCN (Mavrodi et al 2006). The biological activity of the phenazines produced by pseudomonads is due directly to their unusual redox properties. PYO can undergo cellular redox-cycling in the presence of molecular oxygen and various reducing agents including NADH and NADPH, resulting in the accumulation of toxic superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Hassan and Fridovich 1980). Phenazine biosynthesis requires five genes (Figure 2.2 B) encoding the proteins PhzA, PhzD, PhzE, PhzF, and PhzG (*P. fluorescens* nomenclature), with the *phzA* gene duplicated as *phzB* in pseudomonads. Most gene clusters also encode PhzC, which catalyzes the first step of the shikimate pathway and acts to redirect intermediates from primary metabolism into

phenazine biosynthesis (Figure 2.2 C). All phenazine operons also encode additional genes involved either in transcriptional regulation or phenazine modification.

2.5.4.6.1.2. Pyrrolnitrin

Pyrrolnitrin [Prn, 3-chloro-4-(2'-nitro-3'-chlorophenyl) pyrrole] (Figure 2.3 A) and its production by *Pseudomonas pyrrocinia* Imanaka, Kousaka, Tamura & Arima was first discovered in 1964 (Arima et al 1964). Pyrrolnitrin has been reported to be produced by *P. fluorescens* BL195 (Ligon et al 2000), *B. cepacia* B37w (Burkhead et al 1994), *Enterobacter agglomerans* (Chernin et al 1996), *Serratia* spp. (Kalbe et al 1996). Prn is active against a wide range of Deuteromycete, Ascomycete and Basidiomycete fungi. Prn is highly labile in sunlight, but the substitution of the chlorine on the pyrrole ring with a cyano group increased the stability of the compound under light and also the overall antifungal activity. The primary antifungal mechanism of pyrrolnitrin is the interference of the osmotic signal transduction pathway, and the secondary mechanism is probably the inhibition of respiration, which is active at a high dosage of the antibiotic in *N. crassa* and other fungi (Okada et al 2005). An operon consisting of four genes, prnA, prnB, prnC and prnD (Figure 2.3 B), was described, in Pseudomonas fluorescens, to encode the biosynthesis of pyrrolnitrin (Hammer et al., 1997; Kirner et al., 1998). It was found that four genes organized in a single transcriptional unit are required for Prn synthesis. *prnA* gene encodes a tryptophan halogenase that catalyzes the chlorination of tryptophan to form 7-CT; *prnB* encodes the enzyme that catalyzes the complex rearrangement of 7-chlorotryptophan from the indole to the phenylpyrrole form and decarboxylation to form monodechloroaminopyrrolnitrin (MDA); *prnC* encodes an MDA halogenase that catalyzes a second chlorination in the 3 position of the pyrrole ring to form amino-Prn,

and *prnD* encodes an enzyme that oxidizes the amino group of amino-Prn to a nitro group to form Prn (Figure 2.3 C) (Ligon et al 2000).

2.5.4.6.1.3. Lipopeptides Antibiotics

Many *Bacillus subtilis* strains produce a small peptide(s) with a long fatty moiety, the so-called lipopeptide antibiotics. The peptide portions of these compounds contain α-amino acids with a D configuration and are produced nonribosomally with templates of the multifunctional peptide synthetases. The peptide chain grows in a defined sequence by moving on the template of the multifunctional peptide synthetase (Tsuge et al 2001). Based on their structural relationship, the lipopeptides that have been identified in *B. subtilis* are generally classified into three groups: the surfactin group (Peypoux et al 1999), the plipastatin-fengycin group (Umezawa et al 1986, Vanitanakkom et al 1986, Lin et al 1999), and the iturin group (Maget-Dana et al 1994). The members of the surfactin and plipastatin-fengycin groups are composed of one β-hydroxy fatty acid and 7 and 10 α-amino acids, respectively, while the members of the iturin group consist of one β-amino fatty acid and 7 α-amino acids. The presence of the β-amino fatty acid is the most striking characteristic of the iturin A group and distinguishes this group from the other two groups.

Iturin A (Figure 2.4 A) has previously shown strong antifungal activity towards *R. solani* (Yu et al 2002), *Colletotrichum dematium* (Hiradate et al 2002), and *Gleosporium gleosporioides* (Cho et al 2003). Bacillomycin D (Figure 2.4 B) has also been shown to exhibit antifungal activity towards *A. flavus* and *A. parasiticus* (Moyne et al 2004), and *Fusarium oxysporum* (Koumoutsi et al 2004). Iturin A and bacillomycin D affect

membrane surface tension, which causes pore formation and results in leakage of K+ and other vital ions, finally leading to cell death (Yao et al 2003).

The iturin A operon (Figure 2.4 C) is composed of four ORFs, *ituD, ituA, ituB,* and *ituC*, in that order, and closely resembles the mycosubtilin operon (Tsuge et al 2001). ItuA exhibits homology to β-ketoacyl synthetase, amino transferase, and peptide synthetase in one molecule and is probably responsible for synthesis of a β-amino fatty acid accompanied by ItuD dipeptide (β-amino fatty acid–Asn) formation, as has been proposed for the mycosubtilin synthetase MycA (Duitman et al 1999). Genes *ituB* and *ituC*, encode large peptide synthetases that build peptide chains on the precursor from ItuA. The level of homology between *ituC* and *mycC*, the counterpart of *ituC* in the mycosubtilin operon, is relatively low (64%) compared to the levels of homology for other groups (79%), reflecting the difference in the amino acid arrangements in ItuC and MycC. ItuC is predicted to be responsible for the inversion of the sixth and seventh amino acids, D-Asn→L-Ser portion of iturin A, while MycC is thought to synthesize the D-Ser→L-Asn part of mycosubtilin (Tsuge et al 2001).

The bacillomycin D operon (Figure 2.4 C) is made of four ORFs, designated as *bamD, bamA, bamB* and *bamC*, which are transcribed in the same direction and delimited by two putative transcriptional terminators (Moyne et al 2004). A putative ribosomal binding site is present in each intergenic region. The first ORF, *bamD*, encodes a 400 amino acids protein with a predicted mass of 45 kDa. The protein BamD belongs to the malonyl-CoA transacylase family. The second ORF, bamA, codes for a protein with 3980 amino acids and a predicted mass of 448 kDa. Bam A has four domains and is responsible for the integration of the β-amino fatty acid moiety into the bacillomycin D.

The third ORF, *bamB*, codes for a protein of 5363 amino acid with a predicted mass of 607 kDa. BamB exhibits four amino acid activating modules, each of them having the characteristic conserved condensation, adenylation and thiolation domains. Additionally, an epimerization domain is integrated between T and C domains in the first and second modules. The fourth ORF, *bamC*, codes for a protein with 2619 amino acids and a predicted mass of 299.5 kDa. Two amino acid activating modules can be distinguished and an epimerization domain is included in the first module (Moyne et al 2004). The last domain of BamC, the thioesterase domain is required for cyclization and release of the peptide product from the synthetase (Trauger et al 2000).

Surfactin (Figure 2.5 A) exhibits antifungal properties, moderate antibacterial properties and hemolysis; inhibits fibrin clot formation; induces the formation of ion channels in lipid bilayer membranes (Sascha et al 2002); inhibits enzymes, such as cyclic AMP phosphodiesterase; and exhibits antiviral and antitumor activities. The biosynthesis of the lipoheptapeptide surfactin by *Bacillus subtilis* is encoded by the *srf*-operon (Figure 2.5 B) which comprises four open reading frames codifying the protein components SrfA-D of the surfactin synthetase multienzyme system (Nakano et al 1991, Cosmina et al 1993, Van Sinderen et al 1993, Vollenbroich et al 1994). SrfA and SrfB, each comprising three amino acid activating modules. SrfA thioesterifies L-Glu and two leucine residues, while SrfB incorporates L-Asp, L-Val, and L-Leu as thioesters. SrfC is a one-module enzyme that contributes the C-terminal L-leucine-residue. It was found that these three multifunctional proteins assemble the surfactin lipopeptide chain and produce surfactin when complemented with a low molecular weight protein fraction (Ullrich et al 1991, Menkhaus et al 1993). SrfD initiates the formation of surfactin synthesis by the β-

ydroxy fatty acid substrate is transferred from β-hydroxymyristoyl-coenzyme A to the start enzyme SrfA followed by formation of β-hydroxymyristoyl-glutamate (Steller et al 2004). SrfD functions as the thioesterase/acyltransferase enzyme in the initiation process.

2.5.4.6.1.4. Zwittermicin A

Zwittermicin A (Figure 2.6 A) is a linear aminopolyol (He et al 1994) predominantly produced by *B. cereus* UW85 (Silo-Suh et al 1994). It has a broad spectrum of activity, inhibiting certain gram-positive, gram-negative, and eukaryotic microorganisms (Silo-Suh et al 1998). It also potentiates the insecticidal activity of the protein toxin produced by *Bacillus thuringiensis*, increasing mortality of insects that are typically recalcitrant to killing, such as gypsy moths reared on willow leaves (Broderick et al 2000, Broderick et al 2003). The zwittermicin A biosynthetic operon (Figure 2.6 B) is made up of 9 ORFs and the self- resistance gene (*zmaR*), all oriented in the same direction and exhibit an overlapping gene structure, suggesting they are part of an operon (Emmert et al 2004). Orfs 1 to 3 play an important role in the hydroxymalonyl-ACP pathway, while Orfs 4, 5 and 6 are predicted to be an integral part of the aminomalonyl-ACP biosynthesis. Orf7, is a homolog of the adenylation domains of non-ribosomal peptide synthetases, and has an amino acid specificity code for L-serine. Orf 8, due to its homology to non-ribosomal peptide synthetases and polyketide synthetases, along with Orf9 is proposed to have a role in the antibiotic biosynthesis (Emmert et al 2004). The proposed minimum biosynthetic machinery required for the assembly of zwittermicin A (Emmert et al 2004) is presented in figure 2.6 C.

2.5.4.6.1. PCR-based detection of antibiotic biosynthetic genes

Advances in genetic approaches have aided in the cloning and sequencing of antibiotic biosynthetic genes, which in turn has facilitated the development of specific primers and probes that can be used for the PCR-based detection of specific antibiotic producing bacteria (de Souza and Raaijmakers., 2003). These include zwittermicin A produced by *B. cereus,* and 2, 4 – diacetylphloroglucinol, pyrrolnitrin, pyoluteorin and phenazine produced by various *Pseudomonas* species. The use of probes and primers directed against genes involved in antibiotic biosynthesis has proven to be a powerful tool in the study of the distribution and function of indigenous antibiotic-producing bacteria. Understanding the distribution and ecology of naturally occurring strains that harbor biocontrol traits would help in improving the efficacy of existing strains and also identify new strains with regards to their adaptation to specific soils or host-pathogen systems. Knowledge about the distribution of antibiotic-biosynthetic genes in natural environments would help in lessening the concerns regarding the environmental release of non-indigenous strains containing these traits or transgenic biocontrol agents in which these traits have been introduced (Raaijmakers et al 2002).

PCR amplification involves a technique that has revolutionized molecular biology based methodologies. PCR is an enzymatic reaction that allows amplification of DNA through a repetitive process in vitro. Thus, during each cycle the amount of DNA in the reaction theoretically doubles (Pepper and Dowd 2002). In practice, 25 to 30 cycles of PCR results in an approximately 10^6-fold increase in the amount of DNA present. The use of two oligonucleotide primer results in a single amplification product. This amplification of a specific DNA sequence can be visualized after gel electrophoresis and staining. PCR principally involves the repeated cycling of the reagents through a set of

three temperatures: 1) Denaturation – a small amount of double-stranded DNA is pulled apart by heating (91 to 95°C); 2) Annealing – the two sequence-specific oligonucleotide primers are allowed to bind to their target sequences at predetermined sites by cooling the reaction mixture to a set temperature; 3) extension – the reaction mixture is taken to an optimal temperature, where the DNA polymerase makes new DNA that extends from the primer and thus copying the targeted sequence; 4) the temperature cycling is repeated 20 to 40 times.

The choice of the primer sequence is critical for the successful amplification of a specific DNA sequence. The primers are mostly 17 to 30 bases in length and complementary to the template sequence. The distance between the primers determines the size of the amplification product. It is critical that the primers contain different sequences and anneal at different sites of the chromosome, thus preventing self hybridization and formation of primer dimers. Most primers contain high G + C contents, so that their melting temperatures are increased (Pepper and Dowd 2002). Stringency is a critical determinant of sensitivity and specificity in all hybridization- and PCR-based gene detection strategies, as are the structural considerations that must be considered in the design of all PCR primers (Thomashow et al 2002). Primers used for the detection of antibiotic genes in environmental isolates or in community DNA must be sufficiently nonspecific to accommodate templates that may exhibit sequence heterogeneity because of codon degeneracy. To design such primers DNA sequences from several homologues of the potential target needs to be aligned. These sequences can be recovered from publicly accessible databases or they can be determined empirically, from PCR products amplified from known producer strains with primers designed from the sequence of one

of the homologues (McSpadden Gardener et al 2001). The alignment will reveal suitably spaced blocks of conserved sequence from which pairs of consensus primers can be developed that meet the structural criteria for primer design. These candidate primers should then be tested in various combinations to identify pairs that amplify products of the expected size from diverse positive control strains but not from negative control strains indigenous to the same habitats (Thomashow et al 2002).

With change in agronomic practices leading to change in population of *L. maculans*, which in turn has led to the breakdown of resistance in canola cultivars, there has risen the need for a more integrated blackleg management. The inclusion of a natural disease control strategy, in the form of phyllosphere bacterial biological control, along with dependable cultural control could help manage and retard the high potential in the change of the pathogen population to more virulent races, until reliable polygenic cultivar resistance is achieved. The objective of this research is to identify a reliable phyllosphere bacterial biocontrol strategy for the control of *L. maculans*, through: 1) Screening for potential bacterial biocontrol agents through *in vitro* and *in vivo* assays; 2) Identification of potential antifungal antibiotic producing bacteria through detection of antibiotic biosynthetic genes using rapid PCR-screening, and biochemical detection of the corresponding antifungal compounds produced; 3) Investigation of the mechanism of control mediated by the antibiotic producing bacterial biocontrol agents through *in vivo* plant assays.

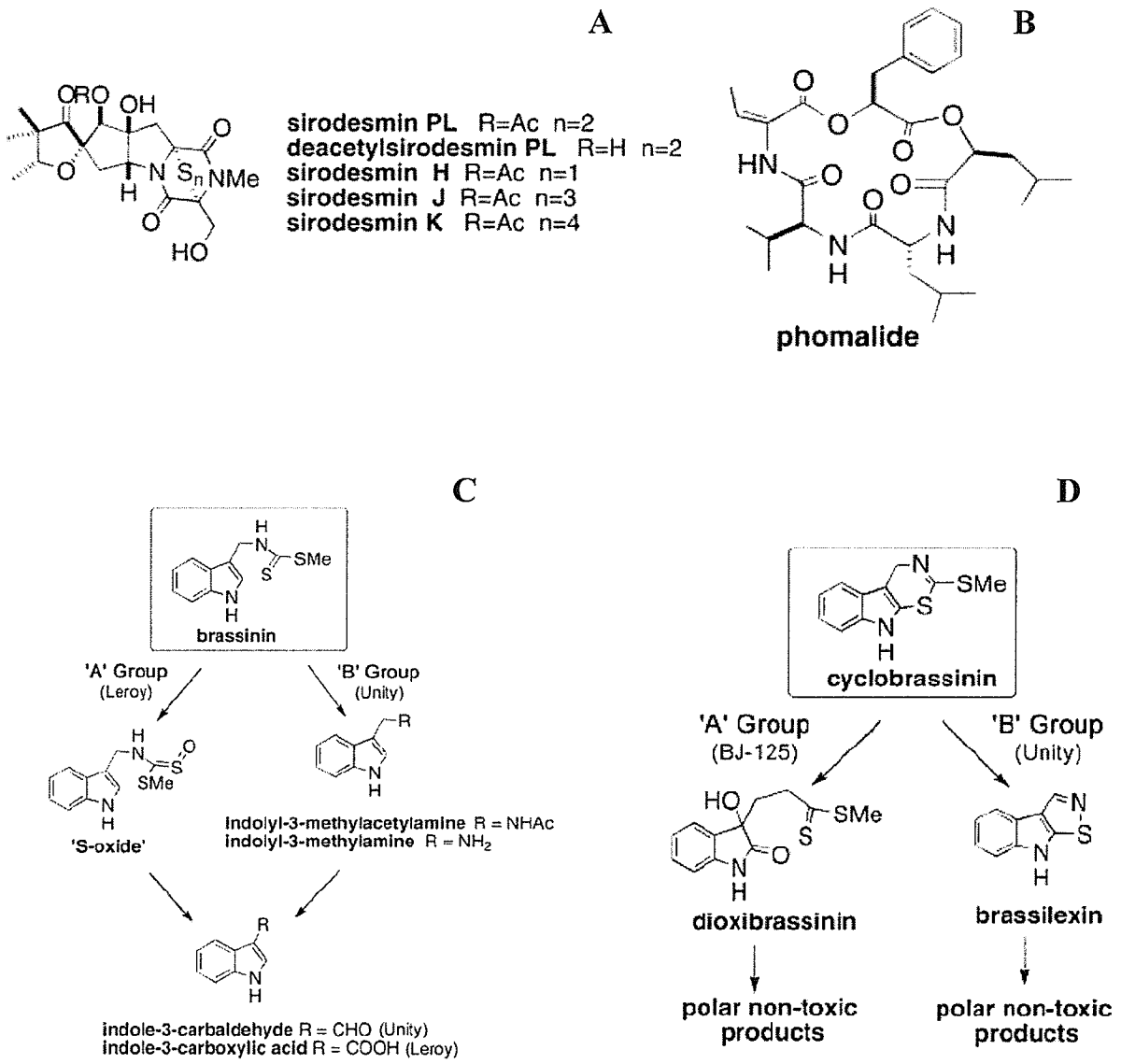

Figure 2.1. (A & B) Chemical structures of toxins, sirodesmin PL and phomalide, produced by *Leptosphaeria maculans*; (C) Biotransformation of the phytoalexin brassinin by "A" pathotype group isolates (e.g., Leroy) and "B" pathotype group isolates (e.g., Unity) of *L. maculan;* (D) Biotransformation of the phytoalexin cyclobrassinin by "A" pathotype group isolates (e.g., BJ-125) and "B" pathotype group isolates (e.g., Unity) of *L. maculans.*. (From Howlett et al 2001)

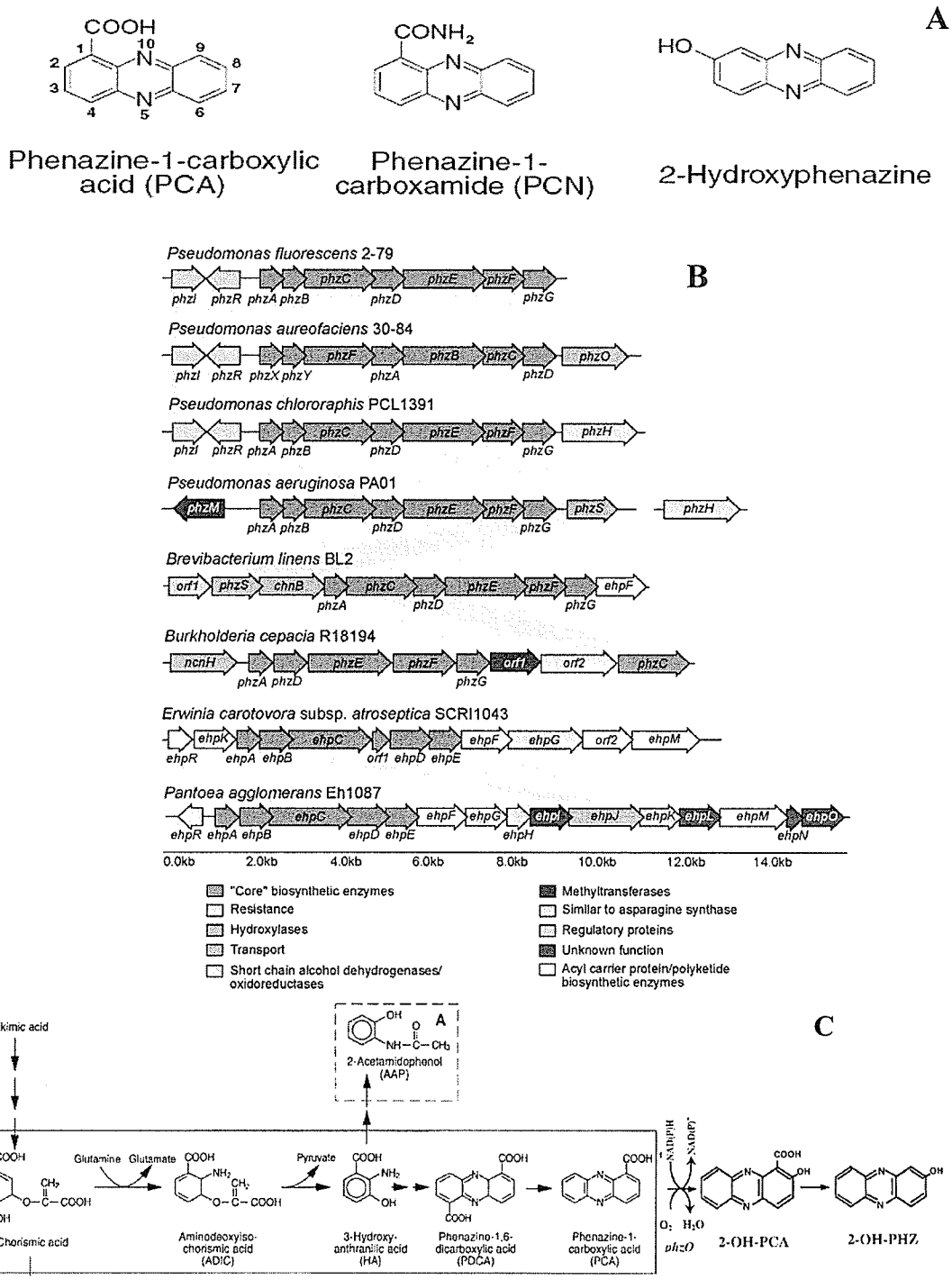

Figure 2.2. (A) Chemical structure of different antifungal phenazines produced by various *Pseudomonas* spp. (B) Comparison of new phenazine biosynthetic loci from *Brevibacterium linens* BL2, *Burkholderia cepacia* R18194,and *Erwinia carotovora* subsp. *atroseptica* SCRI1043 with those from *Pseudomonas fluorescens* 2-79, *P. aureofaciens* 30-84, *P. aeruginosa* PA01, and *P. agglomerans* Eh1087. Genes of similar function are connected with gray lines (Mavrodi et al 2006); (C) Biosynthesis of antifungal phenazines (Slininger et al 2000, Delaney et al 2001)

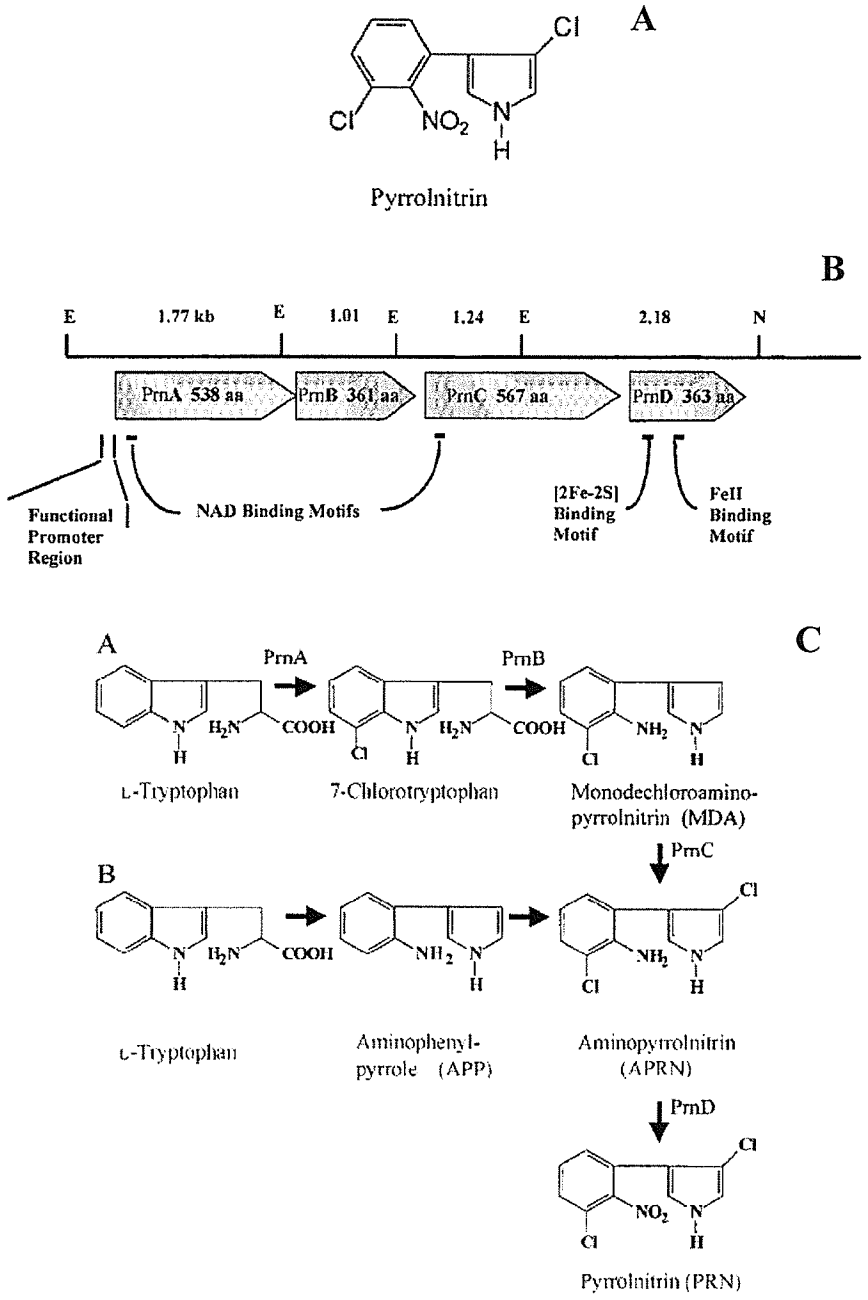

Figure 2.3. (A) Chemical structure of pyrrolnitrin; (B) The genetic organization of the prnABCD gene cluster from the *Pseusomonas fluorescens* strain BL915 (Ligon et al 2000); (C) Biosynthetic pathways for Prn as proposed (A) by van Pee et al (1980) and (B) by Chang et al (1981). The reactions catalyzed by the enzymes encoded by the *prnABCD* genes are indicated at the appropriate reaction arrows.

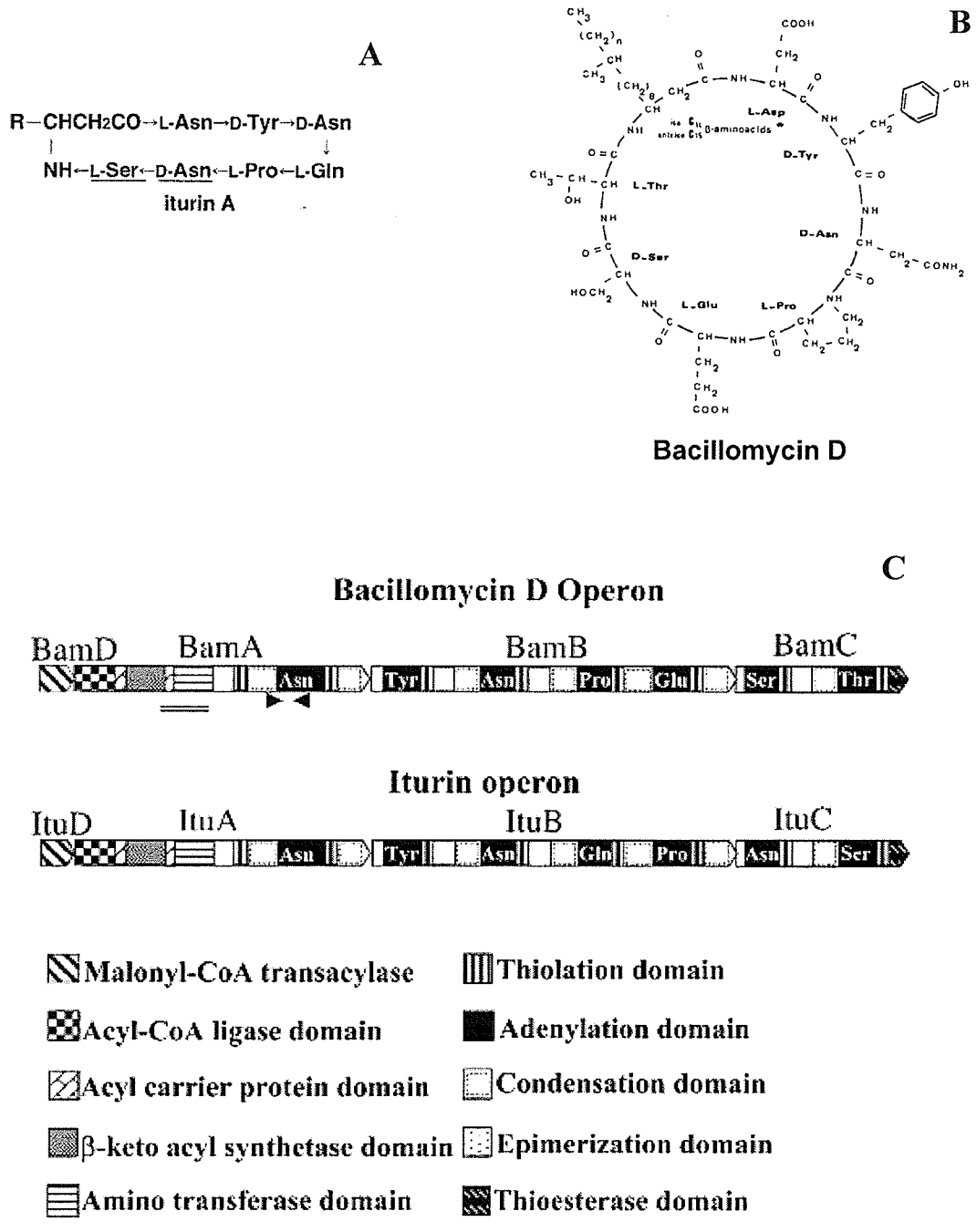

Figure 2.4. Chemical structure of iturin A (A) (Tsuge et al 2001) and bacillomycin D (B) (Peypoux et al 1981); Schematic diagram of the biosynthetic operon of iturin A and bacillomycin D (Moyne et al 2001)

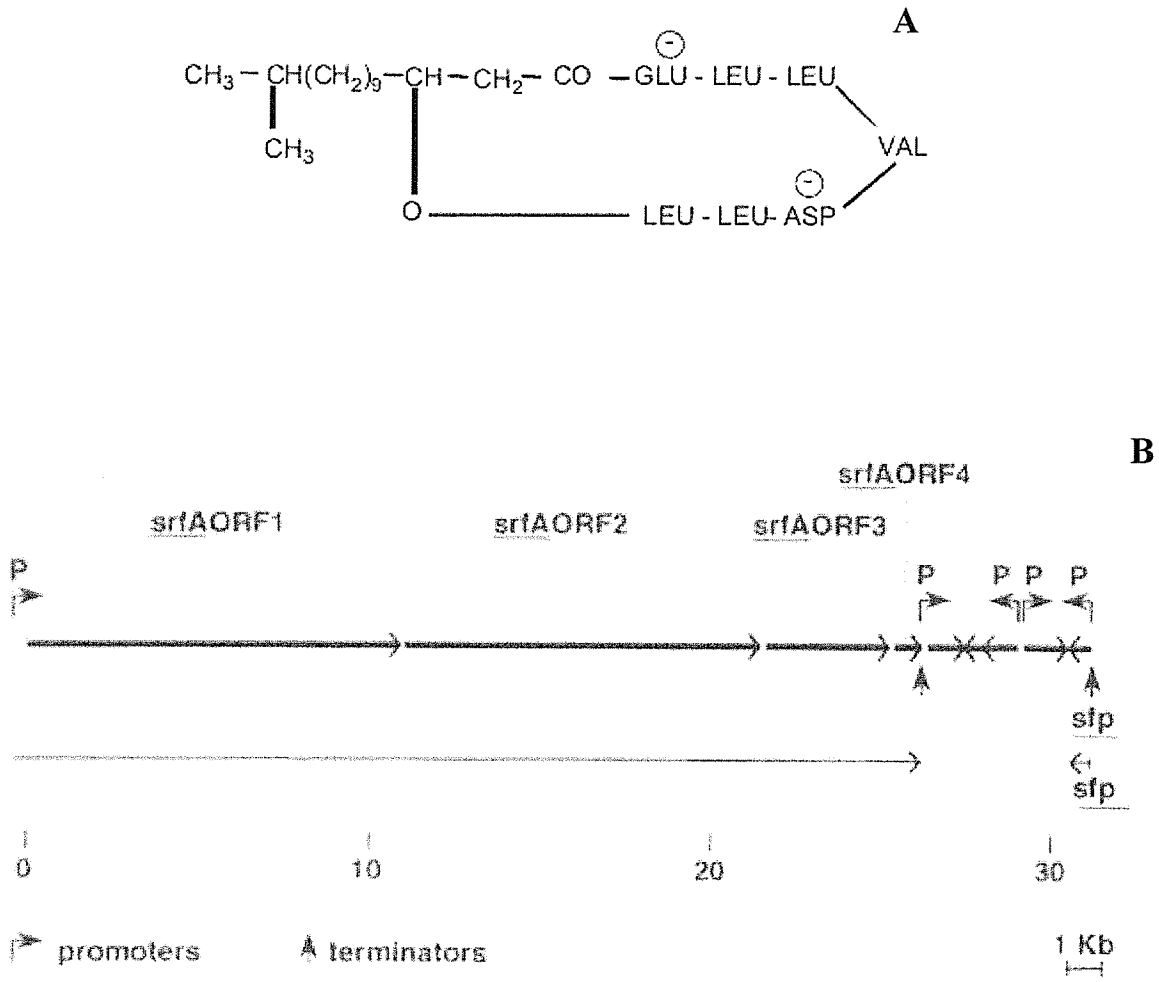

Figure 2.5. (A) Chemical structure of surfactin (Cosmina et al 1993); (B) Organization of the surfactin biosynthetic operon (Cosmina et al 1993).

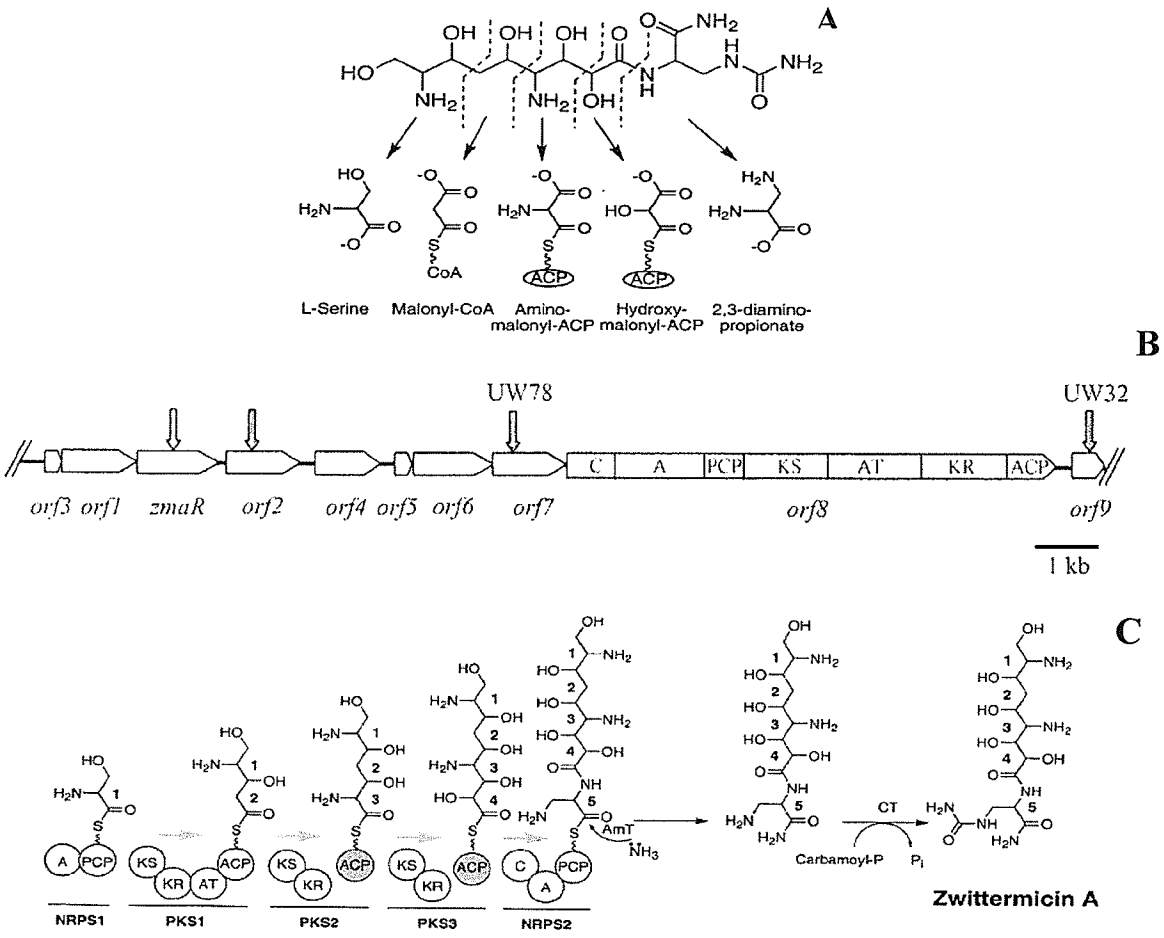

Figure 2.6. (A) Chemical structure of zwittermicin A and the proposed precursors for its biosynthesis. The dashed lines delineate the individual precursors in the final molecule, while the arrows point to the corresponding precursor shown at the bottom; (B) Organization of genes identified in the zwittermicin A biosynthetic cluster of *B. cereus* UW85. Vertical arrows represent mutations within the designated gene. An open arrow indicates that the mutant produced zwittermicin A; a shaded arrow indicates that the mutant did not produce detectable levels of zwittermicin A. The domain organization of *orf8* is indicated. Abbreviations: C, condensation; A, adenylation; PCP, peptidyl carrier protein; KS, ketoacyl synthase; AT, acyltransferase; KR, ketoreductase; ACP, acyl carrier protein; (C) Proposal for the minimum biosynthetic machinery needed for zwittermicin A assembly. The monomers incorporated into zwittermicin A are as follows: 1, serine; 2, malonyl; 3, aminomalonyl; 4, hydroxymalonyl; 5, 2,3-diaminopropionate. Each circle represents an individual domain of the NRPS/PKS megasynthase. The grey circles identify the ACP components of the aminomalonyl- or hydroxymalonyl-ACP precursors. The grey arrows indicate the direction of zwittermicin A assembly. The bars mark the borders between NRPS and PKS portions of the putative megasynthase. Abbreviations: A, adenylation; PCP, peptidyl carrier protein; KS, ketosynthase; KR, ketoreductase; AT, acyltransferase; C, condensation; AmT, amidotransferase; CT, carbamoyltransferase. (Emmert et al 2004)

3.0 SCREENING FOR POTENTIAL BACTERIAL BIOCONTROL AGENTS OF LEPTOSPHAERIA MACULANS, THE BLACKLEG PATHOGEN OF CANOLA

3.1 Abstract

Leptosphaeria maculans causes blackleg disease of canola (*Brassica napus* L.). Bacteria isolated from soil, canola stubble and plant parts were assayed for the suppression of blackleg through *in vitro* (plate) and *in vivo* (greenhouse and field) assays. In plate assays, the bacteria isolated from canola stubble had the highest agar-diffusible antifungal activity (75%) followed by isolates from the stem (59%) and leaf endophytes (59%). The antifungal activity observed was fungitoxic as the mycelial plugs from the inhibitory plates did not grow when re-plated on fresh V8 medium. In plant cotyledon assays, endophytes provided the highest disease suppression. Bacteria with the highest disease suppression in cotyledon assays also had significant disease suppression at the 3-4 leaf stage. In the field assays, bacterial spray treatment at the cotyledon leaf stage was important for the suppression of the disease. Bacterial treatments at cotyledon stage alone, and at both cotyledon and 3-4 leaf stage, suppressed the disease to the levels of control achieved through the fungicide treatment. In contrast, bacterial treatment at the 3-4 leaf stage alone failed to suppress the disease, and was not significantly different from the control. This study is the first large-scale screening of bacterial biocontrol agents against blackleg disease through plate, greenhouse and field assays.

3.2 Introduction

Canola, a member of the *Brassicaceae*, is the largest oilseed crop grown in Canada. It has been a significant contributor to the Canadian net trade balance, with annual cash receipts totaling 1.6 billion dollars in 2002, and the acreage almost doubling between 1988 and 1994 in Canada (Statistics Canada 2003). However, with the increased production of oilseed rape, blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces and de Not (anamoph *Phoma lingam* (Tode: Fr./Desm.)) has become a disease of major economic importance and occurs in epidemic proportions in most of the rapeseed producing regions in Australia, Europe and Canada (West et al 2001).

Several strategies such as crop rotation, stubble management, chemical control, sanitation and resistant cultivars have been implemented for blackleg control (Guo et al 2005). With the introduction of cultivars with major gene resistance to *L. maculans*, a high potential exists for the increased incidence of the aggressive isolates of *L. maculans*, or evolution of new virulent pathotypes of the pathogen (Mayerhofer et al 1997). The report on the appearance of the more aggressive Pathogenicity Group 3 (PG3) and 4 (PG4) isolates in W. Canada and North Central United States, where PG2 is the predominant group, is a good example of this (Fernando and Chen 2003, Bradley et al 2005, Chen and Fernando 2005). With respect to chemical control with fungicides, the perceived health and environmental risks of using these chemicals has enhanced the trend towards alternate disease management strategies (Jacobsen and Backman 1993). Also, the continuous use of fungicides enhances the evolution of resistant races of the pathogen (Fry et al 1993). Biocontrol seems to be a viable alternative to be included in an approach, which involves harnessing disease-suppressive microorganisms to improve

plant health (Handelsman and Stabb 1996). Bacteria, especially the Pseudomonads and Bacilli, and their antibiotics have been shown to play a key role in the suppression of plant pathogens in other cropping systems. Some prominent antibiotic producing bacterial genera, the type of antibiotics produced, and the target pathogen controlled has been reviewed by Whipps (2001) and Raaijmakers et al (2002). Other than antibiotic production, bacteria are known to mediate biocontrol of pathogenic fungi through competition (van-Dijk and Nelson 2000), siderophore production (Loper and Henkels 1999), hyperparasitism (Chernin et al 1995, 1997, Pleban et al 1997), and induction of systemic resistance in the host plant (Bostock 2005). Among the various biocontrol mechanisms of bacteria, the role of antibiotics in disease control has been investigated extensively. The recovery of phenazine-1-carboxylic acid (PCA) from wheat roots and associated rhizosphere soil colonized by *P. fluorescens* 2-79, and the inability of the antibiotic non-producing mutants to suppress disease (Thomashow et al 1990) are direct evidence for the role of antibiotics in disease suppression. The narrow susceptibility period of canola to blackleg favors the use of biocontrol as a viable disease control strategy. The disease is destructive to the canola crop only when the infection occurs early in plant development from cotyledon to 6-leaf growth stage (West et al 2001). If the plant can be protected during this most susceptible period, disease could be managed to avert yield loss. Berg et al (2002) recovered a high number of disease suppressive, antagonistic bacteria from oilseed rape (*Brassica napus* var. *oleracea*). This offers us a positive sign of finding potential bacterial biocontrol agents for the control of *L. maculans*. Stubble, which is a nutrient- rich organic microenvironment, is a suitable site for the production of antibiotics by bacteria (Thomashow et al 1997). Bacteria isolated

from the stubble could act as an excellent pool for the search of antibiotic producing biocontrol agents. This could provide an opportunity to control blackleg at the stubble stage, and prevent the formation of pseudothecia and release of ascospores. Also, endophytic bacteria, whose role in biocontrol has been established earlier (Chen et al 1995; Kloepper et al 1992), could act as excellent phyllosphere biocontrol agents that can prevent infection of the leaves from ascospores and pycnidiospores of *L. maculans*.

The objective of this study was to identify and characterize bacterial agents that could be effective in the phyllosphere biocontrol of *L. maculans*. This study involved the screening for antagonistic, antifungal bacterial biocontrol agents from canola through: (1) plate; (2) greenhouse (cotyledon and 3-4 leaf stage), and; (3) field assays.

3.3 Materials and Methods

3.3.1 Isolation, storage of bacterial strains and culture conditions

Bacteria were isolated from soil, diseased and non-diseased plant parts (flower, leaf, stem and root tip) and canola stubble collected from farmers fields (Savchuk 2002). Each of these were suspended in sterile distilled water and sonicated for 30s. One hundred microlitres of the water suspension was plated on 1/2–strength nutrient agar (NA, Difco Laboratories, Detroit, MI., USA) plates and incubated at 28°C. Single colonies were selected to ensure pure cultures (Savchuk 2002). To isolate bacterial endophytes, leaves of *B. napus* cvs. Westar and Cresor from different growth stages, such as cotyledon, rosette, bud and flowering were cut into 1cm x 1cm pieces. The leaf pieces were surface-sterilized using a modified method of Dobranic et al (1995). The surface sterilized pieces were plated on NA plates, amended with Nystatin (20 ml L^{-1}), and

incubated at 32°C. Pure cultures of each bacteria were maintained in Luria Bertani broth (LBB), amended with 20% glycerol (Fisher Scientific, Fair Lawn, NJ, U. S. A) and stored at -80°C. Bacteria were streaked from the stock onto Luria Bertani agar (LBA) or NA plates and incubated at 28°C for 24h in an incubator. For the plate and plant assays, 5 ml of LBB was inoculated with a bacterial loop and incubated at 28°C for 16 – 18h at 180 rev min^{-1} in an incubator shaker (Jeio Tech SI-600, Seoul-city, S. Korea).

3.3.2 Fungal strain and culture conditions

The fungal culture, *L. maculans* (PL 86-12) from pathogenicity group 2, was stored as concentrated pycnidiospore suspension in sterile water at -20°C. The fungal cultures were initiated by diluting the concentrated stock in sterile distilled water and spreading a few drops onto a V8 agar (200 ml V8 juice, 800 ml distilled water, 17 g agar) plate. The plates were incubated at room temperature under a light bank, until dark pycnidia started forming on the surface of the agar. Pieces of the agar (1cm x 1cm) containing the pycnidia were placed upside down on fresh V8 agar plates. The plates were incubated under light at room temperature, until the mycelial growth reached the periphery of the plates.

3.3.3 Plate inhibition assays

Three different media, V8 agar, NA and PDA, were used for *in vitro* antagonism tests. Five microlitres of 17-18h bacterial culture were dispensed carefully at four equidistant points along the periphery of the plates containing the agar media. The plates were incubated at room temperature for 24h. Mycelial plugs of *L. maculans*, 5 mm dia, were cut out from the edges of an actively growing colony and placed mycelial side down on the agar, at the center of the assay plates. The control plates had only the fungal

mycelial plug. The plates were incubated under a light bank at 25°C and scored for radial mycelial growth, when the radial growth of the mycelia in the control plates reached the periphery of the plate. The mycelial growth between each of the two opposite bacterial spots was measured. The percentage mycelial inhibition (%) was calculated using the equation (Fernando & Pierson III, 1999):

$$R_1 - R_2/R_1 \times 100$$

where,

R_1 - maximum radius of mycelial growth on the control plate.

R_2 – radius of mycelial growth directly opposite to the bacterial growth.

All treatments had five replicates. The PDA inhibition experiment was repeated. Due to the large number of bacteria assayed, the analysis was performed by grouping the bacteria based on their source of origin, such as endophytes, flower, leaf, root tip, soil, stem and stubble.

3.3.4 Greenhouse assays

3.3.4.1 Cotyledon assays

Cotyledons of *B. napus* cv Westar were used for the assays. The seedlings were grown in METRO-MIX® contained in S806 "T" inserts that were placed in rectangular trays. The plants were grown in a controlled growth room and incubated at 22/18° C day/night and 16/8 h photoperiod (280 μE m^{-2} s^{-1}) with daily watering. The 7-8d old seedlings were wounded with forceps, one wound per cotyledon lobe. A 10μl drop of 16 - 18h old bacterial culture (10^8 cfu/ml), grown in LBB, was placed on the wound, 24h prior to inoculation with pycnidiospores of *L. maculans*. A 10 μl drop of pycnidiospores of *L. maculans* ($2x10^7$spores/ml) was placed on each wound site. A drop of Tween 20 was

added to 10 ml of pycnidiospore suspension to enhance the spores to be equally distributed on the surface of the leaves. Control treatments included plants treated with LBB or water 24h prior to the pycnidiospore inoculation. The seedlings were placed in a controlled growth room and incubated at conditions mentioned above. The true leaves were removed on a regular basis, until cotyledons were scored for the disease levels. Disease severity was scored using an interaction phenotype (IP) scale of 0-9, 10-12 days post-pycnidiospore inoculation, where, 0= no darkening around the wounds, as in healthy control; 1= limited blackening around the wound, lesion diameter= 0.5-1.5mm, faint chlorotic halo may be present, sporulation absent; 3= dark necrotic lesions, 1.5-3.0mm chlorotic halo may be present, sporulation absent; 5= non-sporulating 3-5mm lesions, sharply limited by dark necrotic margin, may show gray-green tissue collapse as in IP 7 and 9 or dark necrosis throughout; 7= gray-green tissue collapse, 3-5mm diameter, sharply delimited, non-darkened margin; 9= rapid tissue collapse at about 10 days, accompanied by profuse sporulation in large, more than 5mm, lesions with diffuse margins. The experiment contained 5 plants per treatment and was repeated.

3.3.4.2 Assays at 3-4 Leaf stage

Brassica napus cv Westar plants at 3-4 leaf stage were used for the assay. Seedlings were raised as mentioned in the previous section and allowed to reach the two leaf stage, when they were transplanted into 4.5" pots containing soil mixture (2:1:1 – soil, sand and peat moss, respectively). The plants were grown in controlled growth rooms under conditions mentioned in the previous section. Each treatment contained 10 plants and 2 leaves per plant were picked for the assay. Ten wound sites were made on one leaf and the other leaf was left intact. Both bacteria and pycnidiospores were sprayed

on wounded and intact leaves. The inoculum concentration, time of application and assay conditions were same as mentioned in the cotyledon assays, except that Tween 20 was added to the bacterial suspension, prior to spraying. The experiment had 10 replicates per treatment and 100 lesions scored per treatment. The experiment was repeated.

3.3.5 Evaluation of selected bacterial biocontrol agents under field conditions

Biocontrol of *L. maculans* by bacterial strains DF114, DF190, DF192, DFE4 and DFE16 was evaluated under field conditions at the University of Manitoba Carman Research Station in Carman, Manitoba in 2003. The trial was conducted in a 59 m x 45 m block, which was planted to oats in 2002. The trial consisted of 48 4x4 m plots with 3 m alleyway between the treatments and around the plot. The block was situated on a well drained, clay loam (Denham clay loam) soil. Plots were seeded with the canola cultivar Westar at a seeding rate of 10 kg/ha. Fertilizer (23-24-0) was applied with the seed at a rate of 80 kg/ha. Prior to seeding, the seeds were treated with HELIX, which contains insecticides (thiamoethoxam) and the fungicides (difenoconazole, metalaxyl-M and fludioxonil). The plots were treated with Poast ® Ultra (6.1ml/L, BASF, Missisauga, Ont., Canada) at a rate of 300 g of ai (active ingredient)/ha and Muster® (0.273 g/L, DuPont, Missisauga, Ont., Canada) at a rate of 22 g of ai/ha. The herbicides were sprayed with a bicycle sprayer. Fall rye was used to fill the guard rows.

The trial consisted of 11 treatments in four replicates arranged in a completely randomized design (CRD). The treatments were as follows: (1) DF114, (2) DF190, (3) DF192, (4) DFE4 and (5) DFE16 sprayed at cotyledon stage and 3-4 leaf stage; (6) DF192 and (7) DFE16 sprayed at cotyledon stage only; (8) DF192 and (9) DFE16 sprayed at 3-4 leaf stage only; (10) fungicide spray (TILT, 3.16 ml/64m^2, Syngenta,

Guelph, Ont., Canada) at the recommended rate of 200 ml/ac; (11) pathogen control. The bacteria were sprayed at log 8 cfu/ml concentration. The fungicide was sprayed at rosette stage, between 2nd true leaf and bolting. The pathogen inoculum was in the form of infected corn kernels, which were spread at the rate of 150 g/m^2. Dry corn kernels were soaked overnight in water and sterilized in aluminum trays. Each tray was filled with 1.8 kg of corn kernels. To the sterilized kernels, 100 ml of pycnidiospore suspension (1 x 10^7 spores/ml concentration) was added and mixed thoroughly. The trays were sealed with aluminum foil and incubated at room temperature. The incubation period ranged from 28 – 35 days. The trays were shaken thoroughly once every 5 days, under a flow hood, to enable aeration and mix up the mycelium formed on the kernels. At the end of the incubation period, the kernels had developed pseudothecia and pycnidia of the blackleg pathogen. The inoculum was spread 24 hours after the bacterial spray.

The plants were scored for stem canker levels (disease severity (DS)) by a visual estimation of the circular area covered by the canker at the base of the stem. The DS was scored on a scale of 0 – 5; where: 0 – no infection; 1- 1-25% of the circular area infected; 2– 26-50% infection; 3- 51-75% infection; 4- 76 – 99% infection; 5- 100% infection or plant dead. Thirty plants were scored from each plot. The plants were picked uniformly from all over the plot.

3.3.6 Bacterial Identification

Some of the bacteria demonstrating consistent antifungal activity were selected for further identification, using the Microlog™ system (Biolog Inc., Hayward, Calif.). Single colonies were obtained by the streaking method and the following steps were involved in the process of identification: (i) Gram stain (Biolog Inc.) rated as Gram

positive or Gram negative; (ii) bacteria were streaked onto Biolog universal growth (BUG) agar medium (Biolog Inc.,): (iii) Approximate bacterial number were quantified with a turbidimeter, and 150 μL of the bacterial solution were pipetted into each of the 96 wells in the Biolog microplates; (iv) The plates were incubated for 32 °C for 16-24 h and then read with an automated plate reader (Biolog Inc.), assessed visually and identified to genus or species level.

3.3.7 Data analysis

For the plate assays and greenhouse assays, analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference), at P = 0.05, were performed for interaction within bacterial origin groups , and interaction between bacterial origin groups were tested at 95% confidence limit for the expected mean square. ANOVA and a mean separation test (Fisher's Least Significant Difference), at P = 0.05, were also used for the analysis of the field data. The Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, USA) was used for the statistical analysis.

3.4 Results

3.4.1. Plate inhibition assays

Forty-six bacterial isolates were initially tested for their antifungal activity against *L. maculans* in V8, NA and PDA plate assays. In the V8 assays, only 3 isolates had mycelial inhibition > 50% and were significantly different from the rest of the bacterial treatments. V8 agar seemed to favor growth of the fungus, which was reflected by the overall mean % mycelial inhibition of 36%. *Leptosphaeria maculans* had very slow

growth on NA, where the mycelial growth reached only 40 mm diameter, in contrast to complete growth (80 mm) on PDA (Figure 3.1), for the same incubation period. Mycelial growth on the control was considered as 40 mm for calculating the % mycelial growth. Of the 46 isolates tested, 28 isolates were in the range of 79-100% inhibition of the fungal mycelium, and were not significantly different from each other. The overall mean mycelial inhibition of 78% partially explained the promotion of bacterial growth by NA. One hundred and seventy two bacteria, including 162 isolated by Savchuk (2002) and 10 that were isolated in this study as endophytes (from different growth stages of cultivars Westar and Cresor (cotyledon through flowering)), were assayed for mycelial inhibition of *L. maculans* on PDA, and the mycelial inhibition data for the two experiments of the PDA assays were similar and therefore pooled together for the analysis. Among the isolates grouped based on their origin, the isolates from stubble had the highest mean blackleg-mycelial inhibition (75%) followed by isolates from stem (59%) and isolates of endophytic (59%) origin. In all the plate inhibition assays, where agar diffusible antifungal activity was observed, the mycelial plugs from the inhibitory plates when re-plated on fresh V8 plates failed to grow. The overall mycelial inhibition mean of the PDA group assays and the number of isolates with >50% mycelial inhibition are presented in Table 3.1. All bacterial origin groups had mycelial inhibitions significantly different from the control, and isolates from the stubble had the highest and most significant mycelial inhibition as compared to other origin groups (Table 3.1).

3.4.2 Greenhouse Assays

3.4.2.1 Cotyledon assays

One hundred and seventy two isolates were assayed for disease suppression of *L. maculans* on canola cotyledon leaves. An IP rating of 3 and lower is considered a resistant reaction, when rating cotyledon resistance in canola-blackleg resistance breeding programs. Bacterial treatments with a similar disease rating were considered as good disease suppressive treatments. Bacteria were grouped based on their origin, as grouped for the PDA assays, and were analyzed for their IP ratings. Most of the bacterial treatments were significantly different in their IP ratings as compared to the control, which had a disease rating of 9. Bacteria that were isolated as endophytes, from the internal tissues of leaves, had the highest disease suppression (lowest IP rating mean, 2.76) (Figure 3.2), followed by the isolates from stubble (IP= 4.92) and leaf surface (IP=5.44). The overall IP mean of the individual groups, the number of bacterial treatments with an IP rating less than 3, and the bacteria which were significantly different in their disease control among their respective groups are presented in Table 3.2. All bacterial origin groups had disease suppressive IP ratings significantly different from the control, and the bacteria isolated as endophytes had the highest and most significant disease suppression on cotyledons as compared to other origin groups (Table 3.2).

3.4.2.2 Assay at 3-4 Leaf stage

Bacteria from the cotyledon assays that had an IP rating from 0.2 to 3.4 were tested for blackleg suppression at the 3-4 leaf stage (Table 3.3). The wound inoculation seems very critical, as no disease lesions were observed when pycnidiospores were spray inoculated on intact leaves. The disease ratings were done on the wounded leaves. Of the

42 bacteria tested, all the bacterial treatments had disease suppressive IP ratings, which were significantly different from that of the control. The bacterial treatments had high disease suppression ratings, ranging from 0 – 1.5 (Figure 3.3). ANOVA revealed that the bacterial isolate-mode (*in vitro* vs *in vivo*) interaction was highly significant. Overall, the mycelial inhibition observed in the plate assays was not steadfast with the IP rating observed in the plant assays. Only a few isolates, such as DF121, DF114, DF1, DF190, DF192, DF202, DF205, DF210 and DFE16 had >75% mycelial inhibition in plate assays, which correlated with high disease suppression (lower IP rating) in the plant assays. Only 33 isolates exhibited > 50% mycelial inhibition in plates and lower IP ratings in the plat assays. The results were generally in the pattern, where: 1) isolates had high % mycelial inhibition but low disease suppression in plant assays; 2) isolates had low % mycelial inhibition but high disease suppression; and 3) low mycelial inhibition and low disease suppression.

3.4.3 Evaluation of selected bacterial biocontrol agents under field conditions

Bacterial treatments at both cotyledon and 3-4 leaf stage and at cotyledon stage alone suppressed blackleg disease at levels significantly different from the control (Figure 3.4). On the contrary, when isolates DF192 and DFE16 were applied only at the 3-4 leaf stage, they were not able to suppress the disease levels, which were as high as and not significantly different from the control (Figure 3.4). Bacterial treatments of isolate DF114 at both cotyledon and 3-4 leaf stage, and DFE16 treated at cotyledon stage alone suppressed the pathogen at levels as low as the fungicide treatment. Bacteria application at the cotyledon stage alone had disease suppression which was not significantly different from the fungicide treatment.

3.4.4 Bacterial Identification

Thirteen bacteria, with significant inhibition of the radial mycelial growth in plates and disease suppression in plant assays, were identified using the Micrologᵀᴹ system. The bacteria were identified to the species level, and the results are presented in Table 3.3.

3.5 Discussion

This study investigated large-scale screening of bacteria, isolated from canola, for the suppression of the blackleg fungus. Bacteria isolated from the surface of diseased and non-diseased parts of canola, soil, stubble and from internal leaf tissue, showed very high potential for the use as biocontrol agents (BCA) of *L. maculans*. The bacteria inhibited mycelial growth in plates and prevented infection by pycnidiospores of *L. maculans* in plant assays. Selected bacteria also significantly suppressed the disease under field conditions. The origin of these bacteria from canola, a member of *Brassicaceae*, adds as a positive sign of finding antagonistic bacteria, as found by Berg et al (2002) and Johansson and Wright (2003). Berg et al (2002) isolated a high number of disease suppressive, antagonistic bacteria from oilseed rape (*B. napus* var. *oleracea*). Johansson and Wright (2003), in their search for potential biocontrol agents of wheat seedling blight, isolated high number of disease suppressive bacteria from cruciferous plants, suggesting that crucifers could be a possible source of BCA's. Similarly in this study, we isolated from canola a high percentage of disease suppressive bacteria, out of which 57% had >50% inhibition of the fungal mycelial growth and 24% had a disease suppressive IP rating of 0 - 3.4. Nineteen percent of the bacteria exhibited >50% mycelial inhibition in plates and high disease suppression in plant assays. Many isolates that had high mycelial

inhibition in plates did not suppress the disease in the plants, probably due to lack of production of the antifungal compound in the plant environment. On the other hand, bacteria isolates DF98, DF149, DF137, DF153 and DFE15 had very low mycelial inhibition but were highly effective in suppressing the disease in the plant, thus explaining the potential role of mechanisms such as competition or extracellular lytic enzyme production other than antibiosis in the suppression of the pathogen. Competence of the BCA and synchronization of its activity, in time and space, with the pathogen are key factors that determine the efficiency of a BCA (Folman et al 2003). In our study, we considered testing the bacteria *in vivo* at the most crucial stages of blackleg infection, at the cotyledon and the 3-4 leaf stage of plant growth, which upon infection enables the movement of the fungus to the base of the stem, leading to cankering and death of the stem. This narrow period of susceptibility of the host enables the use of bacterial BCAs, which favor a short duration for establishing their numbers and survival, to combat the pathogen at its course of primary infection of the plant. Timing of bacterial application seems to play an important role in the prevention of blackleg infection. The phenomenon was clearly established in our field study; where bacteria (DF192 and DFE16) applied at the cotyledon stage suppressed the disease as efficiently as the fungicide used. The bacteria seem to prevent the early infection of the cotyledon leaves, reducing the chances for systemic infection, girdling and cankering of the stem. This is further strengthened from our results, where the same bacteria applied only at the 3-4 leaf stage and not at cotyledon stage, failed to suppress the disease, suggesting infection of the plant could have occurred prior to the application of the bacteria. Cotyledon stage of canola is a narrow window of infection and seems to be a potential opportunity to be exploited for

the implementation of biocontrol of blackleg. Similarly, phyllosphere bacteria could act as a potential resource for biocontrol on leaves, as they have naturally evolved to compete and survive in the harsh conditions that prevail in the phyllosphere. Twenty-nine isolates of the 43 bacteria that had high disease suppression in plant assays, were obtained from the surface of flowers, stem, leaves and internal tissues of the leaves. The role of endophytic bacteria in biological control has been previously demonstrated (Chen et al 1995; Kloepper et al 1992). In our study *Bacillus* spp, which are known for their antifungal antibiotics (Edwards et al. 1994) and produce environmental stress-resistant endospores (Sadoff 1972), dominated the endophyte assemblage. These features seem to enable them to colonize the phyllosphere, where they were able to inhibit the pycnidospores of the blackleg pathogen. Four isolates, DFE4, DFE11, DFE13 and DFE16, belong to different *Bacillus* spp., and exhibited high inhibition of radial mycelial growth in plates (>50%), high disease suppression in greenhouse (IP rating <2) and significant disease suppression in field (DFE4 and DFE16). Stubble in the soil is a rich organic source of nutrients that serve as microenvironments, where microorganisms compete with one another for nutrients and space, and in due course produce secondary metabolites, such as antibiotics, which help in the elimination of other microorganisms. Antifungal antibiotics of microbial origin, which are synthesized biologically, have been demonstrated not only to have specific activity against the target organisms but also to be biodegradable (Yamaguchi 1996), overcoming the concern with residual effects of synthetic fungicides. In our study, 21 of the 39 bacteria isolated from stubble (at different depths in the soil), had 80 – 100% inhibition of the radial mycelial growth of *L. maculans*, through agar-diffusible antifungal activity. Therefore, in vitro assays act as a

quick indicator of the mode of antifungal action, especially through antibiosis. The bacteria isolated from the stubble not only had fungal inhibition in plates, but 11 of them had high blackleg suppression (IP ratings < 3.4) in greenhouse assays. Isolates DF190 and DF192 isolated from stubble also had significant disease suppression under field conditions, thus making them potential candidates for biocontrol of blackleg. This possible production of antifungal antibiotics in the phyllosphere leading to the suppression of blackleg needs to be investigated further. Thus, this study has identified potential bacterial biocontrol agents of *L. maculans*, which exhibited strong antifungal activity in plates and also disease suppression in plants in greenhouse and field conditions.

The next step should implement some of the most effective agents under commercial growing conditions. This successful implementation would depend on the selection of the right bacterial strains, application at the right growth stage of the host to prevent infection and disease establishment, mode of delivery of the bacteria, which would involve formulation, and number of such bacterial applications for the successful control of the disease. Better understanding of the biocontrol agent with regards to its ecological survival, the host-pathogen system with regards to the survival of the pathogen, its spread, mode of infection, the susceptible growth stage of the host (duration of the period of susceptibility), would help in designing an application strategy that would enable an economically feasible disease control. With regards to bacterial biocontrol of blackleg, we have identified that the application of the bacteria at either cotyledon stage alone or both cotyledon and 3-4 leaf stage is the most crucial for blackleg control. This period of plant development from emergence of cotyledons to the 3-4 leaf

stage usually takes up to 21 days, depending of weather conditions. During this period of susceptibility, we found that a single or double application of the bacteria was able to achieve disease control similar to the levels of the fungicide. This narrow window seems to be suitable for the growth and survival of the bacteria to bring about disease control. This needs to be further established through bacterial survival studies. We have identified foliar spray of the bacteria to be a potential mode of application. Seed treatment could also be a viable option for the application of the bacteria. Seed coating acts as a source of inoculum for the colonization of above ground plant parts, especially the phyllosphere (Roos and Hattingh 1986), and also help in the establishment of the bacteria in the rhizosphere, which may lead to induction of systemic resistance towards disease control. For both foliar spray application and seed coating the right formulation needs to be achieved for the successful application of the bacteria under commercial growing conditions leading to disease control. The bacteria isolated from the stubble can be looked into their potential for control of the over-wintering pathogen, which would prevent the source of primary inoculum of the pathogen. More studies are needed for the better understanding of the system and designing bacterial application strategies, which include formulation and mode of application, for the successful commercial implementation of biocontrol bacteria for blackleg control.

Table 3.1. Mycelial inhibition means of isolates, from different parts of the canola plant and soil, assayed for the mycelial inhibition of *Leptosphaeria maculans* in PDA plate assays.

Origin based grouping	No. of isolates assayed	Mycelial Inhibition Mean (%)	No. of. isolates > 50% Mycelial Inhibition
Stubble	36	75.36^{a}	29
Stem	30	59.34^{b}	22
Endophytes	10	58.78^{b}	8
Root tip	28	54.63^{c}	17
Leaf	38	42.9^{d}	14
Flower	24	41.77^{d}	10
Soil	6	34.87^{e}	1
Control	-	0^{f}	-

The % mycelial inhibition data were analyzed using analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference) was performed at P=0.05. The PDA assays were repeated. The bacteria were grouped based on their origin for the analysis of the mycelial inhibition on PDA and the data of the respective groups were pooled from the two experiments.
Mycelial inhibition means with the same alphabetical superscript are not significantly different from each other (95% confidence limit). Bacteria isolated from canola stubble had highest mycelial inhibition and were significantly different from other bacterial groups and the control.

Table 3.2. Mean Interaction Phenotype (IP) rating of bacterial treatments assayed for the suppression of blackleg on cv. Westar cotyledons.

Origin based grouping	No. of isolates assayed	Mean IP	No. of isolates with IP < 3	Isolate #*
Endophytes	10	2.76[a]	8	DFE4, DFE13, DFE11, E16
Stubble	36	4.92[b]	9	DF190, DF192
Leaf	38	5.44[c]	9	DF94, DF114, DF117
Soil	6	5.63[c]	1	DF153
Flower	24	5.69[c]	7	DF121, DF181
Stem	30	6.91[d]	2	DF1
Root tip	28	6.96[d]	1	DF14
Control	-	9[e]	-	-

The IP rating data were analyzed using analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference) was performed at P=0.05. The bacteria were grouped based on their origin and the data of the respective groups were pooled from the two experiments.

*- Bacterial treatments, which had an IP rating <3, and were significantly different, in their disease suppression, from the other isolates in the same group.

IP Means with the same alphabetical superscript are not significantly different from each other (95% confidence limit). Bacteria isolated as endophytes had maximum disease suppression and were significantly different from other bacterial groups and the control.

Table 3.3. List of origin of bacterial isolates from canola, with high inhibition of *Leptosphaeria maculans* in *in vitro* and *in vivo* assays

Origin	Isolate#	Bacteria ID	% Mycelial Inhibition	IP rating	
				Cotyledon Stage	3-4 leaf stage
Flower	DF121		77.8	0.4	0.2
Flower	DF181		62.5	1.4	0.0
Flower	DF109		54.5	1.6	0.1
Flower	DF98		29.0	2.0	1.0
Flower	DF151	*P. fluorescens*	52.4	2.6	0.5
Flower	DF100		57.0	3.0	0.7
Flower	DF97		63.3	3.2	1.5
Flower	DF177		41.8	3.2	1.3
Flower	DF120		41.8	3.4	1.2
Flower	DF149		18.0	3.8	0.8
Leaf	DF114	*Staphylacoccus delphii*	84.3	0.8	0.0
Leaf	DF94		44.3	1.4	1.1
Leaf	DF137		29.3	2.2	0.3
Leaf	DF95		41.3	2.6	1.1
Leaf	DF87		52.8	2.8	1.5
Leaf	DF129		50.5	2.8	0.8
Leaf	DF88		55.5	3.0	0.9
Leaf	DF148	*Stenotrophomonas maltophila*	50.0	3.2	0.6
Leaf	DF96		53.8	3.2	1.1
Leaf	DF118		88.5	3.4	0.0
Leaf	DF67		62.8	3.4	0.7
Root tip	DF14	*P. fluorescens* BiotypeG	79.0	3.0	0.3
Soil	DF153		21.5	2.2	0.2
Stem	DF1		81.4	2.0	0.0
	DF187		69.3	1.4	0.4
Stubble-surface	DF190	*P. chlororaphis* BiotypeD	100.0	1.0	0.0
Stubble-surface	DF192	*Cellulomonas cellasea*	100.0	1.0	0.2
Stubble-surface	DF217		71.5	1.4	0.3
Stubble-surface	DF218		64.5	1.8	0.2
Stubble-surface	DF215		51.5	3.4	-
Stubble-5cm	DF205		100.0	1.4	0.2
Stubble-5cm	DF202	*P. chlororaphis* BiotypeD	100.0	1.8	0.0
Stubble-10cm	DF210	*P. chlororaphis* BiotypeD	100.0	1.8	0.0
Stubble-10cm	DF225		93.1	2.6	0.3
Stubble-10cm	DF211	*Rhodococcus fascians*	100.0	3.4	1.1
Cresor Bud	DFE3		-	3.0	0.7
Westar Cotyledon	DFE4	*B. cereus*	69.5	0.6	0.0
Westar Cotyledon	DFE6		53.4	2.4	0.6
Cresor Cotyledon	DFE13	*B. cereus*	58.3	0.2	0.0
Cresor Cotyledon	DFE11	*B. pumilus*	55.6	2.0	0.8
Cresor Cotyledon	DFE12		74.6	2.2	0.6
Westar Bud	DFE15		29.8	2.6	1.5
Cresor Bud	DFE16	*B. amyloliquefaciens*	80.3	1.8	0.0
	Control		0.0	9.0	9.0

Isolates listed in the table have mycelial inhibition >50% and IP rating < 3.4 (or) IP rating < 3.4. These 42 isolates initially selected from the cotyledon assays, and later tested on the 3-4leaf stage assays, had significant inhibition of the pathogen as compared to the control. Bacteria were identified to the species level using Biolog (Micrologᵀᴹ)

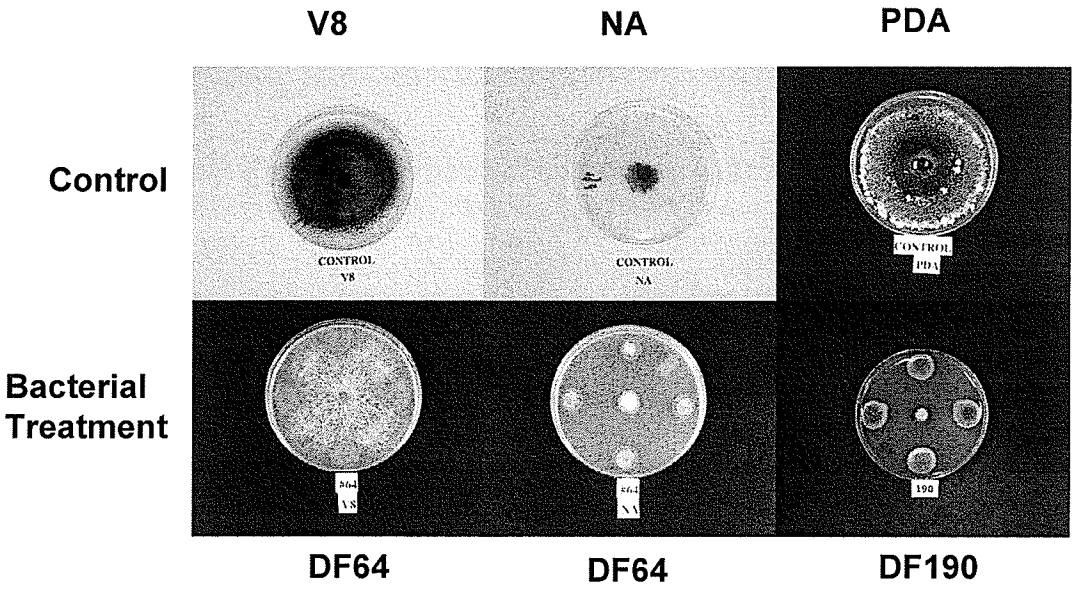

Figure 3.1. Dual plate assays on V8, NA and PDA plates to check for the inhibition of *Leptosphaeria maculans* mycelial growth *in vitro*, by bacteria isolated from canola. Antifungal activity was low in the V8 agar plates (Isolate DF64). NA promoted the bacterial growth but was not conducive for fungal growth. NA control plate has very low fungal mycelial growth. Note isolate DF64 with minimal activity in V8 showing high activity in NA. PDA supported growth of both the bacteria and *L. maculans*, serving as the favorable media for the inhibition assays. *Pseudomonas chlororaphis* strain DF190 had 100% inhibition in conditions favoring both bacterial and fungal growth.

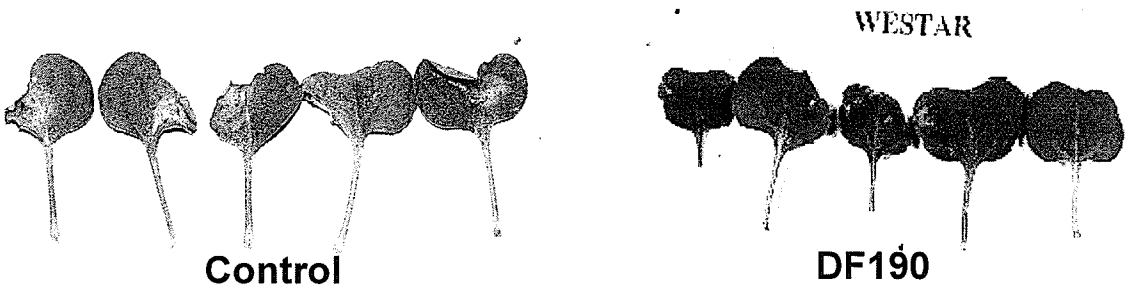

Figure 3.2. Inhibition of *Leptosphaeria maculans* pycnidiospore and development of lesions on cotyledon leaves of canola, when *Pseudomonas chlororaphis* strain DF190 inoculated 24h prior to inoculation of *L. maculans* pycnidiospores. All the cotyledon leaves, when treated with DF190 show minimal or no lesions. IP rating ranging from 0-3 is considered a blackleg resistant rating.

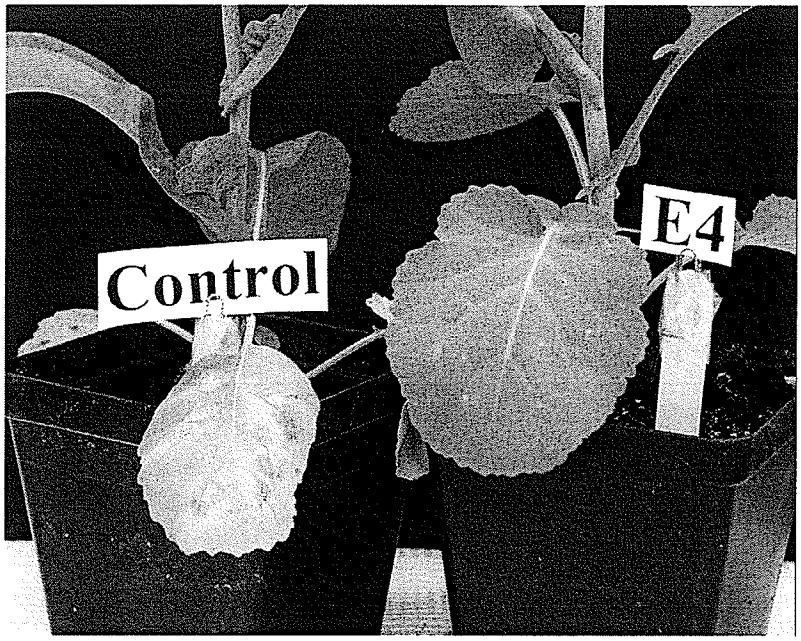

Figure 3.3. Inhibition of infection by pycnidiospore of *Leptosphaeria maculans* and development of blackleg lesions on canola leaves at 3-4 leaves stage by *Bacillus cereus* strain DFE4.

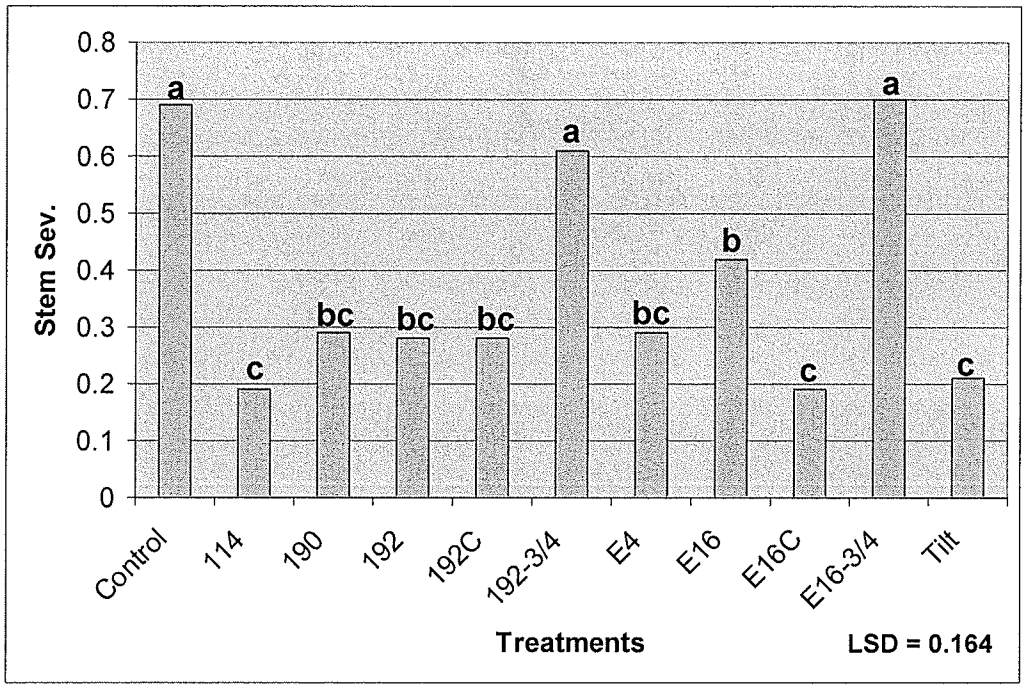

Figure 3.4. Evaluation of selected bacterial biocontrol agents for the suppression of blackleg disease under field conditions. Bacteria were sprayed 24 h prior to the spread of the blackleg-infected corn kernel inoculum. The treatments were as follows: Isolate DF114, *Pseudomonas chlororaphis* strain DF190 (190), *Cellulomonas cellasea* strain DF192 (192), *Bacillus cereus* strain DFE4 (E4) and *Bacillus amyloliquefaciens* strain DFE16 (E16) sprayed at cotyledon stage and 3-4 leaf stage; strain DF192 (192C) and strain DFE16 (E16C) sprayed at cotyledon stage only; strain DF192 (192-3/4) and strain DFE16 (E16-3/4) sprayed at 3-4 leaf stage only; and fungicide spray (Tilt). The stem disease severity is presented in the figure for the different bacterial treatments. The statistical significance was analyzed using ANOVA and a mean separation test (Fisher's Least Significant Difference), performed at P = 0.05. Treatments with the same alphabet are not significantly different from each other.

4.0 PCR- BASED MOLECULAR SCREENING OF GENES INVOLVED IN THE BIOSYNTHESIS OF ANTIBIOTICS AND BIOCHEMICAL DETECTION OF THE CORRESPONSING ANTIFUNGAL COMPOUNDS PRODUCED BY BACTERIA ANTAGONISTIC TO *LEPTOSPHAERIA MACULANS*

4.1 Abstract

Pseudomonas spp. and *Bacillus* spp. mediate disease control against pathogenic fungi through various mechanisms, of which antifungal antibiotics play an important role. PCR screening, using specific-primers, was used to identify antagonistic bacteria harboring the genes involved in the biosynthesis of phenazine, pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, lipopeptide antibiotics (iturin A, bacillomycin D and surfactin) and zwittermicin A (including the self-resistance protein ZmaR). Specific-PCR primers were developed in this study for the detection of genes of the lipopeptide antibiotics and zwittermicin A biosynthetic cluster. Forty bacteria that were isolated from canola, with varying levels of suppression of *L. maculans* in plate and plant assays, were subjected to PCR screening for antibiotic biosynthetic genes. Twenty two of the 40 isolates tested positive for the *prnD* gene of the pyrrolnitrin biosynthetic cluster. Four bacterial isolates tested positive for the presence of *phzC* and *phzD* genes of the phenazine biosynthetic cluster. *Pseudomonas chlororaphis* (DF190, DF202, DF210) and *P. aurantiaca* (DF200) isolates contained genes of the phenazine and pyrrolnitrin biosynthetic cluster, whose PCR products were sequenced and specificity of the amplifications confirmed by n-Blast search. Genes involved in pyoluteorin and 2,4-diacetylphloroglucinol biosynthesis were not detected in any of the bacteria screened. *Bacillus cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6 were

identified tp harbor genes involved in the biosynthesis of iturin A and bacillomycin D. Sequences of *srfDB3* gene of the surfactin biosynthetic operon was also detected in strains DFE4 and DFE16. *Bacillus cereus* isolates DFE4, DFE8 and DFE13 harbored genes involved in the biosynthesis of zwittermicin A and the self-resistance protein (ZmaR). The broth extracts of strain DF190, DFE4 and DFE16 exhibited antifungal activity in *in vitro* filter paper disc assays towards the inhibition of pycnidiospores of *L. maculans*. The GC-MS analysis of the methanol suspension of the broth extract of strain DF190 revealed the presence of phenazine, 2-hydroxyphenazine and 2-acetamidophenol. To our knowledge, this is the first report on the production of 2-acetamidophenol by a *P. chlororaphis* isolate. MALDI-TOF-MS analysis of the cell surface extracts of strains DFE4 and DFE16 detected the production of antifungal lipopeptide antibiotics iturin A, bacillomycin D and surfactin, confirming the PCR detection of genes involved in the biosynthesis of these antibiotics. The production of lipopeptide antibiotics iturin A, bacillomycin D and surfactin by a *B. cereus* isolate has not been reported earlier. This study used specific PCR primers for the detection of potential phenazine and lipopetide antibiotics producing antagonistic bacteria, and confirmed the molecular detection with the biochemical detection of the corresponding antibiotics synthesized by *P. chlororaphis* strain DF190, *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16.

4.2 Introduction

Pseudomonas spp., have been successfully used as biocontrol agents and correlated with disease suppression (Weller et al 1985; Stutz et al 1986; Raaijmakers and Weller 1998; Chapon et al 2002). Biological pesticides, formulated from Pseudomonads, can be incorporated in disease management to reduce the use of chemical pesticides, either alone or in combination with lower doses of chemicals (Chin-A-Woeng et al 2003). *Pseudomonas* spp., mediate crop protection by exerting multiple mechanisms of inhibitory activity such as: production of extracellular enzymes (Dunlap et al 1997); competition (Lugtenberg et al 1999); induced systemic resistance (Yan et al 2002). Bacteria, especially the Pseudomonads, and their antibiotics have been shown to play a key role in the suppression of plant pathogens in many cropping systems. Some prominent examples are: control of *Gaeumannomyces graminis* var *tritici* in wheat by phenazine produced by *Pseudomonas chlororaphis* PCL1391 (Chin-A- Woeng et al 1998); control of *Rhizoctonia solani* in cotton by pyrrolnitrin produced by *P. fluorescens* BL915 (Ligon et al 2000); control of *Thielaviopsis basicola* and *Pythium ultimum* in cotton by pyoluteorin produced by *P. fluorescens* CHA0 (Keel et al 1992); control of *Gaeumannomyces graminis* var *tritici* in wheat by 2-4, diacetylphloroglucinol produced by *P. fluorescens* Q8r1-96 (Raajmakers & Weller 2001); The recovery of phenazine-1-carboxylic acid (PCA) from wheat roots and associated rhizosphere soil colonized by *P. fluorescens* 2-79, and the inability of the antibiotic non-producing mutants to suppress disease (Thomashow et al 1990) are direct evidence for the role of antibiotics in disease suppression.

Bacillus species produce a wide range of secondary metabolites, with a broad spectrum of activity, and very diverse structures. These metabolites range from gene-encoded antibiotics to a variety of small antibiotic peptides, which are synthesized non-ribosomally (Moyne et al. 2004). The lipopeptide antibiotics such as iturin, surfactin, fengycin and plispastatin fall under this category. The lipopeptides have a hydrophilic peptide portion and a hydrophobic fatty acid portion (Roongsawang 2002). Most of them are cyclic in nature, mediated by either β-hydroxy fatty acid (β-hydroxy type) or β- amino fatty acid (β-amino type). Bacillomycin D is a member of the iturin family, along with mycosubtilin and iturinA, and is made of one β-amino fatty acid and seven α-amino acids. Fengycins are cyclic lipodecapeptides containing a β-hydroxy fatty acid with a side chain length of 16 to 19 carbon atoms. Four D-amino acids and ornithine (a non-proteinogenic residue) have been identified in the peptide portion of fengycin (Koumoutsi et al. 2004). The members of the iturin family exhibit strong antifungal and hemolytic activity and limited antibacterial activity (Maget-Dana and Peypoux, 1994). Fengycin shows specific antifungal activity against filamentous fungi and inhibits phospholipase A_2 (Nishikori et. al 1986). The non-ribosomal synthesis of these lipopeptides is achieved through the involvement of large modular multi-enzyme templates, the non-ribosomal peptide synthetases (NRPS). Each module of the multimodular-peptide synthetase is made up of approximately 1000 amino acids and catalyses the incorporation of one amino acid in the lipopeptide product (Stachelhaus et al 1995). Zwittermicin A, is a novel linear aminopolyol produced by *Bacillus cereus* isolates (He et al 1994), possibly involving a five-module NRPS-PKS (Polyketide synthetase) hybrid megasynthase (Emmert et al 2004).

Bacillus spp., especially *B. subtilis*, *B. cereus*, *B. amyloliquiefaciens*, are reported to be effective for the control of plant diseases caused by soil-borne, foliar and post-harvest fungal pathogens (Chiou and Wu 2003; Janisiewicz and Korsten 2002; Raupach and Kloepper 1998; Shoda 2000; Silo-Suh et al. 1994; Tjamos et al. 2004;). Zwittermicin A, produced by *B. cereus* UW85, is known to be involved in the control of *Phytophthora medicaginis* in alfalfa (Silo-Suh *et al.*, 1994). Among the lipopeptide antibiotics, fengycin has been shown to exhibit strong antifungal activity in the biocontrol of damping-off of bean seedling caused by *Pythium ultimum* and gray mold disease of apple caused by *Botrytis cineria* (Ongena 2005). Similarly, bacillomycin D has been reported for exhibiting strong antifungal activity towards aflatoxin producing fungi- such as *Aspergillus flavus* (Moyne et al. 2001). Other lipopeptides such as iturin A have also reported to exhibit strong antifungal activity and potential for biocontrol (Cho et al. 2003; Yoshida et al. 2002).

Specific-PCR primers have been extensively used in the detection of pathogenic bacteria, fungi, viruses, nitrogen-fixing bacteria and other microorganisms from various environmental samples, but for the first time were used for the detection of natural strains of bacteria harboring antibiotic producing genes of phenazine and 2,4-diacetylphloroglucinol by Raaijmakers et al (1997). Specific primers have also been developed for pyrrolnitrin (de Souza and Raaijmakers 2003) and pyoluteorin (Mavrodi et al 2001). However, in the case where discovering strains that produce specific antibiotics is desired, screening candidate strains for particular antibiotic-encoding sequences through PCR-based detection represents a more expeditious approach as compared to the random isolation and screening procedures (de Souza and Raaijmakers 2003).

The objective of this study was to identify potential biocontrol bacteria, with antifungal activity, that harbor genes of the antibiotic biosynthetic cluster and biochemically synthesize the compounds for the effective control of *L. maculans*. This study involved screening of the bacterial biocontrol agents, through PCR-specific primers for the detection of genes involved in the biosynthesis of phenazine, pyrrolnitrin, pyoluteorin, 2,4-diacetylphloroglucinol, lipopeptide antibiotics (iturin A, bacillomycin D and surfactin), and zwittermicin A. The antifungal nature of the culture broths were established through *in vitro* antifungal assays. The biochemical detection of the antifungal compounds produced was achieved through GC-MS and MALDI-TOF-MS analysis.

4.3 Materials and Methods

4.3.1. Bacterial strains and extraction of genomic DNA

The bacteria were stored in -80 °C and streaked onto LBA plates. Five milliliter of LB broth, in sterile culture tubes, was inoculated with a loop of the bacteria. Total genomic DNA was isolated from bacterial strains by a cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol (Ausubel et al 1995), with slight mdifications. Two milliliter bacterial suspension, from a over night culture, was transferred into 1.5 ml sterile microcentrifuge tube. The suspension was microcentrifuged at 7000 rpm for 10 min, until the formation of a bacterial pellet. The pellet was resuspended in 567 µL of TE buffer. Thirty microliters of 10% sodium dodecyl sulfate (SDS) and 3 µL of Proteinase K (20 mg/mL) were added to the bacterial suspension and mixed thoroughly. This was followed by incubation at 37 °C for 1 h. Following the incubation, 100 µL of NaCl was

added to the suspension and mixed thoroughly, followed by 80 µL of CTAB/NaCl. The suspension was mixed thoroughly and incubated at 65 °C for 10 min. Following the incubation, 0.7 to 0.8 mL of chloroform/isoamyl alcohol mixture (24:1) was added, mixed thoroughly and centrifuged at 7000 rpm for 10 min. The supernatant was transferred to a fresh tube, equal volume of phenol/chloroform/isoamyl alcohol mixture (25:24:1) was added, mixed thoroughly and centrifuged at 7000 rpm for 10 min. The supernatant was transferred to a fresh tube and 0.6 volume of isopropanol was added and mixed gently until a stringy white DNA precipitate formed. The DNA was pelleted by brief centrifugation and resuspended in 70% ethanol. The pellet was washed twice with 70% ethanol, re-pelleted by centrifugation at 10,000 rpm for 5 min, and resuspended in 100 µL of sterile de-ionized distilled water. The concentration of the DNA samples were estimated using a spectrophotometer and working solution of concentration 20 ng/µL prepared. The stock solution was stored in -20 °C and the working solution stored in 4 °C.

4.3.2 PCR analysis

4.3.2.1 Specific PCR primers

List of primers used for the detection of antibiotic biosynthetic genes of phenazine, pyrrolnitrin, pyoluteorin, and 2,4-DAPG are presented in Table 4.1.

In this study, we designed primer pairs for the detection of biosynthetic genes for zwittermicin A, and iturin A, bacillomycin D and surfactin synthetase biosynthetic genes using the web-software Primer3 (Rozen and Staletsky, 2000). A 1218 bp region (GenBank accession # AF155831 (Region: 2630 – 3847)), an *orf2* - predicted to be involved in the biosynthesis of hydroxymalonyl-ACP, which is necessary for the biosynthesis of zwittermicin A (Emmert et al 2004), was used for designing the

zwittermicin A primers zwitF2 and zwitR1. A 1203 bp region (GenBank accession # AB050629 (Region: 1 - 1203) of *B. subtilis*), of *ituD* gene - predicted to be involved in the biosynthesis of a putative malonyl-CoA transacylase (Tsuge et al 2001) was used for the design of the ITUD1F and ITUD1R primer pair. The designing of bacillomycin D primer pair, BACC1F and BACC1R, was done with a 7.8 Kb region (GenBank accession # AY137375.1 (Region: 1 - 7860) of *B. subtilis* strain ATTCAU195) involved in the synthesis of bacillomycin D synthetase C was used (Moyne et al 2004). Finally for the surfactin primers, SUR3F/3R, a 732 bp region (GenBank accession # AY040867 (Region: 1 - 732) of *B. subtilis* strain B3), of *srfDB3* gene - proposed to be involved in the biosynthesis of a putative thioesterase (Yao et al 2003) was used. The details of the specific primer pairs are presented in Table 4.2. The specificity of the primers were checked with a nucleotide to nucleotide BLAST search, and also by including non – related bacterial species in the PCR analysis.

4.3.2.2 PCR amplification

PCR amplifications were performed in a 25 µl reaction mixture containing 20 ng of template DNA, 1 x PCR buffer, 1.5 mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP and dTTP (Invitrogen Life Technologies, CA, USA), 20 pmol of each primer (50 pmol for primers 677 and 678) (Invitrogen Life Technologies, CA, USA) and 2.0 U of Platinum®Taq (Invitrogen Life Technologies, CA, USA). The following bacterial strains were used as positive controls for the PCR analysis: *P. fluorescens* Pf-5 for pyrrolnitrin and pyoluteorin (Dr. Joyce Loper's lab); *P. fluorescens* 2-79 and *P. fluorescens* Q2-87, for phenazine and 2, 4- DAPG respectively (Dr. Linda Thomashow's lab); *Bacillus*

subtilis strain ATCC 13952 for iturin A; *B. subtilis* strain ATCC 6633 for surfactin; *B. subtilis* strain Bs49 for bacillomycin D; and *B. cereus* strain UW85 for the detection of zwittermicin A. Amplifications were carried out with a PTC-100^{TM} programmable thermal controller. The list of PCR programs used for the amplification of sequences of antibiotic biosynthetic genes is presented in Table 4.3. Eight microlitres of each sample was loaded onto a 1% or 1.5% agarose gel, containing ethidium bromide and electrophoresed in 1 x Tris-Borate EDTA (TBE) buffer at 100V for 1-2h. The gels were visualized with a UV illuminator and digitally recorded.

4.3.3 Purification, sequencing and n-Blast search of PCR products for homology to sequences in GenBank database

The bacterial DNA that tested positive with a specific amplification product, were re-amplified with the specific primers and the desired bands were purified with a High Pure PCR product purification kit (Roche Applied Science, IN, USA). Following PCR, the reactions were verified for amplification by electrophoresis in a 1% agarose gel and viewed under UV light. The volume of the PCR reactions were adjusted to 100 µL and 500 µL of the binding buffer (Roche Applied Science) was added, and mixed well. The solution was transferred into a High Pure Filter Tube, which was fitted into a collection tube, and centrifuged for 1 min at 13,000 g. The flowthrough was discarded and 500 µL of washing buffer (Roche Applied Sceince) was added to the upper reservoir, followed by centrifugation at 13,000 g for 1 min. The flowthrough was discarded and 200 µL of washing buffer was added and centrifuged at 13,000 g. The upper reservoir of the filter tube was placed in a 1.5 ml centrifuge tube and 50 – 75 µL of sterile de-ionized water was added and centrifuged at 13,000 g for 1 min. The upper reservoir was discarded and

the tube centrifuged again at 13,000 g for 1 min to pellet any glass fibres, from the filter and primer residues. The supernatant was transferred into a fresh tube. The purified products were quantified in agarose gel with the 1kb-ladder, and were sequenced at the University of Calgary, Biotechnology Lab. The sequences obtained were searched for homology, with sequenced genes in the GenBank database, through the NCBI-Blast search for nucleotides.

4.3.4 Assay for antifungal activity of bacterial broth extracts towards inhibition of pycnidiospores of *L. maculans*

Five milliliter of Luria Bertani Broth (LBB) in 15 mL culture tubes was inoculated with a loop of strain DF190 and incubated at 28°C. The cells were pelleted and the bacterial culture broths were extracted with equal volume of chloroform. The chloroform extracts were dried under a nitrogen stream and re-suspended in 2.5 mL methanol. The control was plain LBB extracted and suspended in methanol. The assay plates were prepared by spreading 100 µL of *L. maculans* pycnidiodpore suspension (2 x 10^7 spores /mL) on V8 agar, with the help of a hockey stick. The plates were allowed to dry for 1-2 h. Sterile filter paper discs (2 cm dia) were placed in the centre of the plate and spotted with 50 µL of the bacterial methanol extract. The methanol suspension was spotted in portions of 10 µL to allow the filter paper to absorb the solution and prevent run-off. The plates were observed for antifungal activity, when the control discs were covered with the fungal mycelium and pycnidia of the pathogen.

4.3.5 Extraction and GC-MS identification of antifungal compounds from broth extract of *P. chlororaphis* strain DF190

The antifungal metabolites from the broth of strain DF190 were extracted following the protocol of Rosales et al (1995). A loop of strain DF190 was inoculated in 25 mL of Kanner defined medium broth (KMB – 20 g proteose peptone, 1.9 g K_2HPO_4. $3H_2O$, 1.5 g $MgSO_4$. $7H_2O$, 15 mL glycerol, 985 ml distilled H_20), and incubated at 30°C for 48 h in a rotary shaker at 180 rpm. The cells were pelleted by centrifugation at 3500 rpm, and the pellet was resuspended in 25 mL of pigment production media broth (PPM – 20 g peptone, 20 g glycerol, 5 g NaCl, 1 g KNO_3, 7.2 pH, 1000 mL distilled water) for 4 days at 30°C in a rotary shaker at 180 rpm. The culture broth was extracted with equal volume of benzene. The benzene phase was removed to a clean glass tube and evaporated. The residue was re-suspended in methanol. The methanol suspension was subjected to GC-MS analysis. Gas chromatography was performed using a Varian Star 3400 CX series GC with a flame ionization detector, available in the Department of Chemistry, University of Manitoba, Winnipeg. The samples were separated on a 15 m DB-1 megabore column of 100% dimethylpolysiloxane (Shojania et al. 1999). The trap contents were absorbed at 22°C from a unijector (SGE) on to a fused silica column (BP1, 25 m length x 0.22 mm i.d., 0.25 μm film thickness), which was cryogenically focused with dry ice and acetone for 2 min. The column was attached to a Hewlett-Packard 5890 Gas Chromatograph attached to a Hewlett-Packard mass selective detector. Helium was used as the carrier gas and the flow rate was maintained at 1 ml/min. Column temperatures were programmed from 30°C to 200°C at 4oC/min ramp rate. The mass

spectra of the unknown compounds were compared with those in the NIST/EPA/NIH Mass Spec. Library (Version 2.0).

4.3.6 MALDI-TOF-MS analysis of the antifungal metabolites present in the cell-surface extracts of *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16

Strains DFE4 and DFE16 had earlier tested positive for the presence of genes involved in the biosynthesis of antifungal lipopeptides iturin A, bacillomycin D and surfactin. Bacterial surface extracts of strains DFE4 and DFE16 were subjected to MALDI-TOF-MS analysis for the detection of antifungal lipopeptides, iturin A, bacillomycin D and surfactin. *Bacillus subtilis* strain ATCC 13952 was used as the positive control for iturin A and surfactin, and *B. subtilis* strain Bs49 for bacillomycin D. Bacterial surface extracts were prepared based on the methodology of Vater et al (2003). Both strains DFE4 and DFE16 were grown on Landy agar (LA- 20 g glucose, 5 g L-glutamic acid, 0.5 g $MgSO_4$, 0.5 g KCl, 1 g KH_2PO_4, 0.15 g $Fe_2(SO_4)_3.6H_2O$, 5 mg $MnSO_4$. H_2O, 0.16 mg $CuSO_4$, $5H_2O$, 15 g agar, 1000 mL distilled water, pH 6) and at 28°C for 2 days. Surface extracts were prepared by suspending 1-2 loops of bacterial cells from the LA plates in 500 µL of 70% acetonitrile with 0.1% trifluoroacetic acid for 1-2 min. The bacterial cells were vortexed gently for homogeneous suspension of the cells. The bacterial cells were pelleted by centrifugation and the cell-free surface extract was transferred to a new microcentrifuge tube and stored in 4°C, until further analysis. For the MALDI-TOF-MS analysis, 1 µL of the surface extract was spotted onto the target with an equal volume of matrix solution. Dihydroxy benzoic acid (DHB) was used as the matrix (50mg of DHB dissolved in 1 mL of 70% acetonitrile/0.1% trifluoroacetic acid).The sample spots were air dried and subjected to MALDI-TOF-MS analysis. The

MALDI-TOF-MS analysis was performed using the Manitoba/Sciex prototype quadrupole/TOF (QqTOF) mass spectrometer, at the Department of Physics and Astronomy, University of Manitoba. In this instrument, ions are produced by irradiation of the sample with photon pulses from a 20-Hz nitrogen laser (VCL 337ND; Spectra-Physics, Mountain View, CA, USA) with 300 mJ energy per pulse. The sample procurement time was 20 s. The *m/z* ratio range was 500-4000. The mass spectra were analyzed and *m/z* ratio identified using the *moverz* software.

4.4 Results

4.4.1 PCR Analysis

The criteria for the selection of the bacteria for the PCR assays and the results of the PCR analysis are presented in Table 4.4 and Table 4.5, respectively. Forty bacterial DNA templates were screened for the presence of the *prnD* gene and 22 isolates yielded the specific 786bp amplification product (55% of the total bacteria screened), along with the positive control *P. fluorescens* Pf-5 (Figure 4.1). Four amplified products from *P. chlororaphis* BiotypeD (DF190, DF202 and DF210) and *P. aurantiaca* (DF200) were sequenced, with the products from the *P. chlororaphis* yielding higher similarity with the GenBank# U74993 (*prnABCD* genes of *P. fluorescens)* than the *P. aurantiaca* product (Table 4.5). For phenazine (10% of bacteria screened), isolates DF190, DF200, DF202 and DF210 yielded the specific 1408bp amplification product, as seen in the positive control *P. fluorescens* 2-79 (Figure 4.2). The products when sequenced and searched with the blast nucleotide search yielded very high similarity with the *phzIRABCDEFGH* genes of *P. chlororaphis* (AF195615) (Table 4.5). Screening for the presence of the *pltB* gene

of the pyoluteorin biosynthetic cluster, yielded a ~773bp band in 6 bacteria, along with the positive control *P. fluorescens* Pf-5 (Figure 4.3). The amplification product of the isolates DF190, DF200, DF202 and DF210, when sequenced and blast searched yielded very high similarity with the *P. aeruginosa papB* gene (AJ277639) and not the pyoluteorin biosynthetic cluster (Table 4.5). None of the isolates, except DF151 and the positive control (*P. fluorescens* Q2-87) yielded the 535bp band, representing a portion of the *phlD* of the 2, 4- DAPG biosynthetic cluster (Figure 4.4). The amplification product of isolate DF151, when sequenced and searched in the GenBank did not yield any significant match (Table 4.5).

The specificity of the primers that were designed in this study for the detection of biosynthetic genes of the lipopeptide antibiotics and zwittermicin A was checked with a nucleotide to nucleotide BLAST search (to see if the primer would hybridize to any other known sequence). The specificity of the primer sequence to its corresponding target sequence in the GenBank and the E-value are presented in Table 4.6. The zwit F2/R1 primers yielded an 779 bp amplicon for *B. cereus* isolates DFE4, DFE8 and DFE13 (Figure 4.5), which on the BLAST search showed high similarity with the zwittermicin A biosynthetic cluster in the GenBank (AF155831) (Table 4.7). The iturin A specific primers, ITUD1F/1R, yielded a 647 bp product for *B. cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6, and *B. subtilis* strain ATCC 13952 (Figure 4.6). The purified amplicons showed high similarity to sequence of *ituD* gene of the iturin A operon (AB050629) of *B. subtilis* strain RB14, when searched with n-BLAST (Table 4.7). During the detection of the bacillomycin D biosynthetic cluster using specific primer pairs BACC1F/1R, of the 10 isolates tested only bacterial strains, *B. cereus* strain

DFE4 (DFE4), *B. amyloliquefaciens* strain DFE16 (DFE16) and *B. amyloliquefaciens* strain BS6 (BS6) yielded the 875 bp product along the positive control Bs49 (Figure 4.7). The NCBI-nBlast database search for the sequenced PCR products of strains DFE4, DFE16, BS6 and Bs49 showed very high homology to the sequence *bamC* gene of the bacillomycin D operon of *B. subtilis* strain ATTCAU195 (AY137375) (Table 4.7). The surfactin specific primers, SUR3F/3R, yielded a 441 bp product for strains DFE4, DFE16 along with the positive control ATCC 6633 (Figure 4.8), which on the NCBI-nBLAST search showed high homology to the sequence of the *srfDB3* gene of the surfactin operon of *B. subtilis* strain B3 (AY040867) (Table 4.7). Other *Bacillus* spp. and gram positive bacteria included in the analysis did not yield any PCR product, thus establishing the specificity of primer pairs designed in this study. Isolates DFE4, DFE8 and DFE13 yielded a 1kb amplification product along with the positive control *B. cereus* UW85 (Figure 4.9), while screening for the presence of *zmaR*- the zwittermicin A resistance gene. Isolates DFE11 and E15 yielded a distinct 600bp product. The products of isolates DFE4, DFE8, DFE13, DFE11 and DFE15 were sequenced and searched in the GenBank using the nucleotide blast. The 1kb products of isolates DFE4, DFE8 and DFE13, all *B. cereus* isolates, yielded very high similarity with the *zmaR* resistance gene of *B. cereus* (U57065). But, the 600bp products of DFE11 and DFE15 did not yield any significant match in the GenBank (Table 4.7).

4.4.2 Assay for antifungal activity of bacterial broth extracts towards inhibition of pycnidiospores of *L. maculans*

The methanol suspensions of broth extracts of strains DF190, DFE4 and DFE16 were tested for antifungal activity on V8 plates towards germination of pycnidiospores of

L. maculans. All three bacterial broth extracts showed inhibition of the pycnidiospores around the sterile filter paper discs they were spotted on. The presence of the zone of inhibition around the filter paper discs, as compared to the control that had the mycelial growth and pycnidia formation on the discs, was indicative of the presence of antifungal compounds in the broth extracts of strains DF190, DFE4 and DFE16 (Figures 4.10 - A, B and C, respectively).

4.4.3 GC-MS identification of antifungal compounds from broth extract of *P. chlororaphis* strain DF190

The methanol suspension of the benzene-broth extract of strain DF190 was subjected to GC-MS analysis and the peaks of the mass spectrum were searched for homology to chemical compounds in the NIST database. Matches with similarity index greater than 700 were considered as significant matches. The mass spectrum of DF190 broth extract (Figure 4.11A) shared similarities and dissimilarities with the spectrum of *P. fluorescens* strain 2-79 (strain 2-79) (Figure 4.11B). The characteristic phenazine and 2-acetamidophenol of strain 2-79 were present in the broth extract of strain DF190. Strain DF190 also showed the presence of 2-hydroxyophenazine, which was absent in the spectrum of strain 2-79. 2-acetamodipheonol (Figure 4.12A), phenazine (Figure 4.12B) and 2-hydroxyphenazine (Figure 4.12C) had retention times of 14:06.36 min, 16:44.41min and 21:54.24 min, respectively. Peaks, which corresponded to compounds such as pyrrolo[1,2-a] piperazine -3,6-dione, methyl ester hexadecanoic acid, hexahydro-3-(2-methylpropyl)-pyrrolo(1-2,a) piperazine-1,4-dione, 3-dodecyl-2,5-furandione, dihyroergotamine were present in the bacterial broth extract and also in the LB control broth extract.

4.4.4 MALDI-TOF-MS analysis of the antifungal metabolites present in the cell-surface extracts of *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16

The bacterial cell surface extracts of strains DFE4 and DFE16 were subjected to MALDI-TOF-MS analysis for the detection of antifungal lipopeptides, iturin A, bacillomycin D and surfactin. The mass spectra of strains DFE4 (Figure 4.13A) and DFE16 (Figure 4.13B) showed a group of mass peaks at *m/z* = 1032.7, 1046.7, 1060.7, and 1074.7, which correspond to the potassium adducts of C13-C15-surfactins, which were also detected in the mass spectrum of the positive control strain ATCC 13952 (Figure 4.13C). A second group of peaks at the range of *m/z* = 1081.8, 1095.8, and 1109.8, which correspond to the potassium adducts of C14-C16-iturin A, were identified in the mass spectra of strains DFE4 (Figure 4.14A), DFE16 (Figure 4.14B) and the positive control strain ATCC13952 (Figure 4.14C). Peaks in the range of *m/z* = 1069.5, 1083.7, 1097.6, and 1111.6 were detected in the surface extracts of DFE4 (Figure 4.15A), DFE16 (Figure 4.15B) and the positive control B49 (Figure 4.15C), which can be attributed to the potassium adducts of C14-C17 bacillomycin D.

4.5 Discussion

To our knowledge the following findings of this study have not been reported before: 1) PCR-screening of for detection gene sequences involved in the biosynthesis of antibiotics and biochemical confirmation of the antifungal compounds produced by antagonistic bacteria isolated from canola; 2) design of specific- PCR primers for the detection of genes of multi-modular enzymes, the peptide synthetases, involved in the synthesis of the antifungal antibiotics iturin A, bacillomycin D, surfactin, and zwittermicin A; 3) *Bacillus cereus* strain to harbor biosynthetic genes of iturin A,

bacillomycin D and surfactin and also to produce them; and 4) production of 2-acetamidophenol by a *P. chlororaphis* strain.

Selection and identification of antibiotic producing bacteria from natural environments through random isolation and screening procedures is time-consuming and laborious (de Souza & Raaijmakers, 2003). The partial or complete cloning and sequencing of the antibiotic biosynthetic and regulatory genes has facilitated the development of specific primers and probes that can be used for the PCR-based detection of specific antibiotic producing bacteria (de Souza & Raaijmakers., 2003). Garbeva et al (2004) found that the PCR-screening assay was very useful for the initial screening of soils for *prnD* gene of the pyrrolnitrin biosynthetic cluster, as well as for the screening and identification of Pseudomonas isolates with the capacity to produce pyrrolnitrin. Also, they strongly recommend the use of this assay when pure cultures have to be analysed for the presence of *prnD.* McSpadden Gardener (2007) recognizes that, currently, screening for the genes involved in antibiotic biosynthesis as a useful technique for the directed selection procedure of strains that synthesize them. Also, it has helped to identify novel but functionally related strains, which could rapidly accelerate the process of strain discovery. Moreover, understanding the distribution of antibiotic biosynthetic genes in natural environments reduces the concerns about their environmental release through indigenous strains containing these traits or biocontrol agents in whom these traits have been introduced (de Souza and Raaijmakers 2003).

In our screening, 18 isolates, other than the 3 isolates of *P. chlororaphis* and 1 isolate of *P. aurantiaca,* tested positive for the presence of pyrrolnitrin biosynthetic genes. This result is in support of earlier findings on the wide distribution of pyrrolnitrin

and its wide range of producers (Hammer et al 1999). Isolate DF1, with a mycelial inhibition of 81%, IP rating of 2 and a positive result for the pyrrolnitrin screening could be a pyrrolnitrin producer and a potential BCA of blackleg. Similarly, isolates DF190, DF202, DF210 (identified as *P. chlororaphis* BiotypeD) and isolate DF200 (identified as *P. aurantiaca*), that tested positive for both phenazine and pyrrolnitrin can be considered as potential BCAs, owing to their multiple antibiotic mechanisms. These isolates showed significant inhibition of the pathogen both in the plate assays (100%) and cotyledon assays (IP <2). Being native colonizers of the stubble, these isolates may have potential in inhibiting the fungus on the stubble, and prevent the formation of pathogen's reproductive structures; pseudothecia and pycnidia on stubble. Absence of the pyoluteorin and 2,4-diacetylphloroglucinol producers in the screening, further supports earlier finding on the limited distribution of these genes and co-occurrence of their biosynthetic pathways only in a very specific group of 2, 4- DAPG producers (Mavrodi et al 2001). Also, pyoluteorin production seems to be limited to a select group of *Pseudomonas* strains, most of which are genotypically very similar or even identical (Keel et al 1996; Sharifi-Tehrani et al 1998; McSpadden-Gardener et al 2000). Some bacteria (DF14, DF88, DF118, DF121, DF192, DFE6 and DFE12) (Table. 4.4), though tested negative for the antibiotics screened, exhibited significant agar-diffusible antifungal activity in plates and high disease suppression in plants, suggesting their potential as sources of other antifungal mechanisms or presence of novel antibiotics. This also helps us to look at other mechanisms of disease suppression, such as competition for nutrients, lytic enzymes and siderophore production useful in antifungal activity.

The primer pairs developed in this study, target the genes involved in the biosynthesis of the multi-modular iturin A, bacillomycin D, surfactin synthetases, and the zwittermicin A megasynthase. With the help of these primer pairs, *B. cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6, isolated from canola as endophytes, were identified harboring the biosynthetic genes of peptide synthetases of iturin A, bacillomycin D. Strains DFE4 and DFE16 also tested positive for the presence of the biosynthetic genes of surfactin synthetase. The zwittermicin A specific primers detected the presence of the zwittermicin A biosynthetic genes in *B. cereus* strains DFE4, DFE8 and DFE13, not reported before, and also the biosynthetic genes of the zwittermicin A resistant protein were identified to be present in these strains. Earlier studies had identified the biosynthetic gene of the zwittermicin A self-resistance protein (ZmaR) as the molecular marker for the presence and production of zwittermicin A (Raffel et al 1996; Stohl et al 1999). But, the detection of the biosynthetic gene of the zwittermicin A megasynthase (Emmert et al 2004) would be a more rational way of molecular detection, because Raffel et al (1996) reported strains that tested positive for the self resistance protein but did not produce any zwittermicin A. Therefore, we designed the zwittermicin A biosynthetic primers that targeted the *orf2*- of the zwittermicin A biosynthetic cluster, which is proposed to be involved in the biosynthesis of hydroxymalonyl-ACP, a core component of zwittermicin A. These *Bacillus* spp., (strains DFE4, DFE16 and BS6), isolated from canola phytosphere, with their high disease suppressive and antifungal activity add further support to the findings of Berg et al (2002) and Johanssen and Wright (2003) who isolated a high number of disease suppressive, antagonistic bacteria from the *Brassicaceae*. The role of endophytic bacteria in biological control has been previously

demonstrated (Chen et al 1995; Kloepper et al 1992). In our study *Bacillus* spp, which are known for their antifungal antibiotics (Edwards et al 1994) and environmental stress-resistant endospores (Sadoff 1972), dominated the endophyte assemblage. These features seem to enable them to colonize the phyllosphere, where they were able to suppress the pycnidospores of the blackleg pathogen. This potential of the bacteria was demonstrated, when they were sprayed on the leaves, in greenhouse and field conditions, thus resulting in suppression of the disease.

According to McSpadden-Gardener et al (2001) and Hsieh et al (2004) the presence of the biosynthetic operon usually correlates with synthesis of the compounds, unless there is a mutation in the regulatory genes. Similarly, in our study we find the presence of the genes involved in the biosynthesis of the antibiotics corresponding with the production of the compounds, which has been detected through GC-MS and MALTI-TOF-MS analysis. Initial evidence for the role of antibiotics in the suppression of the pathogen was observed when the culture filtrates or the purified antibiotics provided similar levels of control as achieved by the antibiotic-producing wild-type strain (Kang et al 1998; Nakayama et al 1999). Similarly in our study, the bacterial broth extracts of strains DF190, DFE4 and DFE16 exhibited antifungal activity towards the pycnidiospores of *L. maculans* in *in vitro* filter paper disc assays. Extracellular glucanases and chitinases have earlier been established for their role in the biocontrol of plant pathogens (Valois et al 1996; Pleban et al 1997). In our study, we presume that the crude broth extraction procedure, with organic solvents, would be too hostile for these large extracellular enzymes to be present in their active form. Therefore, we assume that the antifungal activity in these broths would be due to the presence of organic low

molecular weight compounds such as heterocyclic pyrrolnitrin, phenazines etc., or small peptides like lipopeptide iturins, surfactin. These compounds are generally extracted with organic solvents during the process of purifying and characterizing them. The GC-MS analysis of the broth extract of DF190 detected the presence of phenazine, 2-hydroxyphenazine and 2-acetamidophenol. The detection of phenazine and 2-hydroxyphenazine was in correlation and a confirmation of the detection of the phenazine biosynthetic gene in strain DF190. Also, 2-hydroxyphenazine was earlier detected in *P. chlororaphis* strain PA23 (Zhang et al 2006) through HPLC analysis. 2-hydroxyphenazine exhibits higher antifungal activity than phenazine-1-carboxylic acid (PCA), which was demonstrated by Delaney et al (2001), where *P. aureofaciens* 30-84 mutated at the *phzO* gene, responsible for the production of 2-hydroxyphenazine, and produced PCA alone, had lower antifungal activity than the wild type and the *P. fluorescens* 2-79 transformed with *phzO*. This is the first report on the production and detection of 2-acetamidophenol in the broth of a *P. chlororaphis strain*. 2-acetamidophenol was earlier reported to be produced by *P. fluorescens* 2-79 (Slininger et al 2000). 2-acetamidophenol is derived from either 3-hydroxyanthranilic acid, a precursor of the phenazine biosynthetic pathway, or N-acetyl anthrananilic acid, which is derived from anthranilic acid of the tryptophan biosynthetic pathway. 2-acetamidophenol has been earlier shown to exhibit antifungal activity against *Aspergillus niger, Aspergillus oryzae, Myrothecium verrucaria, Trichoderma viride, Mucor cirinellaoides, and Trychophyton mentagrophytes* (Gershon et al 1993). The antifungal activity of 2-hydroxyphenazine and 2-actamidophenol towards *L. maculans* needs to be established by

extracting purifying and testing these compounds individually and in combination in *in vitro* and *in vivo* assays.

MALDI-TOF-MS analysis of cell surface extracts of strain DFE4 and DFE16 detected the presence of lipopeptide antibiotics iturin A, bacillomyin D and surfactin. This confirms and supports the PCR-based detection of the genes involved in the biosynthesis of the lipopeptide antibiotic, where the presence of the gene of the biosynthetic cluster correlates with the synthesis of the compounds. Bacillomycin D and iturin A belong to the iturin family of lipopeptides (Moyne et al 2004), which are heptapeptides containing β-amino fatty acid. Iturin A and bacillomycin D affect membrane surface tension, which causes pore formation and results in leakage of K+ and other vital ions, finally leading to cell death (Yao et al 2003). The antifungal activity of iturin A and bacillomycin D has been mentioned earlier in the introduction. It has been recently documented that the colonization of plant roots by *B. subtilis* is associated with surfactin production and biofilm formation, and strikingly, surfactin protected the plant against the infection by the pathogen *Pseudomonas syringae* (Bais *et al.*, 2004). Earlier results of these bacteria exhibiting strong agar-diffusible antifungal activity in plates, which corresponds to disease suppression in plants, as seen in strains DFE4 (Ramarathnam and Fernando 2006), DFE16 (Fernando et al 2006; Ramarathnam and Fernando 2006) and BS6 (Fernando et al 2006), suggests that antibiosis could be the potential mechanism of control mediated by the bacteria. Strains DFE4, DFE16 and BS6 could be potential multiple antibiotic producers and likely strong candidates as biocontrol agents. Also, this opens up avenues to investigate if a synergistic activity of these antibiotics is involved in disease suppression or if it is a single antibiotic effect. *Bacillus*

subtilis strain M4 produces multiple lipopeptides, such as surfactins, fengycins and iturins, but only fengycins were recovered from the disease suppressed apple tissue, where the bacterium was challenge inoculated with the pathogen (Ongena et al 2005). Similarly, *B. amyloliquefaciens* strain FZB42 produces fengycin, bacillomycin D and surfactin and is involved in the biological control of *Fusarium oxysporum*. It was observed that the single mutants of surfactin or fengycin still suppressed the fungus at levels similar to that of the wild type, but the single mutant of bacillomycin D was less efficient in fungal growth inhibition, suggesting that bacillomycin D is contributing to the antifungal activity of *B. amyloliquefaciens* strain FZB42 (Koumousti et al 2004). Surprisingly, it was also observed that a double mutant of bacillomycin D and fengycin completely lost its ability to suppress the fungus, indicating a synergistic action of both antibiotics. Moreover, the synergistic action of multiple antibiotics helps in competing with other microbes, as it enables the members of the "sessile" actinomycetes to compete with other microorganisms for food and space (Challis and Hopwood 2003). The effect of the individual antibiotics towards suppression of *L. maculans* needs to be tested by isolation, purification, and *in vitro* and *in vivo* testing and/or mutagenesis studies. Also, many of these metabolites exhibit various biological activities, thus making them useful targets for biotechnological and biopharmaceutical interest (Vater et al 2002). The antifungal activity of fengycin (Ongena et al 2005) and bacillomycin D (Moyne et al 2001); antiviral and antimicrobial activity of surfactins (Peypoux et al 1999); and strong antifungal, hemolytic and limited antibacterial activity of the iturins (Maget-Dana and Peypoux 1994) are few good examples. The natural pools of these lipopeptides can be extended in a directed manner by biocombinatoric efforts, involving recombinant peptide

synthetases, the enzymes that synthesize these lipopeptides non-ribosomally (Vater et al 2002). Recombinant peptide synthetases can be achieved by swapping their module and domain, and also by site-specific mutagenesis of the amino acid residues involved in the intermediate steps of peptide synthesis. The *Bacillus* species which are natural producers of these lipopeptides could act as a rich genetic source for the construction of large biocombinatorial libraries of genetically engineered peptide synthetases for novel peptide design.

Table 4.1. List of primers used for the PCR analysis in the detection of bacterial antibiotic biosynthetic genes involved in the biosynthesis of antibiotics for biocontrol of plant diseases

Antibiotic	Primer	Primer Sequence (5' – 3')	Product Length (bp)	Target Gene	Positive Strain	Reference
Phenazine	PHZ1 PHZ2	GGCGACATGGTCAACGG CGGCTGGCGGCGTATTC	1408	*phzF* and *phzA* *phzC* and *phzD*	*P. aureofaciens* 30-84 *P. fluorescens* 2-79	Delaney *et al.* 2001
Pyrrolnitrin	PRND1 PRND2	GGGGCGGGCCGTGGTGATGGA YCCCGCSGCCTGYCTGGTCTG	786	*prnD*	*P. fluorescens* strain BL915	De Souza and Raaijmakers 2003
Pyoluteorin	PLTBf PLTBr	CGGAGCATGGACCCCCAGC GTGCCCGATATTGGTCTTGACCGAG	773	*pltB*	*P. fluorescens* Pf5	Mavrodi *et al.* 2001
2,4- diacetyl phloroglucinol	BPF2 BPR4	ACATCGTGCACCGGTTTCATGATG CCGCCGGTATGGAAGATGAAAAAGTC	535	*phlD*	*P. fluorescens* Q2-87	McSpadden Gardner *et al* 2001

Table 4.2. Characteristics of specific-primers developed for the detection of biosynthetic genes of iturin A, bacillomycin D, surfactin and zwittermicin A.

Primer Name	Primer Sequence (5' to 3')	Length (bp)	G+C (%)	Tm (oC)	Positiona	Product length (bp)	Positive Strain
ZWITF2	ttgggagaatatacagctct	20	40	56	2906	779	*Bacillus cereus* strain UW85
ZWITR1	gacctttgaaatgggcgta	20	45	58	3685		
ITUD1F	gatgcgatctccttggatgt	20	50	60	4041	647	*B. subtilis* strain RB14
ITUD1R	atcgtcatgtgctgcttgag	20	50	60	4687		
BACC1F	gaaggacacggcagagagtc	20	60	60	3382	875	*B. subtilis* strain ATTCAU195
BACC1R	cgctgatgactgttcatgct	20	50	60	4256		
SUR3F	acagtatgggaggcatggtc	20	55	62	313	441	*B. subtilis* strain B3
SUR3R	ttccgccacttttcagttt	20	40	56	743		

Table 4.3. List of PCR Programs used for the detection of sequences of antibiotic biosynthetic genes in bacteria antagonistic to *Leptosphaeria maculans*

Antibiotic	PCR Cycles						Source
	Initial Denaturation	Denaturation	Annealing	Extension	Final extension	# Cycles	
Phenazine	94 °C, 2 min	94 °C, 1 min	56 °C, 45 s	72 °C, 1.75 min	75 °C, 1 min	30	Delaney et al (2001)
Pyrrolnitrin	95 °C, 2 min	95 °C, 1 min	68 °C, 1 min	72 °C, 1 min		30	de Souza and Raaijmakers (2003)
Pyoluteorin	94 °C, 2 min	94 °C, 1 min	56 °C, 45 s	72 °C, 1 min		29	Mavrodi et al (2001)
2,4-diacetylphloroglucinol	95 °C, 3 min	94 °C, 1 min	60 °C, 1 min	72 °C, 1 min	72 °C, 5 min	35	McSpadden Gardner et al (2001)
IturinA, bacillomycin D and surfactin	94 °C, 3 min	94 °C, 1 min	60°C, 30 s	72 °C, 1 m 45 s	72 °C, 6 min	36	This study
Zwittermicin A	94 °C, 5 min	94 °C, 1 min	52 °C, 1 min	72 °C, 2 min	72 °C, 2 min	45	This study
Zwittermicin A self-resistance protein (ZmaR)	94 °C,5 min	94 °C, 1 min	56 °C, 1 min	72 °C, 2 min	72 °C, 5 min	45	Raffel et al (1996)

Table 4.4. List of bacterial isolates from canola screened for the presence of antibiotic biosynthetic genes using specific primer-based PCR.

Origin	Isolate#	Bacteria ID	% Mycelial Inhibition	IP rating Cotyledon Stage	PRN	PYO	PHZ	2, 4-DAPG
Stubble-surface	DF190[a]	*P. chlororaphis* BiotypeD	100.0	1.0	+	+*	+	
Stubble-surface	DF192	*Cellulomonas cellasea*	100.0	1.0				
Stubble- 5cm	DF202	*P. chlororaphis* BiotypeD	100.0	1.8	+	+*	+	
Stubble - 10cm	DF210	*P. chlororaphis* BiotypeD	100.0	1.8	+	+*	+	
Stubble - 10cm	DF211	*Rhodococcus fascians*	100.0	3.4	+			
Stubble - 10cm	DF225		93.1	2.6	+			
Leaf	DF118		88.5	3.4				
Stem	DF1		81.4	2.0	+			
Cresor Bud	DFE16		80.3	1.8				
Root tip	DF14	*P. fluorescens* BiotypeG	79.0	3.0				
Flower	DF121		77.8	0.4				
Cresor Cotyledon	DFE12		74.6	2.2				
Stubble-surface	DF217		71.5	1.4	+			
Westar Cotyledon	DFE4	*B. cereus*	69.5	0.6				
Stubble-surface	DF218		64.5	1.8	+			
Leaf	DF97		63.3	3.2	+			
Flower	DF181		62.5	1.4	+			
Cresor Cotyledon	DFE13	*B. cereus*	58.3	0.2				
Cresor Cotyledon	DFE11	*B. pumilus*	55.6	2.0				
Leaf	DF88		55.5	3.0				
Westar Cotyledon	DFE6		53.4	2.4				
Flower	DF151	*P. fluorescens*	52.4	2.6	+			+*
Stubble-surface	DF215		51.5	3.4	+			
Leaf	DF148	*Stenotrophomonas maltophila*	50.0	3.2	+			
Soil	DF25[b]		79.1	3.8				
Soil	DF20		76.5	4.0				
Soil	DF17		77.5	4.4				
Stubble-surface	DF200	*P. aurantiaca*	100.0	4.6	+	+*	+	
Stem	DF2		74.6	4.6	+			
Leaf	DF36		52.3	4.6	+			
Westar Bud	DFE8	*B. cereus*	68.8	4.8				
Root tip	DF13		100.0	5.0				
Stubble-10cm	DF212		100.0	5.0	+	+*		
Stubble-10cm	DF227		100.0	5.0	+			
Stem	DF5		67.5	5.0				
Stubble-5cm	DF228		57.8	5.0	+			
Flower	DF177[c]		41.8	3.2	+			
Westar Bud	DFE15		29.8	2.6				
Soil	DF153		21.5	2.2	+			
Leaf	DF115[d]		10.8	6.0	+			

[a] Isolates DF190 to DF148 exhibited high mycelial inhibition in plates and high disease suppression on cotyledons; [b] Isolates DF25 to DF228 exhibited high mycelial inhibition in plates and low disease suppression on cotyledons; [c] Isolates DF177 to DF153 exhibited low mycelial inhibition in plates and high disease suppression on cotyledons. [d] Isolate DF115 exhibited low mycelial inhibition and low disease suppression on cotyledons. Bacteria were identified to the species level using Biolog (Micrologᵀᴹ). The isolates were selected, for the PCR assays, to have a representation of all the categories. PRN – pyrrolnitrin; PYO – pyoluteorin; PHZ – phenazine; DAPG – 2,4 – diacetylphloroglucinol. *Isolates DF190, DF200, DF202, DF210 and DF212 yielded a 773 bp product, which had similarity with the gene sequence of *papB* of *P. aeruginosa*. Isolate DF151, though tested positive for 2,4-DAPG primers BPF2/BPR4, did not yield significant match on the blast search.

Table 4.5. Blast results of the sequenced products obtained from PCR amplification using gene-specific primers for biosynthetic genes of bacterial antibiotics involved in biocontrol of plant diseases.

Antibiotic	Isolate	Primer	Product size (bp)	GenBank Accession #	Obtained GenBank Match	Score	E value
Phenazine	*P. chlororaphis* (DF190, DF202, DF210);	PHZ1/PHZ2	1408	L48339	*phzFABCD* genes of *P. aureofaciens*	1651	0
	P. aurantiaca (DF200)			L48339	*phzFABCD* genes of *P. aureofaciens*	1639	0
Pyrrolnitrin	*P. chlororaphis* DF190	PRND1/PRND2	786	U74493	*prnABCD* genes of *P. fluorescens*	345	$8e^{-92}$
Pyrrolnitrin	*P. aurantiaca* DF200	PRND1/PRND2	786	U74493	*prnABCD* genes of *P. fluorescens*	76	2^{e-11}
Pyoluteorin	*P. chlororaphis* (DF190 DF202, DF210) *P. aurantiaca* DF200	PLTBf/PLTBr	773	AF081920	*P. aeruginosa papB* gene (AJ277639)*	391	e^{-105}
2,4-DAPG	*P. fluorescens* DF151	BPF2/BPR4	~600	U41818	insignificant	-	-

*GenBank Accession number AJ277639 for the gene sequence of *papB* of *Pseudomonas aeruginosa*.

Only *P. chlororaphis* (DF190, DF202 and DF210) and *P. aurantaica* isolateDF200 tested positive for PRN and PHZ, which were confirmed with sequencing and blast searches. None of the isolates tested positive for 2,4-DAPG and PYO.

Table 4.6. Nucleotide -Blast search results of the specific primers developed for the detection of iturin A, bacillomycin D, surfactin and zwittermicin A.

Lipopeptide	Primer	GenBank Accession #	Position in GenBank Sequence	Positive Strain	E-Score
Zwittermicin A	ZWITF2	AF155831	2906-2925	*Bacillus cereus* strain UW85	0.02
Zwittermicin A	ZWITR1	AF155831	3685-3666	*B. cereus* strain UW85	0.02
Iturin A	ITUD1F	AB050629	4041-4060	*B. subtilis* strain RB14	0.02
Iturin A	ITUD1R	AB050629	4687-4668	*B. subtilis* strain RB14	0.02
Bacillomycin D	BACC1F	AY137375	34274 - 34293	*B. subtilis* strain ATTCAU195	0.02
Bacillomycin D	BACC1R	AY137376	35148 - 35129	*B. subtilis* strain ATTCAU195	0.02
Surfactin	SUR3F	AY040867	313-332	*B. subtilis* strain B3	0.02
Surfactin	SUR3R	AY040867	743-724	*B. subtilis* strain B3	0.02

Table 4.7. Blast results of the sequenced products obtained from PCR amplification using gene-specific primers for biosynthetic genes of iturin A, bacillomycin D, surfactin and zwittermicin A.

Antibiotic	Isolate	Primer	Product size (bp)	GenBank Accession #	Obtained GenBank Match	Score	E value
Zwittermicin A	*B. cereus* DFE4	ZwitF2/R1	779	AF155831	*orf2*- biosynthetic cluster of *B. cereus*	1351	0
Zwittermicin A	*B. cereus* DFE8	ZwitF2/R1	779	AF155831	*orf2*- biosynthetic cluster of *B. cereus*	1382	0
Zwittermicin A	*B. cereus* DFE13	ZwitF2/R1	779	AF155831	*orf2*- biosynthetic cluster of *B. cereus*	1336	0
Zwittermicin A	*B. cereus* UW85	ZwitF2/R1	779	AF155831	*orf2*- biosynthetic cluster of *B. cereus*	1402	0
Bacillomycin	*B. cereus* strain DFE4	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	1447	0
Bacillomycin	*B. amyloliquefaciens* strain DFE16	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	1463	0
Bacillomycin	*B. amyloliquefaciens* strain BS6	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	848	0
Bacillomycin	*B. subtilis* strain 49	BACC1F/1R	875	AY137375	Bacillomycin D operon of *B. subtilis* ATTCAU195	1495	0
Iturin A	*B. cereus* strain DFE4	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	1031	0
Iturin A	*B. amyloliquefaciens* strain DFE16	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	1035	0
Iturin A	*B. amyloliquefaciens* strain BS6	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	789	0
Iturin A	*B. subtilis* strain ATTC13952	ITUD1F/1R	647	AB050629	*ituD* - iturin A operon of *B. subtilis* strain RB14	724	0
Surfactin	*B. cereus* strain DFE4	SUR3F/3R	441	AY040867	*srfDB* - surfactin operon of *B. subtilis* strain B3	678	0
Surfactin	*B. amyloliquefaciens* strain DFE16	SUR3F/3R	441	AY040867	*srfDB* - surfactin operon of *B. subtilis* strain B3	684	0
Surfactin	*B. subtilis* strain ATTC6633	SUR3F/3R	441	AY040867	*srfDB* - surfactin operon of *B. subtilis* strain B3	694	0

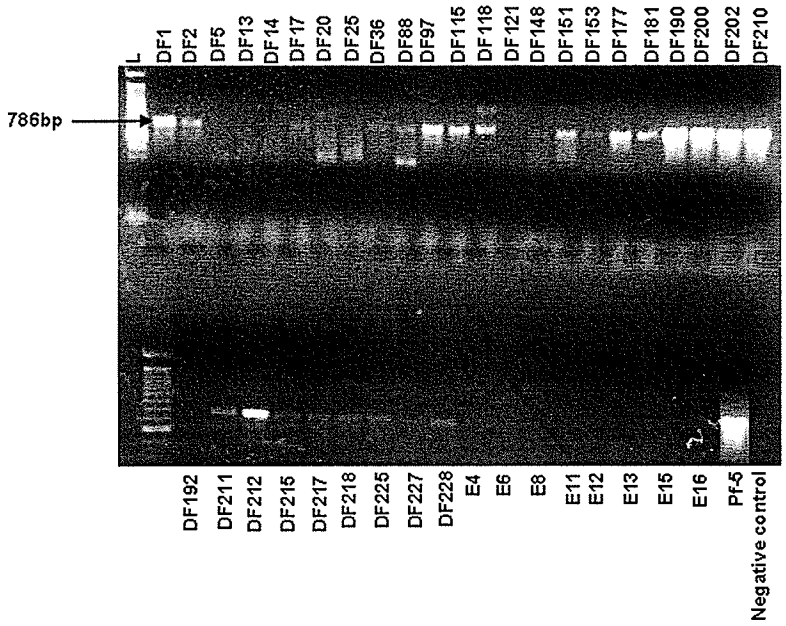

Figure 4.1. PCR- based screening of bacterial isolates from canola for the presence of *prnD* gene. *prnD* is a member of the pyrrolnitrin biosynthetic cluster and primers PRND1 and PRND2 produced the 786bp band for the isolates that seem to have the biosynthetic cluster. The amplified product of *Pseudomonas chlororaphis* strains DF190, DF202 and DF210, and *P. aurantiaca* strain DF200 were sequenced and confirmed for the *prnD* sequence through the GenBank search.

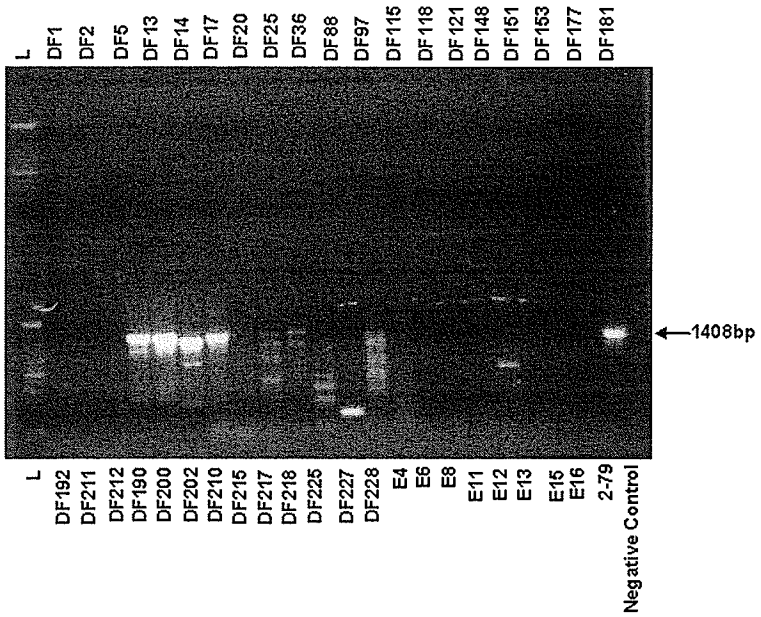

Figure 4.2. Screening for the presence of sequences of genes *phzF* and *phzA* (*Pseudomonas aureofaciens* 30-84) or *phzC* and *phzD* (*P. fluorescens* 2-79) of the phenazine biosynthetic cluster with specific primers PHZ1/PHZ2. Four isolates (*Pseudomonas chlororaphis* strains DF190, DF202 and DF210, and *P. aurantiaca* strain DF200) produced the 1408bp band. The amplified product on sequencing and GenBank searching showed high similarity with the phenazine biosynthetic sequences of *P. aureofaciens* 30-84 and *P. fluorescens* 2-79.

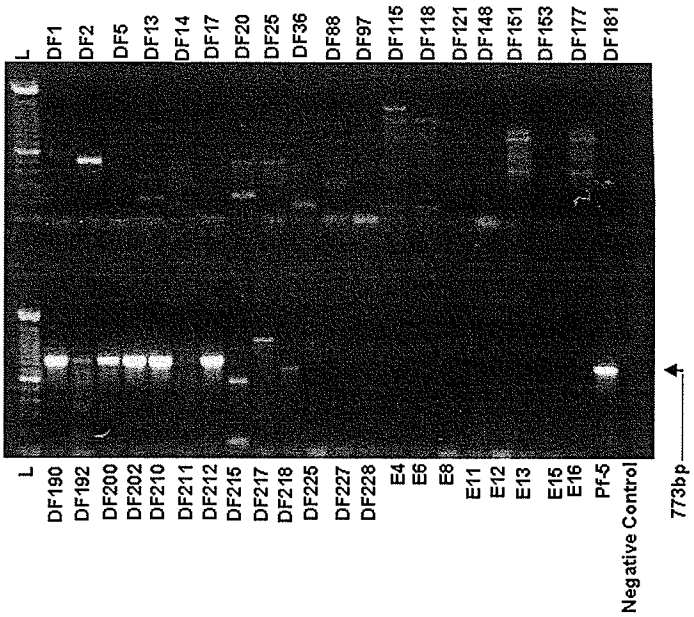

Figure 4.3. Screening for *pltB* gene of the pyoluteorin biosynthetic cluster. Four isolates (*Pseudomonas chlororaphis* strains DF190, DF202 and DF210, and *P. aurantiaca* strain DF200) produced the 773bp product, which on sequencing and the GenBank search matched the *papB* gene of *P. aeruginosa* and not the *pltB* gene sequence.

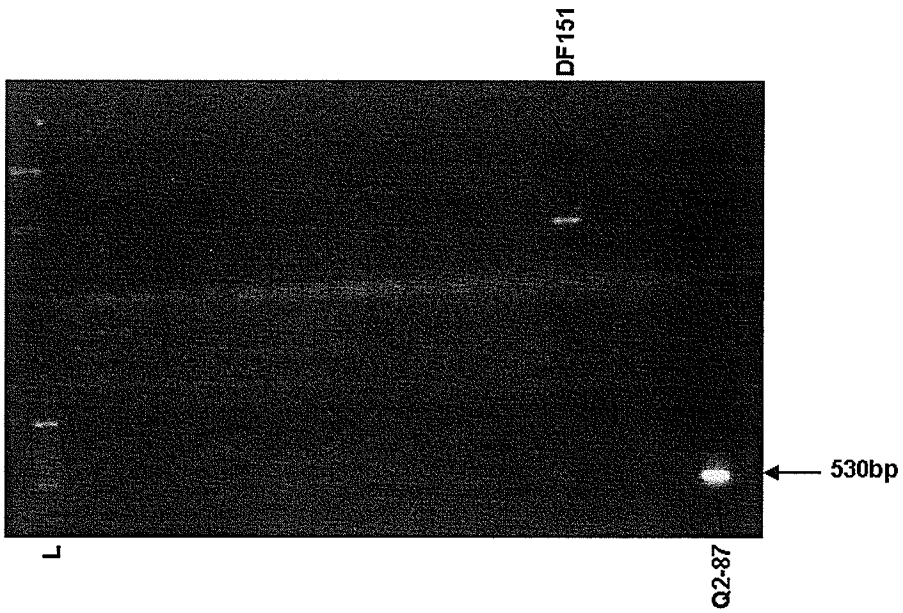

Figure 4.4. Screening for presence of *phlD* gene of the 2,4-diacetylphloroglucinol biosynthetic cluster. None of the isolates screened, except *Pseudomonas fluorescens* strain DF151 and the positive control *P. fluorescens* Q2-87, produced the 535 bp band. The purified product of DF151, when sequenced and n-Blast searched did not yield any significant result.

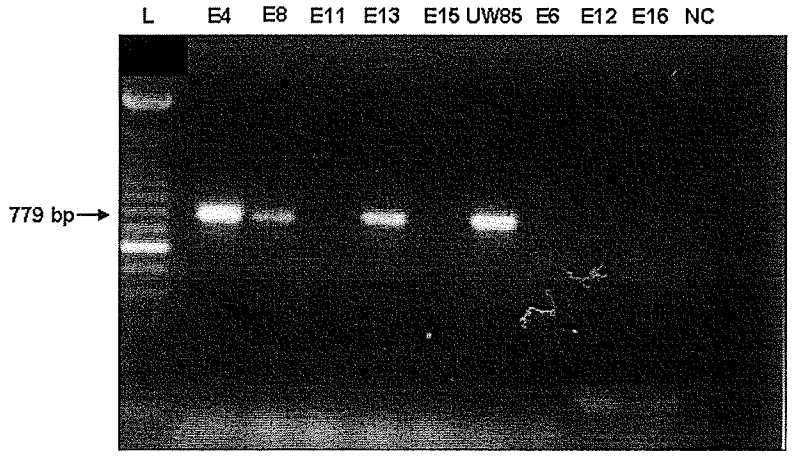

Figure 4.5. PCR - detection of *orf2* of the zwittermicin A biosynthetic cluster from antagonistic endophytic bacteria, using specific primers ZWITF2/R1. The primers amplified a 779 bp band, from *Bacillus cereus* strains DFE4, DFE8 and DFE13, along with the positive control, *B. cereus* UW85. The amplified products were purified, sequenced and confirmed for homology to the sequence of *orf2*- of the zwittermicin A biosynthetic cluster in the GenBank. *orf2* is predicted to be involved in the biosynthesis of hydroxymalonyl-ACP, which is necessary for the biosynthesis of zwittermicin A (Emmert et al 2004)

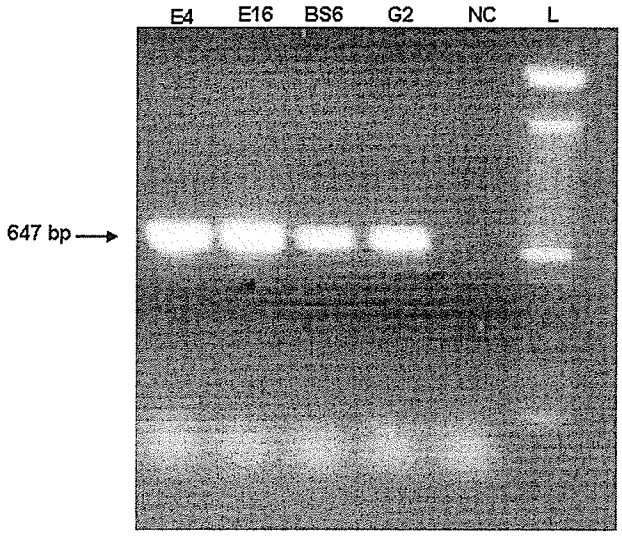

Figure 4.6. PCR - detection of *ituD* gene of the iturin A synthetase biosynthetic operon from antagonistic endophytic bacteria, using the specific primers ITUD1F/1R. The primers amplified a 647 bp product from *Bacillus cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6, along with the positive control, *B. subtilis* strain ATTC13952 (G2). The products were purified, sequenced and confirmed for homology to the *ituD* sequence of the iturin A biosynthetic operon in the GenBank database.

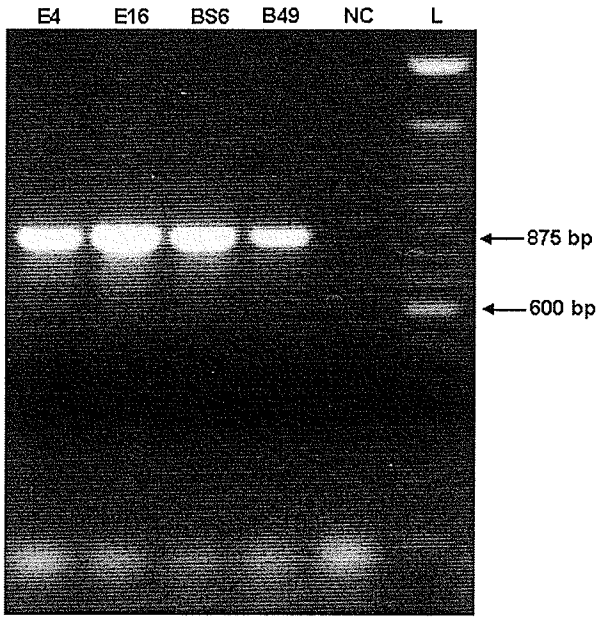

Figure 4.7. PCR - detection *bamC* gene of the bacillomycin D synthetase biosynthetic operon from antagonistic endophytic bacteria, using specific-primers BACC1F/1R. The primers amplified a 875 bp product from *Bacillus cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16 and BS6, along with the positive control, *B. subtilis* strain B49. The products were purified, sequenced and confirmed for homology to the *bamC* gene sequence of the bacillomycin D biosynthetic operon in the GenBank database.

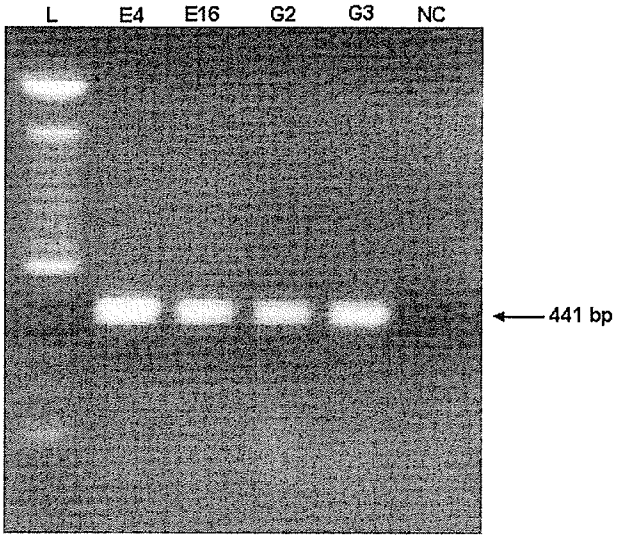

Figure 4.8. PCR - detection of *srfDB3* gene of the surfactin synthetase biosynthetic operon from antagonistic endophytic bacteria, using the specific-primer set SUR3F/3R. The primer set amplified a 441 bp product from *Bacillus cereus* strain DFE4, *B. amyloliquefaciens* strains DFE16, along with the positive controls, *B. subtilis* strain ATTC6633 (G2) and *B. subtilis* strain ATTC13952(G3). The products were purified, sequenced and confirmed for homology to the *srfDB* gene sequence of the surfactin biosynthetic operon in the GenBank database.

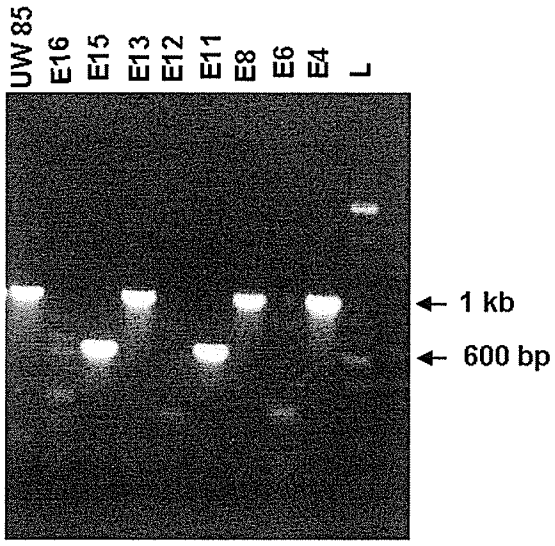

Figure 4.9. PCR - detection of *zmaR* gene of the zwittermicin A self-resistant protein biosynthetic operon from antagonistic endophytic bacteria, using specific-primer set 677/678. The primer set amplified a 1 kb product from *Bacillus cereus* strains DFE4, DFE8 and DFE13, along with the positive control *B. cereus* strain UW85. The primers also amplified a 600 bp product from isolates DFE11 and DFE15. The 1 kb products were purified, sequenced and confirmed for homology to the *zmaR*- gene sequence of the ZmaR biosynthetic cluster in the Genbank database. The 600 bp products did not yield any match in the database.

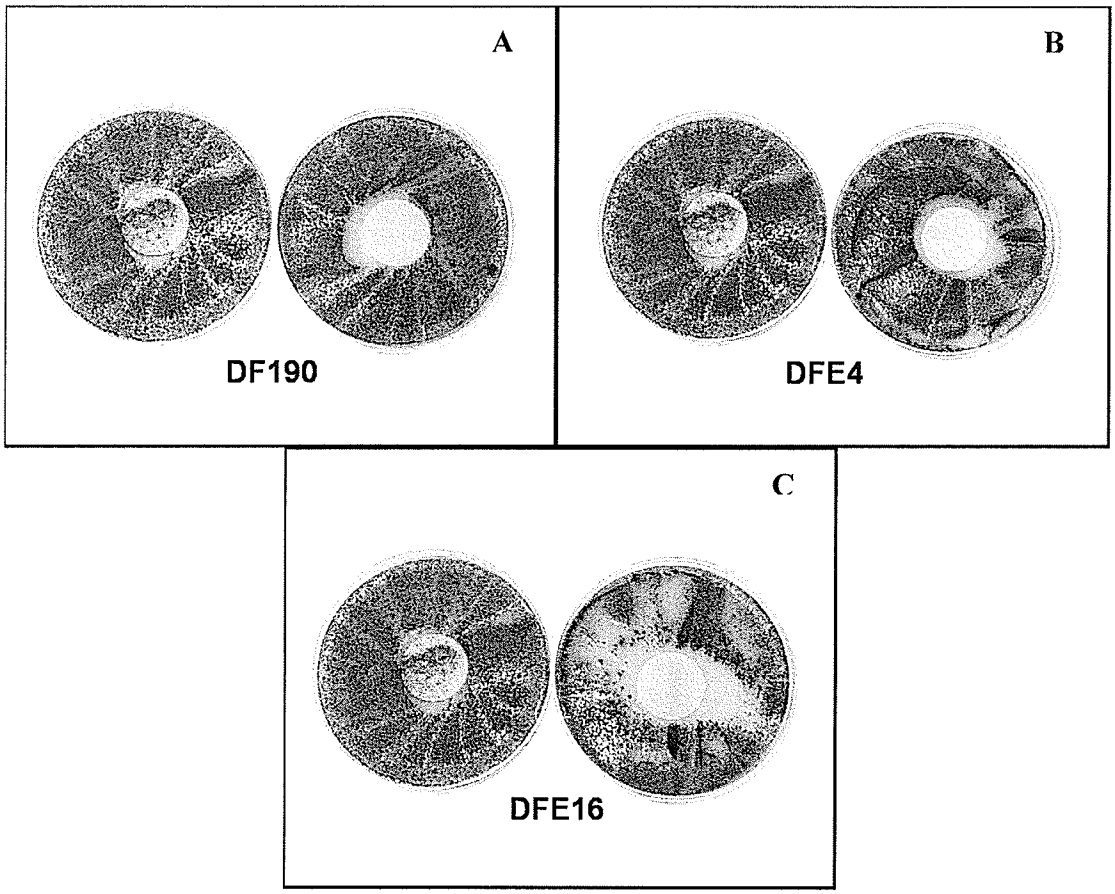

Figure 4.10. *In vitro* antifungal activity of bacterial broth extracts towards inhibition of germination of pycnidiospores of *Leptosphaeria maculans* on V8 agar plates. Plain LB broth was extracted as the control. The control plates are on the left and broth treated plates on the right. A – Broth extract of *P. chlororaphis* strain DF190; B - Broth extract of *Bacillus cereus* strain DFE4; C - Broth extract of *B. amyloliquefaciens* strain DFE16.

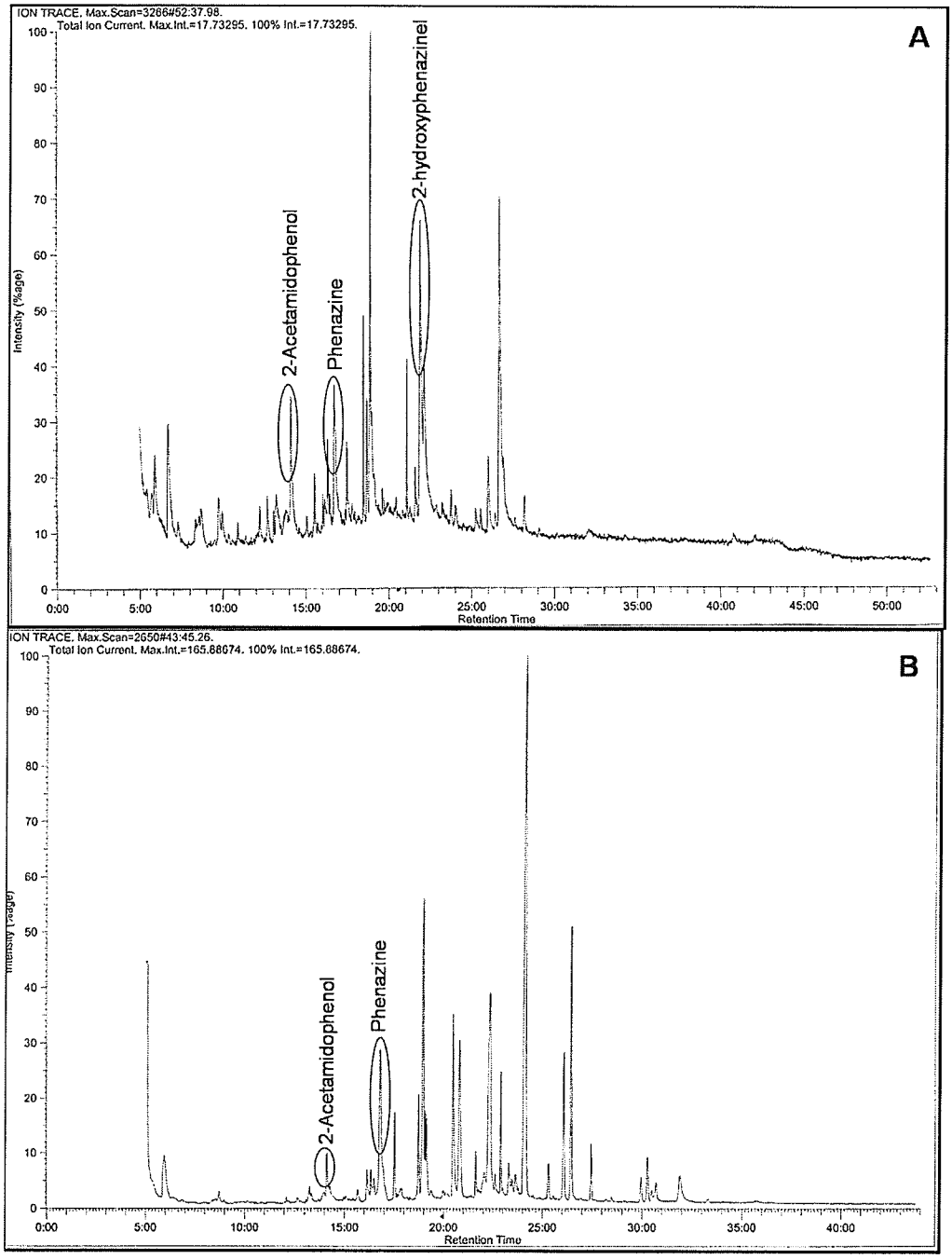

Figure 4.11. GC-MS analysis of methanol-bacterial broth extract for the detection and identification of antifungal metabolites. The peaks were searched for suitable matches and identified in the NIST database. A – Methanol-broth extract of strain *Pseudomonas chlororaphis* strain DF190; B - Methanol-broth extract of strain *P. fluorescens* strain 2-79

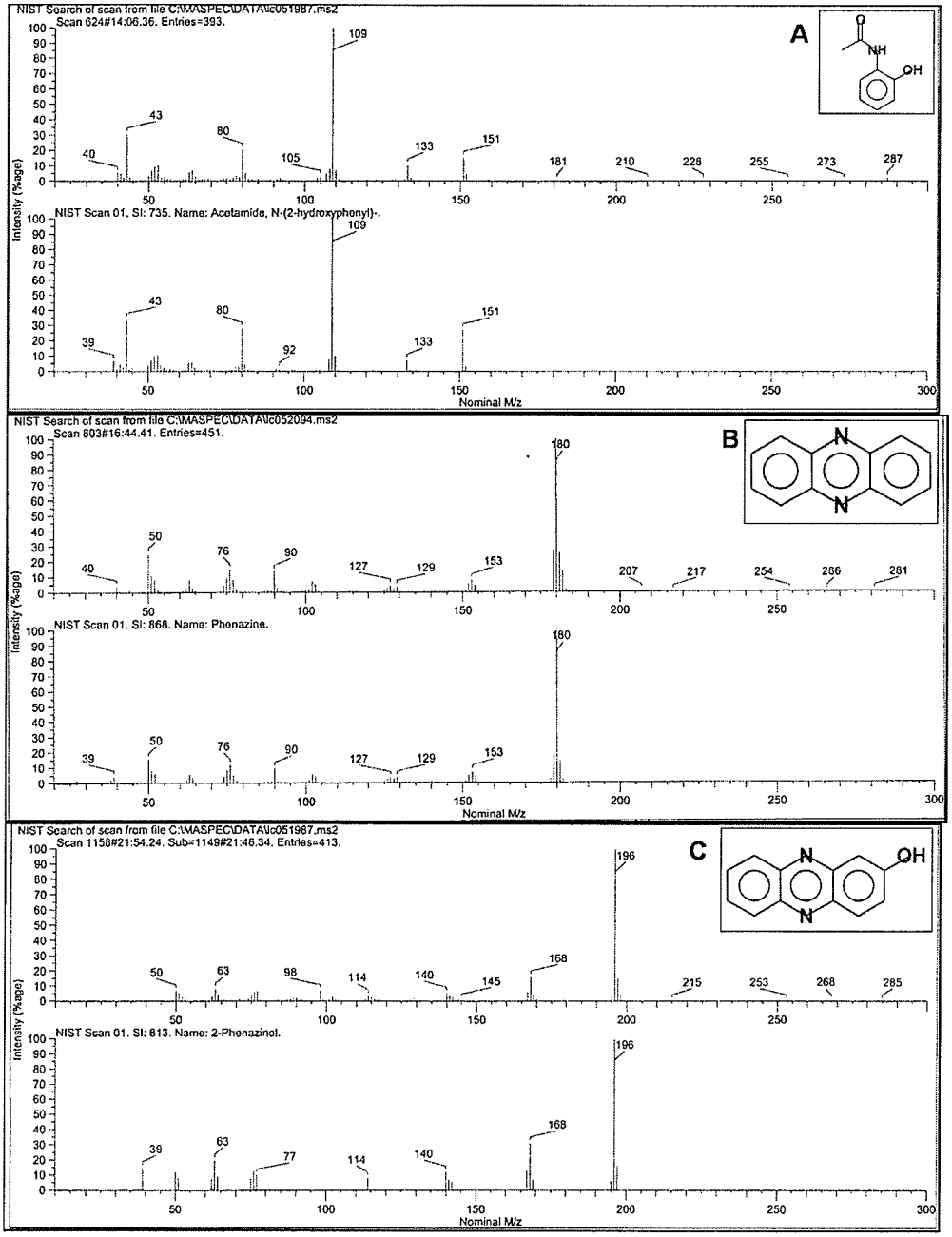

Figure 4.12. NIST-database search and identification of peaks corresponding to antifungal compounds present in broth of *Pseudomonas chlororaphis* strain DF190. A – 2-acetamidophenol (RT- 14:06.36 min); B – Phenazine (RT- 16:44.41min); C – 2-hydroxyphenazine (RT- 21:54.24 min).

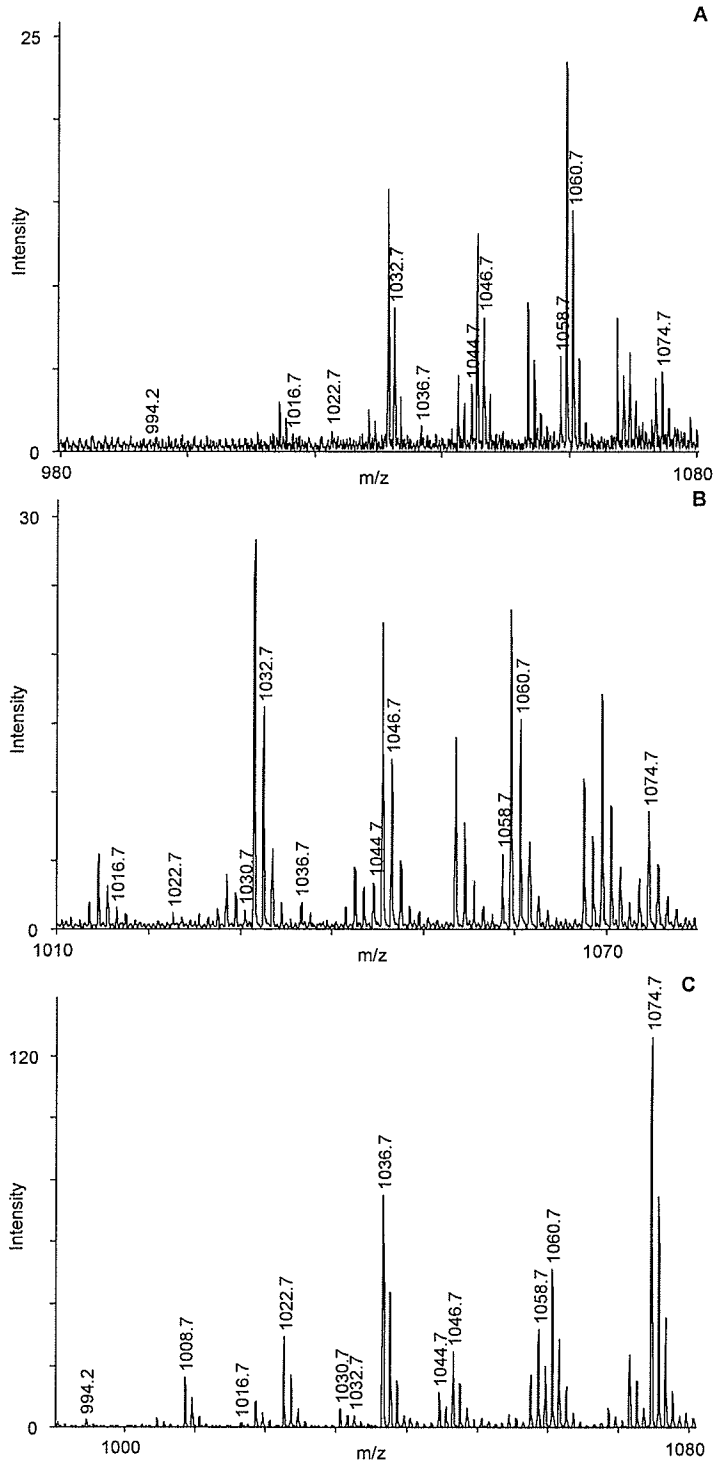

Figure 4.13. MALDI-TOF-MS mass spectra of bacterial cell-surface extracts analyzed for the presence of lipopeptide antibiotic surfactin. Surfactin peaks were observed in the (m/z) range of 990 – 1080. A – *Bacillus cereus* strain DFE4; B – *B. amyloliquefciens* strain DFE16; C- *B. subtilis* ATCC13952 (positive control).

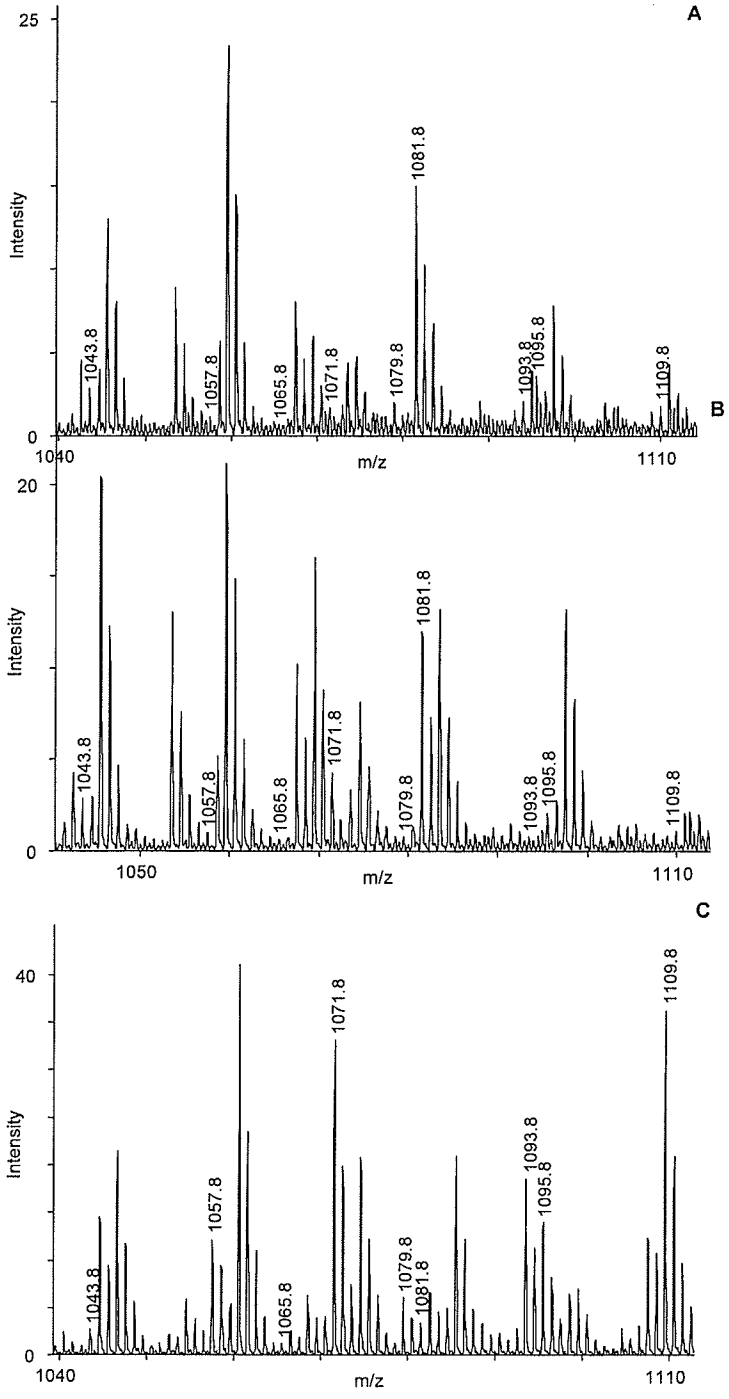

Figure 4.14. MALDI-TOF-MS mass spectra of bacterial cell-surface extracts analyzed for the presence of antifungal lipopeptide antibiotic iturin A. Iturin A peaks were observed in the (m/z) range of 1040 – 1110. A – *Bacillus cereus* strain DFE4; B – *B. amyloliquefciens* strain DFE16; C- *B. subtilis* ATCC13952 (positive control).

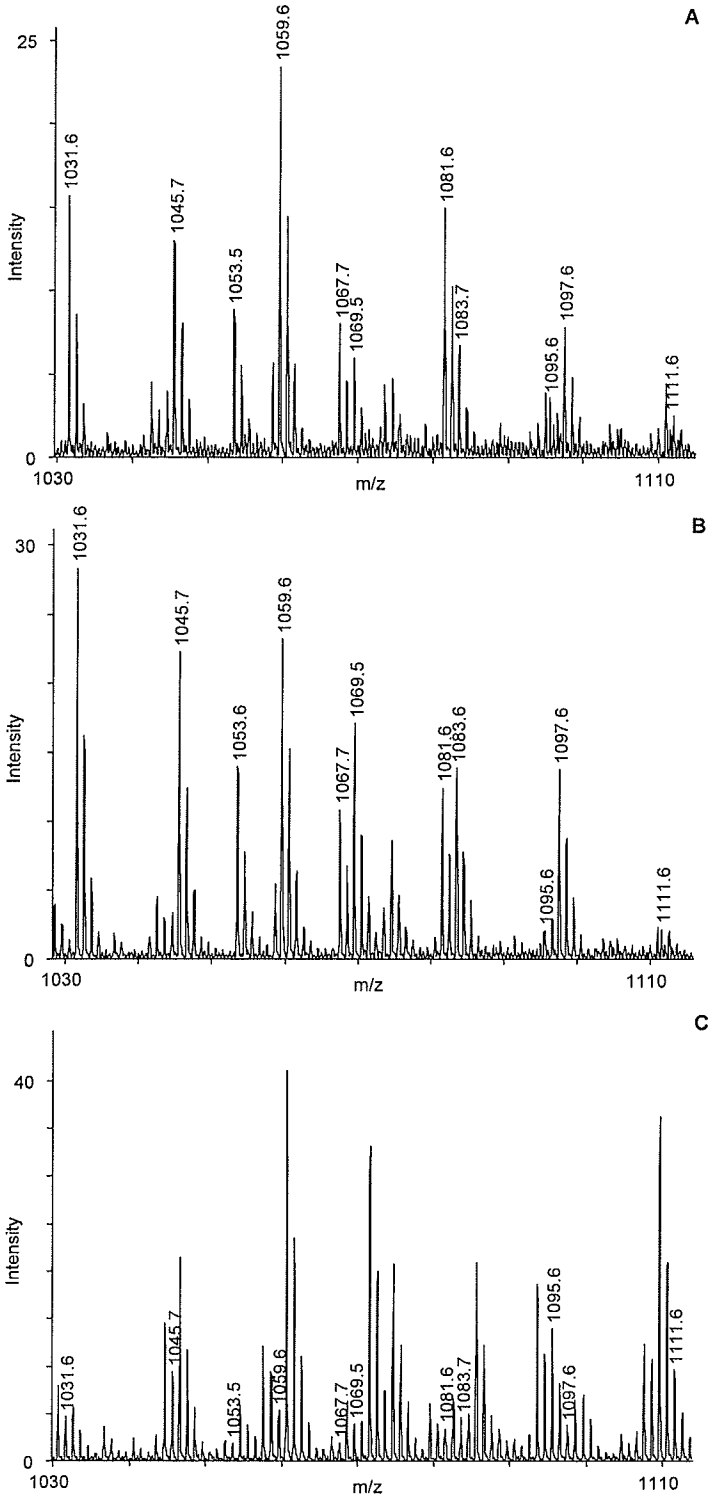

Figure 4.15. MALDI-TOF-MS mass spectra of bacterial cell-surface extracts analyzed for the presence of antifungal lipopeptide antibiotic bacillomycin D. Bacillomycin D peaks were observed in the (m/z) range of 1030 - 1115. A – *Bacillus cereus* strain DFE4; B – *B. amyloliquefciens* strain DFE16; C- *B. subtilis* strain B49 (positive control).

5.0 UNDERSTANDING THE ROLE OF ANTAGONISTIC BACTERIA-MEDIATED MECHANISMS IN BIOCONTROL OF BLACKLEG IN *BRASSICA NAPUS* L.

5.1. Abstract

In addition to antibiosis, hyperparasitism and competition, antagonistic bacteria also induce systemic resistance in the host plant for the successful biocontrol of plant pathogens. Bacterial siderophores, lipopolysaccharides, flagella and antibiotics have been shown to elicit induced systemic resistance (ISR). *Pseudomonas chlororaphis* strains DF190 and PA23, phenazine producers, and *Bacillus cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16, surfactin iturin A and bacillomycin D producers, were tested for elicitation of ISR in blackleg control. The crucial application time of the bacterial biocontrol agent was determined to be the inoculation of the bacteria 24 and 48 h prior to the application of the pathogen inoculum. This early application helps the bacteria colonize and establish itself in the wound tissue and facilitate the suppression of the pathogen pycnidiospores. The bacteria varied in their siderophore production, with DF190 and PA23 showing more siderophore activity than DFE4 and DFE16. The bacteria, when applied on a cotyledon different from the pathogen inoculated cotyledon, did not induce any systemic suppression of the blackleg lesion in the pathogen inoculated cotyledon. Similarly, the bacterial broth extracts, containing antifungal metabolites, also did not induce systemic resistance, when applied on a cotyledon different from the pathogen inoculated cotyledon. But, the bacterial broth extracts, when inoculated at the same wound site as the pathogen pycnidiospore significantly reduced the blackleg lesion development on the cotyledons to very low levels. To further ascertain the mechanism

involved in the localized suppression of the pathogen, localized induction of defense-related enzymes upon bacterial inoculation and pathogen challenge inoculation was tested. Strains DF190, DFE4 and DFE16 were tested for the induction of chitinase, β-1,3 glucanase and peroxidase activity. Except for strain DF190, which showed higher level of β-1,3 glucanase activity, when challenge inoculated with the pathogen, none of the other strains showed any positive induction of enzyme activity. All bacterial treatments had lower activity of chitinase and peroxidase. With establishment of the absence of systemic disease suppression and localized induction of defense enzymes, this study indicates the potential role of direct antifungal activity, both from the bacteria and the culture broth extract, as the mechanism of suppression of *L. maculans* at the infection site on the cotyledons. Tn5 mutagenesis generated a *gacS* mutant of strain PA23 that lost its antifungal and biocontrol activity. This was restored with the complementation of the *gacS* gene. Interestingly, a phenazine non-producing mutant of strain PA23, producing same levels of pyrrolnitrin as the wild type, exhibited same or better levels of blackleg disease suppression, when compared to the wild type. We postulate that phenazine may not be involved in the biocontrol of blackleg by *P. chlororaphis*, but pyrrolnitrin could mediate the antifungal activity of the bacterium towards the pathogen.

5.2. Introduction

In addition to direct antagonism, competition and hyperparasitism, non-pathogenic bacteria also mediate plant disease control through indirect mechanisms, such as induction of resistance in the host plant. Most of the work on induced systemic resistance (ISR), the term used for bacterially induced resistance (Pieterse et al 1996), has focused on non-pathogenic rhizosphere colonizing *Bacillus* and *Pseudomonas* species in systems where the inducing bacteria and the challenging pathogen remained spatially separated for the duration of the experiment, and no direct interaction between the bacteria and the pathogen was possible (Sticher et al 1997; Van Loon et al 1997). Prevention of direct interaction between the inducing-bacteria and the pathogen can be achieved through experimental methods such as, split root inoculations, as seen in control of *Fusarium oxysporum* in radish (Leeman et al 1996a), application as seed treatments for control of foliar pathogens (Ramamoorthy et al 2001; Van Loon et al 1998). Bacteria differ in their ability to induce resistance which seems to be determined by the host plant and also by variability within the host plant species (Van Loon 1997). *Pseudomonas putida* strain WCS358 induced resistance to *F. oxysporum* in *Arabidopsis*, but not in radish (Van Loon 1997). Similarly, *P. fluorescens* strain WCS417 induced systemic resistance in *Arabidopsis* ecotypes Colombia and Landsberg *erecta*, but no induction activity was seen in the ecotype RLD (Van Wees et al 1997).

Bacteria mediated ISR involves, elicitation of ISR pathway, generation of ISR signal, translocation of ISR signal, and ISR signal transduction leading to ISR-related gene expression and resistance (Pieterse et al 2001). Even though, the full range of inducing agents produced by bacteria is not yet known, lipopolysaccharides (LPS) of the

bacterial cell wall and iron-chelating siderophores have clearly been shown to elicit systemic resistance in plants (Whipps 2001). Most of the work relating to the role of LPS, siderophores and flagellin in inducing systemic resistance has been done with *Pseudomonas* species, and to our knowledge very little or none with *Bacillus* species. Among the *Pseudomonas* species, the elicitors of ISR are the fluorescent siderophore pseudobactin, the outer membrane lipopolysaccharide (LPS), and the flagella of *P. putida* WCS358 (Bakker et al., 2003; Meziane et al., 2005); the pseudobactin, other iron-regulated metabolites and LPS of *P. fluorescens* WCS374 (Leeman et alb., 1995, 1996), and the LPS and iron-regulated metabolites other than pseudobactin for *P. fluorescens* WCS417 (van Peer and Schippers, 1992; Leeman et al., 1995a, 1996). Other than the above mentioned elicitors, antifungal antibiotics such as 2,4-diacetylphloroglucinol and phenazine-1-carboxylic acid have been shown to induce ISR. Audenaert et al. (2002) demonstrated that phenazine-1-carboxylic acid and a siderophore, pyochelin, produced by *P. aeruginosa* strain 7NSK2 both contributed to the ability of this isolate to induce systemic resistance in tomato against *Botrytis cinerea*. In *Arabidopsis thaliana,* ISR by *P. fluorescens* strain CHA0 depends on the production of the antibiotic 2,4-diacetylphloroglucinol (Iavicoli et al., 2003). Among the lipopeptide antibiotics produced by *Bacillus* spp., treatment of potato tuber cells with purified fengycins resulted in the accumulation of some plant phenolics involved in or derived from the phenylpropanoid metabolism (Ongena et al 2005). Similarly, in a study by Ongena et al (2007) purified surfactin of *B. subtilis* S499 was shown to induce systemic resistance against B. cinerea in bean and tomato plants. In tomato cells, key enzymes of the lipoxygenase pathway appeared to be activated in resistant plants following induction by surfactin

overproducers. Also, with respect to canola, a double application of *P. chlororaphis* strain PA23 on canola petals induced the activity of chitinase and β-1,3 glucanase leading to the suppression of *Sclerotinia sclerotiorum* (Fernando et al 2007). ISR expression functions through the wound signaling intermediates jasmonic acid (JA) and ethylene (ET), not through local and systemic changes in the production of these signal molecules, but rather with an increased sensitivity to these hormones. ISR-expressing plants are primed to react faster and more strongly to JA and ET produced as a result of pathogen infection (Van Loon et al 1998; Van Wees et al 1999; Conrath et al 2002).

Initial evidence for the role of antibiotics in the suppression of the pathogen was observed when the culture filtrates or the purified antibiotics provided similar levels of control as achieved by the producing wild-type strain (Kang et al 1998; Nakayama et al 1999). Mutagenesis has been successfully used to demonstrate the role of antibiotics in biological control. The inactivation of antibiotic production by mutagenesis has resulted in the reduction of antagonistic activity by the mutants as compared to the wild-type. The re-complementation of the wild-type DNA not only restored production but also, the antifungal activity, thus demonstrating the role of antibiotics in biocontrol of the pathogen. Mutagenesis has been used for the establishment of the role of: phenazine-1-carboxylic acid in the control of *G. graminis* var *tritici* (Thomashow et al 1988); phenazine-1-carboxamide in the control of *F. oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng et al 1998); zwittermicin A in the control of *P. medicaginis* (Silo-Suh et al 1994). The GacS/GacA system controls the expression of genes required for the synthesis of secondary metabolites, such as antibiotics, in many plant-associated fluorescent

Pseudomonas species. GacS/GacA global two-component regulatory systems are comprised of a membrane-bound environmental sensor, GacS, and a transcriptional response regulator, GacA. Antibiotics like, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol are regulated directly or indirectly by this system, as well as hydrogen cyanide, chitinase, and exoproteases (Chancey et al 2002). Mutation in the *gacS* region of biocontrol strains *P. chlororaphis* and *P. aureofaciens* led to deficiency in secondary metabolite production and loss of biocontrol activity (Schmidt-Eisenlohr et al 2003; Chancey et al 2002). Similar effects were also observed in the *gacS* mutants of the PRN producer *P. fluorescens* BL915 (Ligon et al 2000).

Identifying different mechanisms of action enables to hit pathogens with a broader spectrum of microbial weapons, and facilitate the combination of strains, bacteria with bacteria or bacteria with fungi (de Boer et al 1999, Olivain et al 2004). We selected four potential antagonistic bacterial strains, *P. chlororaphis* strains PA23 (PA23) and DF190 (DF190), phenazine producers, and *B. cereus* strain DFE4 (DFE4) and *B. amyloliquefaciens* strain DFE16 (DFE16), surfactin, iturin A and bacillomycin D producers, to be tested for their ability to induce systemic resistance towards *Leptosphaeria maculans* in canola. *P. chlororaphis* strain PA23, earlier exhibited *in vitro* antifungal activity (plate assays on PDA) – percentage mycelial inhibition of 96.75%, and *in vivo* disease suppression (Westar cotyledon assays) – IP rating of 1.8 (not presented in this thesis (chapter 3)). Also, strain PA23 tested positive both for the presence of antibiotic biosynthetic genes of phenazine and pyrrolnitrin, and the biochemical detection for the production of these compounds (Zhang et al 2006). Hence, strain PA23 was used in this study along with strain DF190 for the induced resistance and direct antifungal

activity experiments. The study demonstrated the absence of induced systemic resistance elicitation by the antibiotic producing bacteria, and the involvement of direct antifungal activity in disease suppression. Direct antifungal activity could result from the production of antifungal metabolites and lytic enzymes, whose production of controlled by the GacS/GacA global regulatory system. Tn5 mutagenesis was used to generate *gacS* mutants of *P. chlororaphis* strain PA23, which demonstrated the inability of the *gacS* mutants in the in vivo suppression of *L. maculans*, as compared to the wild type and the complemented strain. This established the role of direct antifungal activity mediated by the antagonistic bacteria in blackleg suppression in canola cotyledons.

5.3. Materials and Methods

5.3.1. General growth conditions

The bacteria, stored in -80 °C, were streaked onto a Luria Bertani agar (LBA) plate and incubated at 28 °C for strains PA23 and DF190, and 32 °C for strains DFE4 and DFE16. For the plant assays, 5 ml of LBB was inoculated with a bacterial loop and incubated at 28 °C (PA23 and DF190) and 32 °C (DFE4 and DFE16) for 16 – 18h at 180 rev min^{-1} in an incubator shaker (Jeio Tech SI-600, Seoul-city, S. Korea). The bacterial cells from 16-18 h cultures were transferred to a 15 ml centrifuge tube and pelleted at 7000 rpm for 15 mins. The bacterial pellets were washed once and re-suspended in sterile distilled water. Ten microlitres of the bacterial suspension ($1x10^8$ CFU/mL) was used as inoculum in the plant assays.

The fungal isolate *L. maculans* (PL 86-12) from pathogenicity group 2 was stored as a concentrated pycnidiospore suspension in sterile water at -20°C. The inoculum was

prepared by thawing and adding a few drops of the stock to sterile distilled water. The spore concentration was adjusted to $2x10^7$ spores/mL. Ten microlitres of the pycnidiospore suspension was used as the fungal inoculum in the plant assays.

Cotyledons of *B. napus* cv Westar were used for the assays. The cotyledons were grown in METRO-MIX® contained in S806 "T" inserts that were placed in rectangular trays. The plants were grown in a controlled growthroom and incubated at 22/18° C day/night and 16/8 h photoperiod (280 μE m^{-2} s^{-1}) with daily watering. The 7-8d old seedlings were wounded with forceps, one wound per cotyledon lobe.

5.3.2. Effect of time of inoculation of the bacteria on the suppression of pycnidiospores of *L. maculans*

Bacterial strains PA23. DF190 and DFE4 were tested in this experiment. Ten microlitres of the bacterial suspension ($1x10^8$ CFU/mL) was inoculated on wounded canola cotyledons of the cultivar Westar. Fungal inoculum (10 μL suspension of pycnidiospores ($2x10^7$ spores/mL)) was placed 24 h following the bacterial inoculation. In this experiment, both the bacteria and the pathogen were inoculated in the same wound spot (local/single point inoculation (SPI)). The following are the different treatments, which indicate the sequence of inoculation of the bacteria and the pathogen:

1. Bacteria inoculated 24 h prior to inoculation of the pathogen.
2. Bacteria inoculated 48 h prior to inoculation of the pathogen.
3. Bacteria and pathogen inoculated at the same time (co-inoculation).
4. Pathogen inoculated 24 h prior to inoculation of bacteria.
5. Pathogen inoculated 48 h prior to inoculation of bacteria.

6. Control – Sterile water inoculated in place of the bacteria along with the pathogen, following the same sequence of inoculation.

The seedlings were left on the counter to allow the pycnidiopore suspension to dry (6-8 h) and were then placed in a controlled growthroom and incubated at conditions mentioned above. Emerging true leaves were removed to maintain nutrienta at the cotyledons, until cotyledons were scored for the disease levels, using an interaction phenotype (IP) scale of 0-9, 10-12 days post-pycnidiospore inoculation. Each treatment consisted of 12 cotyledon plants and the experiment was repeated once.

5.3.3. Assay for Siderophore Production

Siderophore production was assayed by spotting 5 μL of overnight culture of the bacteria on Chrome azurol S agar (Schwyn and Neilands 1987) plates. The plates were incubated for 16 h at 28 °C. The production of siderophores was indicated by the conversion of the blue agar to an orange halo around the bacterial colony. The FeIII attached to the dye when cleaved off by a strong iron chelator (siderophore) converts the color of the dye from blue to orange.

5.3.4. Effect of split inoculation of bacterial cells on the suppression of *L. maculans* on cotyledons

Bacterial strains PA23. DF190, DFE4 and DFE16 were tested in this experiment for their ability to induce ISR for suppression of *L. maculans*. Ten microlitres of the bacterial suspension ($1x10^8$ CFU/mL) was inoculated on one wounded cotyledon. Ten microlitres of the pathogen pycnidiospore suspension ($2x10^7$ spores/mL) was placed, 24 h post bacterial inoculation, on the other cotyledon that was freshly wounded. The seedlings were left on the counter to allow the pycnidiopore suspension to dry (6-8 h) and were

then placed in a controlled growthroom and incubated at conditions mentioned above. Disease severity was scored as described above. Each treatment consisted of 12 cotyledon plants and the experiment was repeated once.

5.3.5. Effect of split and local inoculation of culture broth extracts on the suppression of *L. maculans*

Two bacterial strains, PA23 (phenazine and pyrrolnitrin producer) and DFE4 (iturin A, bacillomycin D and surfactin producer) were used in this experiment. The split inoculation was performed to check if broth extracts were capable of inducing ISR, and the local inoculation (SPI) was performed to check if the broth extracts had direct antifungal activity leading to the suppression of the pycnidiospores of the pathogen. The effect of the age of culture broth was tested for pathogen suppression in both types of inoculations, as antifungal metabolites accumulate in the broth over a period of time (2 – 5 days post inoculation of bacteria. Five milliliter of start culture for each bacteria was initiated in 15 mL culture tubes, incubated at 28 °C (PA23) and 32 °C (DFE4). A sample (100μL) from the overnight cultures was used to inoculate 5 tubes each containing 10 mL of LBB, and each representing the age of the culture at 1, 2, 3, 4 and 5 days, respectively. The tubes were incubated as above and removed for extraction on the specified day. The cells were pelleted and the bacterial culture broths were extracted with an equal volume of chloroform. The chloroform extracts were dried under a nitrogen stream and re-suspended in 5 mL water:methanol (50:50) mixture. The control was plain LBB extracted and suspended in water:methanol mixture. For the SPI, 10 μL of the broth extract was inoculated on a wounded Westar cotyledon leaf and 10 μL of pathogen pycnidiospore suspension ($2x10^7$spores/mL) was inoculated in the same spot 24 h later. For the split

inoculation, 10 µL of the broth extract was inoculated on one of the wounded Westar cotyledon leaves, while 10 µL of pathogen pycnidiospore suspension was inoculated on the other freshly wounded cotyledon leaf 24 h later. The seedlings were left on the counter to allow the pycnidiospore suspension to dry (6-8 h) and were handled for disease rating, as described previously. Each treatment consisted of 12 cotyledon plants and the experiment was repeated once.

5.3.6. Data Analysis

For the plant assays, analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference), at P = 0.05, were performed, using The Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, USA).

5.3.7. Assays for induction of pathogenesis-related enzyme activity upon single point inoculation of bacterial cells and pycnidiospores of *L. maculans*

Single point inoculation of the bacteria or bacterial broth, and pathogen pycnidiospore, bacteria or bacterial broth inoculated 24 h prior to pathogen, suppressed disease. This led us to investigate if the localized suppression of the pathogen was due to the direct antifungal activity of the bacteria or localized induction of plant pathogenesis-related (PR) proteins by the bacteria. One week old Westar cotyledons were inoculated and assayed for the activity of PR enzyme activity. The treatments included:1) bacteria alone; 2) bacteria inoculated with the pathogen 24 h post bacterial inoculation; 3) pathogen alone and 4) uninoculated control (water). The bacterial and pathogen inoculum was prepared as mentioned earlier in section 6.3.1. Ten microlitre each of the bacterial (1 x 10^8CFU/mL) and pycinidiospore (2 x 10^7 spores/mL) suspension was used as the respective inoculums. Twelve cotyledons per treatment were collected at time intervals of

0, 24, 48, 72, 96 and 120 h post-bacterial inoculation for treatment 1 and at the same time intervals post-.pathogen inoculation for treatments 2, 3, and 4. The samples were collected in plastic bags and stored in -80 °C, The cotyledon samples were removed from -80 °C and homogenized with liquid nitrogen using a cold mortar and pestle. The powdered samples were stored in -80 °C until assayed.

5.3.7.1. Chitinase Assay

One gram of the powdered cotyledon tissue was ground in 1 ml of 0.1 M sodium citrate buffer (pH 5) at 4 °C. The homogenate was transferred to a 1.5 mL Eppendorf centrifuge tube and centrifuged at 4 °C for 10 min at 10, 000g. The chitinase activity was determined following the colorimetric method of Boller and Mauch (1988). The chitinase activity was estimated by the release of N acetyl glucosamine (GlcNac) from the substrate, colloidal chitin, and expressed as nmol of equivalent GlcNac/min/mg protein.

5.3.7.2. β-1,3 Glucanase activity

One gram of the powdered cotyledon tissue was ground with 1 mL of 0.05M sodium acetate buffer (pH 5) at 4 °C. The homogenate was allowed to react with 0.4 % laminarin (EC2327124, Sigma) for 10 min at 40 °C. The colorimetric estimation of the release of reducing glucose equivalent from laminarin was performed as described by McCleary and Shameer (1987), and β-1,3 glucanase activity was expressed as μg of equivalent glucose/min/mg of protein.

5.3.7.3. Peroxidase activity

One gram of the powdered cotyledon tissue was ground in 1 mL of 0.1 M phosphate buffer (pH 7) using a cold pestle and mortar (4 °C). The homogenate was

transferred to a 1.5 mL centrifuge tube and centrifuged at 15,000 g at 4 °C for 15 min, and the supernatant was immediately used for the assay. Peroxidase activity was determined using pyrogallol (EC210-762-9 Sigma) as the hydrogen donor and measuring the rate of color development colorimetrically at 420 nm at 30 s intervals for 3 min (Hammerschmidt et al 1982). The peroxidase activity was expressed as change in absorbance/min/mg protein.

5.3.8 Biocontrol of *L. maculans* using Tn5 mutants of *P. chlororaphis* strain PA23

Tn5 mutagenesis work was carried out by Nicole Poritsanos, a Masters student in Dr. Teri DeKevit's lab, at the Department of Microbiology, University of Manitoba. *Pseudomonas chlororaphis* strain PA23 was used for the Tn5 mutagenesis work and mutants generated through insertion of the Tn5-OT182 transposon (Poritsanos 2005). The bacterial strains used for the plant assays are: *P. chlororaphis* strain PA23 – wild type (produces phenazines, pyrrolnitrin, autoinducer and siderophores); mutant PA23–314 – Tn5 insertion in the *gacS* gene (lack of phenazine, pyrrolnitrin, autoindcer, and extracellular enzyme production; but enhanced production of siderophores); recomplemented mutant PA23-314*gacS* – recomplemented with the *gacS* gene from the wild type using the pUCP23 vector (restoration of phenazine, pyrrolnitrin and autoinducer, extrcacellular enzymes levels equal to that of wild type); mutant PA23-63 – Tn5 insertion in the *phzE* gene of the phenazine biosynthetic operon (lack of phenazine production; pyrrolnitrin production similar to wild type levels; siderophore production same as wild type); strains PA23-wildtype with pUCP23 and mutant 314-pUCP23 were used as controls. Bacteria were cultured in LBB, amended with the following antibiotic: PA23-wildtype – rifampicin (25μg/mL); strains PA23-pUCP23, PA23-314-pUCP23 and

PA23-314*gacS* – gentamycin (25μg/mL); strains PA23-314 and PA23-63 – tetracycline (15μg/mL). Westar cotyledons, 7-8 days old, were wound inoculated with 10 μL of bacterial suspension (1 x 10^8 CFU/mL) followed by 10 μL of pycnidiospore suspension (2 x 10^7 spores/mL) 24 h post bacterial inoculation. The growing and incubation conditions were the same as mentioned in previous chapters. The plants were scored 10-12 days post-pycnidiospore inoculation. Each treatment consisted of 12 cotyledon plants and the experiment was repeated twice. Analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference), at P = 0.05, were performed, using The Analyst procedure of SAS, Version 8.1 (SAS Institute, Cary, NC, USA).

5.4. Results

5.4.1. Effect of time of inoculation of bacteria on the suppression of pycnidiospores of *L. maculans*

The effect of time of bacterial inoculation was tested at 24 and 48 h pre- and post-pycnidiospore inoculation, and co-inoculation. The pre-pycnidiospore inoculation of the bacteria at 24 and 48 h was the most effective for the suppression of pycnidiospores of *L. maculans* and prevention of blackleg lesions on cotyledons (Figure 5.1). The pre-pycnidiospore inoculation of the bacterial strains DF190, PA23 and DFE4 had IP rating values <2 (resistant rating), and were significantly different and lower than the co-inoculation, post-pycnidiospore bacterial inoculation and control treatments (Figure 5.1; Figure 5.2- A, B and C). The IP ratings for the co-inoculation, post-pycnidiospore bacterial inoculation and the respective control treatments ranged from 6.2 to 8.9 (Figure 5.1), which were moderately to highly susceptible disease rating.

5.4.2. Assay for Siderophore Production

Bacterial strains DF190, PA23, DFE4 and DFE16 were tested for the production of siderophores on CAS agar plates. All four strains exhibited siderophore production, with strains DF190 and PA23 (Figure 5.3 - A and B, respectively) showing more siderophore production than strains DFE4 and DFE16 (Figure 5.3 – C and D, respectively).

5.4.3. Effect of split inoculation of bacterial cells on suppression of *L. maculans* on cotyledons

Living cells of bacterial strains DF190, PA23, DFE4 and DFE16 were tested for their ability to induce ISR, when split inoculated on a cotyledon different from that of the pathogen inoculated cotyledon. Although, the split inoculated bacterial treatments had significantly lower disease levels than the control, their IP ratings ranged from 6.4 to 7.6 (Figure 5.4). The pathogen control had an IP rating of 8.8. IP ratings >6 are considered as susceptible ratings on the disease scale and hence, the split inoculated bacterial treatments were incapable of disease suppression on the cotyledons (Figure 5.5).

5.4.4. Effect of split and local inoculation of culture broth extracts on the suppression of *L. maculans*

The effect of split (inducing ISR) and local inoculation (direct antifungal activity) of culture broth, with respect to the age of the broth, of strains PA23 and DFE4 were tested for suppression of *L. maculans*. Since the focus of this experiment was on the antifungal metabolites, which accumulate in the culture broth, for their ability to induce ISR or direct antifungal activity, strains PA23 and DFE4 were selected as representatives

of the phenazine and pyrrolnitrin producers and lipopeptide antibiotics producers, respectively.

In the split inoculation assays, bacterial broth treatments - PA23 (D3, D2, D5, D4) and DFE4 (D3 and D5) were significantly different from the control treatment with regards to their disease IP ratings (Figure 5.6). The rest of the broth treatments were not significantly different from the control. All the split bacterial broth inoculation had IP ratings ranging from 6.5-7.9 (Figure 5.7), which are considered as susceptible disease ratings incapable of inducing ISR for the suppression of the pycnidiospores and disease lesion on cotyledons.

In the local (SPI) inoculation assays, all bacterial broth treatments of PA23 and DFE4 (D1, D2, D3, D4, D5) had significantly lower disease levels than the control (Figure 5.8). The bacterial treatments had disease IP ratings ranging from 0.3 – 2.8, which are considered resistant IP ratings as compared to the control IP rating of 8.4 (Figure 5.9). Four and five day-old bacterial broth extracts exhibited more antifungal activity, as seen in the low disease levels on the cotyledons.

5.4.5. Assays for induction of pathogenesis-related enzyme activity upon single point inoculation of bacterial cells and pycnidiospores of *L. maculans*

5.4.5.1. Chitinase Assay

Bacterial strains DF190, DFE4 and DFE16 were tested for localized induction of chitinase enzyme activity at the site of inoculation for the suppression of *L. maculans*. None of the bacterial treatments, either alone or challenge inoculated with the pathogen, induced chitinase activity. All treatments, including the pathogen, had chitinase activity

levels less than the water control at 48, 72, 96 and 120 h after inoculation of the pathogen (Figure 5.10).

5.4.5.2. β-1,3 Glucanase activity

Bacterial strains DF190, DFE4 and DFE16 were tested for localized induction of β-1,3 glucanase enzyme activity at the site of inoculation. Of the three bacterial strains, only DF190 when challenge inoculated with the pathogen induced higher levels of β-1,3 glucanase activity at 48, 72, 96 and 120 h post-pathogen inoculation, as compared to the pathogen and water control (Figure 5.11A). The activity peaked at 72 and 96 h after pathogen inoculation. The enzyme activity for strain DFE4, when inoculated alone or with the pathogen, was always lower than the water control (Figure 5.11B). Strain DFE16 inoculated alone had higher enzyme activity than other treatments at 48 and 96 h, but the bacterial treatment alone or when challenge inoculated with the pathogen at other time intervals had enzyme activity lower than the water control (Figure 5.11C).

5.4.5.3. Peroxidase activity

Bacterial strains DF190, DFE4 and DFE16 were tested for localized induction of peroxidase enzyme activity at the site of inoculation for the suppression of pycnidiospores of *L. maculans*. All treatments, bacteria inoculated alone, bacteria challenged with the pathogen and pathogen alone had peroxidase activity levels greater than the water control. For strain DF190, inoculated alone, peroxidase activity was higher than other treatments at 48, 72 and 96 h. The bacterium, when challenged with the pathogen, had enzyme activity at its highest than other treatments at 120 h (Figure 5.12 A). The enzyme activity for strain DFE4, when challenge inoculated with the pathogen, was higher than the other treatments at 48 and 96 h (Figure 5.12 B). Strain DFE16, when

inoculated alone had peroxidase activity higher than other treatments at 48 and 72 h, and when challenged with the pathogen the activity peaked at 96 h (Figure 5.12 C). The overall peroxidase activity in all the treatments was low (<0.8).

5.4.6 Biocontrol of *L. maculans* using Tn5 mutants of *P. chlororaphis* strain PA23

Tn5 mutants of *P. chlororaphis* strain PA23 were assayed on canola cotyledons for the suppression of the pycnidiospores of *L. maculans*. The treatments with PA23-wild type, PA23-pUCP23, PA23-314*gacS* complemented and PA23-63-phenazine mutant had significantly lower disease levels than the pathogen control, and treatments with PA23-314 (*gacS* mutant) and PA23-314pUCP23 (Figure 5.13). The disease suppressive treatments of PA23-wild type (Figure 5.14A), PA23-pUCP23 (Figure 5.14B), PA23-314*gacS* complemented (Figure 5.14D) and PA23-63-phenazine mutant (Figure 5.14E) had IP ratings of 1.5, 1.8, 1, and 0.6, respectively. The pathogen control (Figure 5.14F) had an IP rating of 8.6, and the mutant treatments PA23-314 (*gacS* mutant) (Figure 5.14C) and PA23-314pUCP23, though significantly different from the pathogen control, had highly susceptible ratings of 8 and 7.8, respectively.

5.5. Discussion

This study examined the ability of the antibiotic producing bacterial antagonists to induce systemic resistance towards the suppression of pycnidiospores and blackleg disease lesions on cotyledons of canola. Lack of ISR and role of direct antifungal activity was established towards the control of the pathogen. The correct timing of application of the biocontrol agent, suitable application strategy and establishment of the biocontrol agent at the target area are the critical elements that determine the success of management

of plant pathogens through biocontrol (Baker and Cook 1974; Campbell 1989; Weller 1988). In our experiment, the application of the bacteria 24 or 48 h prior to the application of the pathogen seem to be an important factor in the prevention of blackleg lesions on the cotyledons, thus stressing the importance of the time of application. Also, the early application seems to provide the bacteria with ample time for the establishment of its population number and successful colonization of the infection court of the pathogen, which helps in prevention of the germination of pycnidiospores and thus, the penetration and establishment of the pathogen in the host. This is also supported by our results, which shows that co-inoculation or post-pycnidiospore inoculation of the bacteria did not suppress the disease in the cotyledon, as the pathogen pycnidiospores could have germinated and penetrated the wound area before the establishment and colonization by the bacteria. The blackleg pathogen needs natural or wound openings for the successful penetration and colonization of the host (Hammond et al 1985). The establishment and colonization of the biocontrol bacteria in the wound tissue presumably could have prevented the germination, penetration and establishment of the pathogen in the cotyledon. Earlier studies by Bull et al (1991) on the colonization of root by *P. fluorescens* in the suppression of *Gaeumannomyces graminis* var. *tritici,* and Parke et al (1990) on the successful colonization of the pea spermosphere in the prevention of *Pythium* infection, are good examples that stress the importance of colonization of biocontrol agents at the target site as a prerequisite for suppression of plant pathogens.

In our study, we inoculated the bacteria on one cotyledon and the pathogen on the other cotyledon to test for the induction of ISR by the bacteria. Similar phyllosphere inoculation studies have been carried out earlier for the induction of ISR with *B.*

mycoides strain Bac J for the control of *Cercospora* leaf spot in sugar beet (Bargabus et al 2002), and *P. putida* WCS358r and *P. fluorescens* WCS374r for the control of bacterial wilt caused by *Ralstonia solanacearum* in *Eucalyptus urophylla* (Ran et al 2005). The fluorescent siderophore pseudobactin, the outer membrane lipopolysaccharide (LPS), and the flagella of *P. putida* WCS358 (Bakker et al., 2003; Meziane et al., 2005) have been demonstrated as ISR inducers. Siderophore production in *Bacillus* spp. has not been looked into in detail as in the *Pseudomonas* spp. Recently, *Bacillus cereus* was demonstrated to produce catechol type siderophores (Park et al 2005), but the role of siderophore production by *Bacillus* spp. in inducing ISR has not been established yet. In our study, the two strains of *P. chlororaphis*, PA23 and DF190, showed higher siderophore production levels (larger orange halo around the bacterial colony) than the *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16. *P. chlororaphis* strains are known to produce pyoverdine, ferribactin and azobactin-type siderophores (Hohlneicher et al 1995). In our study, none of the bacteria, when split inoculated on a different cotyledon other than the pathogen inoculated cotyledon, induced ISR for the suppression of *L. maculans*. With regards to the *P. chlororaphis* strains it could be possible that siderophores are not involved in the induction of ISR in the *L. maculans* –canola system, or that the siderophore-type that could be involved in the induction of ISR is not produced by these strains, as the structures and type of siderophore produced is species or even strain specific (Hohlneicher et al 1995). As for strains DFE4 and DFE16, their siderophore production on CAS plates was very low, and presumably were either not produced in the plant leaf surface or produced at levels not sufficient for the induction of any ISR. From our study, the LPS (DF190, PA23) or the peptidoglycan (DFE4 and

DFE16) also did not play any role in induction of ISR as the bacterial cells did not induce any systemic resistance.

Other than the role of siderophores, LPS and flagella of as bacterial determinants of ISR, antifungal antibiotic metabolites produced by bacteria have also been shown to induce ISR. Examples of ISR inducing antibiotics are phenazine-1-carboxylic acid (Audenaert et al. 2002), 2,4-diacetylphloroglucinol (Iavicoli et al., 2003), and lipopeptide antibiotics fengycin (Ongena et al 2005) and surfactin (Ongena et al 2007). Identifying different mechanisms of action enables to hit pathogens with a broader spectrum of microbial weapons, and facilitate the combination of strains, bacteria with bacteria or bacteria with fungi (de Boer et al 1999, Olivain et al 2004). From our earlier study, *P. chlororaphis* strains PA23 and DF190 were shown to produce phenazines, and *B. cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16 were identified as producers of lipopeptide antibiotics iturin A, bacillomycin D and surfactin. Strains PA23 and DFE4 were selected as representative of each bacterial antibiotic group, and tested for mode of action of the bacterial broth extracts through split (for induction of ISR) and local (for direct antagonism) inoculations. The split inoculation of the bacterial broth did not induce a resistance reaction for suppression of blackleg lesion on the cotyledon, which was consistent with the split inoculation of the bacterial cells. The local (SPI) inoculation of the broths significantly reduced the disease levels, which is also consistent with the SPI of the bacterial cells, establishing a possible role of the antifungal metabolites present in the broth for the direct suppression of *L. maculans*. To further ascertain the mechanism involved in the localized suppression of the disease, the activity of PR-enzymes was studied. None of the bacterial treatments induced chitinase activity, but seemed to repress

enzyme activity as compared to the water control. Similarly, β-1, 3 glucanase activity, except for strain DF190, was not induced for strains DFE4 and DFE16. Also, none of the bacterial strains had a distinct induction of peroxidase when challenge-inoculated with the pathogen. This lack of localized induction of chitinase, β-1, 3 glucanase (except for DF190), and peroxidase indicates that these enzymes may not be involved in the suppression of *L. maculans* at the site of infection. In this case, the "priming" effect, which is the enhanced capacity of induced tissues for rapid and effective activation of cellular defense responses after infection with a challenging pathogen (Conrath et al 2002), characteristic of bacteria mediated ISR (Hase et al 2003), seems to be absent in the control of blackleg in cotyledon of canola. The lack of systemic induction of resistance, both by the bacterial cells and broth extracts, and the lack of localized induction of PR-enzymes by bacterial cells, indicates the lack of induced resistance in the control of blackleg in the cotyledons of canola. This suggests the potential of these bacteria to exhibit direct antifungal activity at the site of infection. Direct antifungal activity could result from the production of antibiotics or extracellular lytic enzymes. *L. maculans* can cause infection of leaf only through penetration of natural openings or wounds, which act as the sites of infection (Hammond et al 1985). Wounds and natural openings of the leaf are nutrient rich microenvironments, which favor the production of bacterial secondary metabolites, like antibiotics (Thomashow et al 1997). The localized inhibition of the pycnidiospores by the bacteria is enabled by successful colonization of the infection site, which in turn most probably acts as a suitable delivery system for the antifungal metabolites. This phenomenon was observed in the suppression of *Fusarium oxysporum*

f. sp. radicis-lycopersici by the successful root colonization and production of phenazine-1-carboxamide by *P. chlororaphis* PCL1391 (Chin-A-Woeng et al 2000).

The GacS/GacA system controls the expression of genes required for the synthesis of secondary metabolites, such as antibiotics, in many plant-associated fluorescent *Pseudomonas* species. Antibiotics like, phenazines, pyrrolnitrin, 2,4-diacetylphloroglucinol are regulated directly or indirectly by this system, as well as hydrogen cyanide, chitinase, and exoproteases (Chancey et al 2002). Mutation in the *gacS* region of biocontrol strains, *P. fluorescens* BL915, pyrrolnitrin producer and *P. chlororaphis,* phenazine producer, led to deficiency in secondary metabolite production and loss of biocontrol activity (Ligon et al 2000; Schmidt-Eisenlohr et al 2003). Similarly, with regards to *P. chlororaphis* strain PA23, the *gacS* mutation led to the deficiency in secondary metabolite production and inability to control *S. sclerotiorum* in canola (Poritsanos et al 2006). Similar results were observed in our study as well. Mutation in the *gacS* gene, led to loss of biocontrol activity in the mutant PA23-314 as compared to the wild type strain PA23. Mutant PA23-314 was incapable of suppressing the pycnidiospores of *L. maculans* and development of blackleg lesion in canola cotyledons. Mutant PA23-314 exhibited enhanced siderophore production, as compared to the wild type (Poritsanos 2005). This phenomenon was earlier observed in *P. fluorescens* strain CHA0, where *gacS* and *gacA* mutations led to the increased production of pyochelin and pyoverdin siderophores (Duffy and Défago 2000; Schmidli-Sacherer et al. 1997). This enhanced siderophore production and lack of biocontrol activity in PA23-314 rules out the role of siderophores in the biocontrol of *L. maculans* by strain PA23. The antifungal activity and biocontrol ability was restored when the *gacS* gene from the

wild type was complemented in mutant PA23-314. The complemented mutant PA23-314*gacS* exhibited enhanced antifungal activity and biocontrol ability probably due to the insertion of multiple copies of the *gacS* gene. Ligon et al (2000) introduced multiple copies of the *gacA* gene into *P. fluorescens* strain BL915 resulting in a 2.5 fold increase in pyrrolnitrin production. We presume that the introduction of multiple copies in PA23-314*gacS* could have resulted in the enhanced production of antifungal metabolites and lytic enzymes, and therefore the enhanced biocontrol activity. The phenazine mutant PA23-63 exhibited antifungal and biocontrol activity similar to that of the wild type. Mutant PA23-63 also produced pyrrolnitrin at levels equal to that of the wild type (Dr. Paulitz, personal communication). The biocontrol activity mediated by mutant PA23-63, despite its lack of production of phenazine, establishes the lack of role for phenazine in the biocontrol of *L. maculans*. It's possible that even in the wild type, which produces both phenazine and pyrrolnitrin, no synergistic activity between phenazine and pyrrolnitrin exists. We postulate that pyrrolnitrin and extracellular lytic enzymes could be responsible for the antifungal activity of *P. chlororaphis* strain PA23 towards *L. maculans*. We presume that the inability of phenazine to control *L. maculans* could be due to the resistance mechanism of the fungus. In a study by Schoonbeek et al (2002), ATP-binding cassette (ABC) transporter BcatrB from the plant-pathogenic fungus *B. cinerea* provides protection against phenazine antibiotics produced by *Pseudomonas* spp. The phenazines PCA and PCN strongly induced BcatrB expression, and BcatrB mutants were more sensitive to phenazine antibiotics than their parental strain. BcatrB, which is responsible for the efflux of phenazine, is not induced by pyrrolnitrin. In *L. maculans,* a similar ABC transporter, SirA, was discovered recently, which contributes to self-

protection against the toxin sirodesmin (Gardiner et al 2005). We assume that the SirA gene or a similar efflux system could be responsible for the resistance of *L. maculans* towards phenazine antibiotics. Pyrrolnitrin, produced by strain PA23, could play a more significant role in the biocontrol of *L. maculans*. The primary antifungal mechanism of pyrrolnitrin is the interference of the osmotic signal transduction pathway, and the secondary mechanism is probably the inhibition of respiration, which is active at a high dosage of the antibiotic in *N. crassa* and other fungi (Okada et al 2005). This explains the antifungal activity of pyrrolnitrin over a wide range of basidiomycetes, deuteromycetes, and ascomycetes (Ligon et al). To clearly establish the role of pyrrolnitrin in biocontrol of *L. maculans*, pyrrolnitrin knock-out mutants need to be generated and tested for their ability to control the blackleg pathogen, which is currently being carried out in Drs. De Kievit's and Fernando's labs.

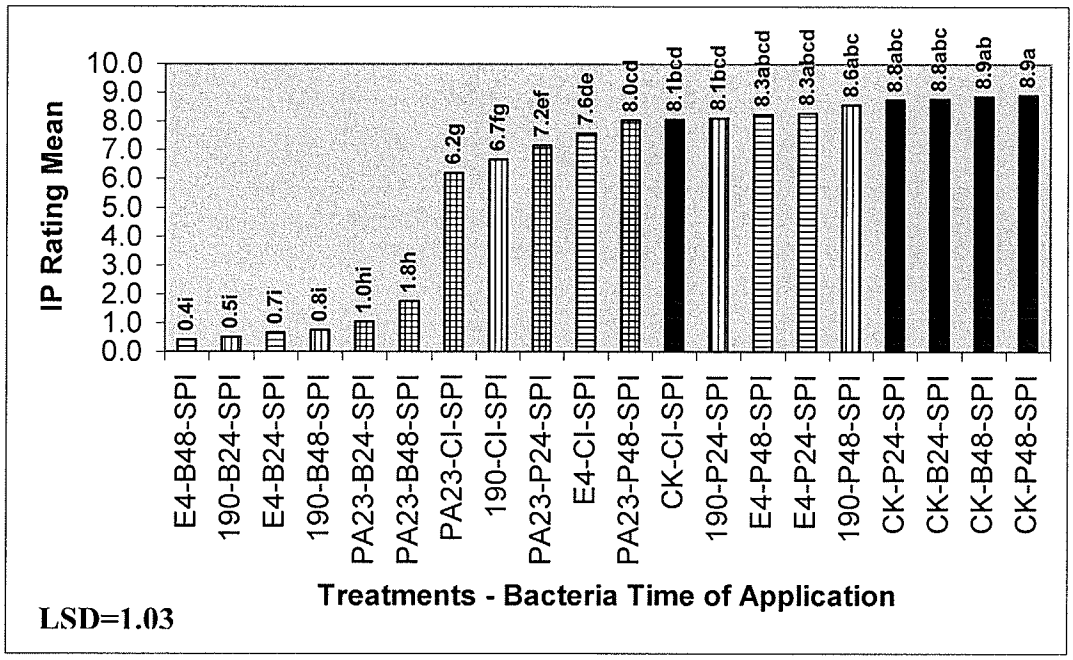

Figure 5.1. Effect of time of inoculation of bacteria on the suppression of pycnidiospores of *Leptosphaeria maculans* on cotyledon leaves of canola cultivar Westar.

Bacteria, *Pseudomonas chlororaphis* strains DF190 and PA23, and *Bacillus cereus* strain DFE4, were inoculated 24 and 48 h pre-pycnidiospore inoculation (B24 and B48, respectively), co-inouclated with pycnidiospore (CI), and 24 and 48 h post-pycnidiospore inoculation (P24 and P48, respectively). Single-point inoculation (SPI) was carried out in all treatments. Disease was scored 10-12 days post pathogen inoculation. Analysis of variance and a mean separation test (Fisher's Least Significant Difference), at $P = 0.05$, were performed.

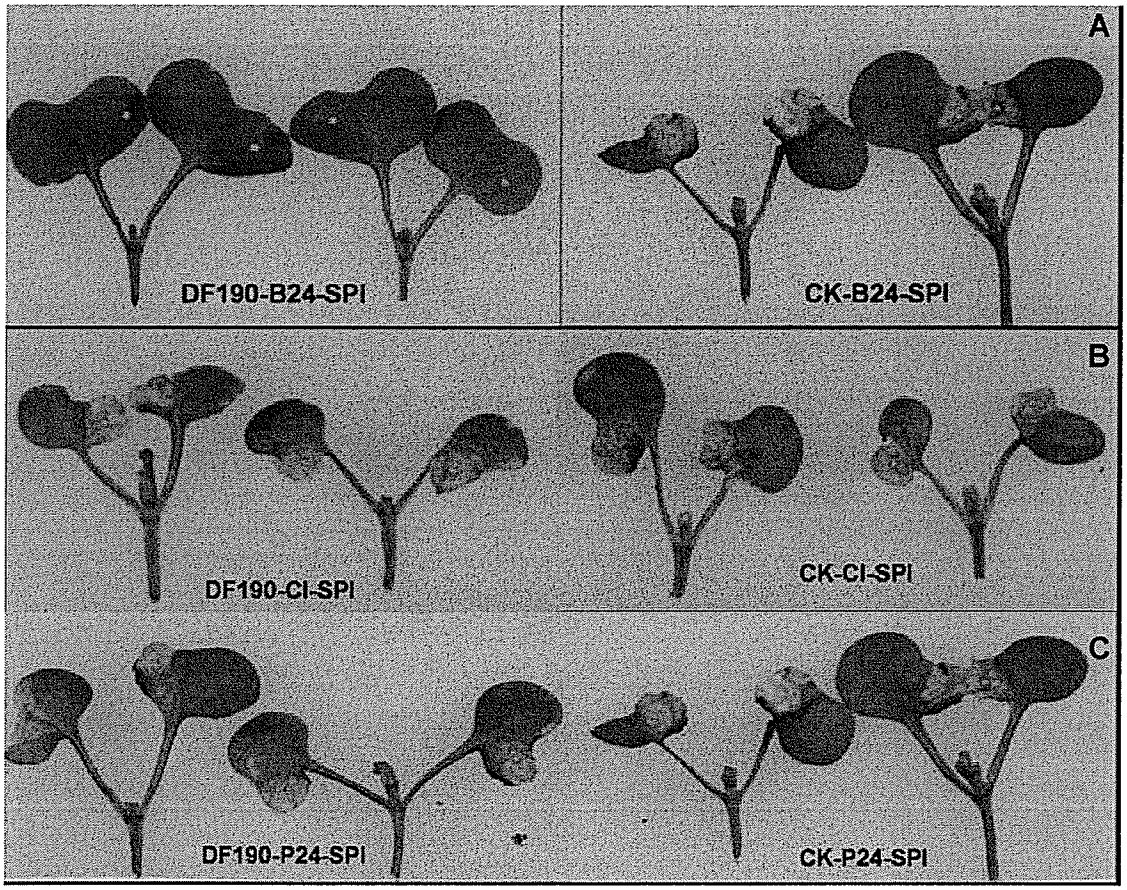

Figure 5.2. Effect of time of inoculation of bacteria on the suppression of pycnidiospores of *Leptosphaeria maculans* on cotyledon leaves of canola cultivar Westar.

All treatments had single-point inoculation (SPI). A – *Pseudomonas chlororaphis* strain DF190 inoculated 24 h prior to pycnidiospore inoculation; B – strain DF190 co-inoculated with pycnidiospores; C – strain DF190 inoculated 24 h post-inoculation of pycnidiospore. Significant disease control achieved in A, and no disease suppression in B and C.

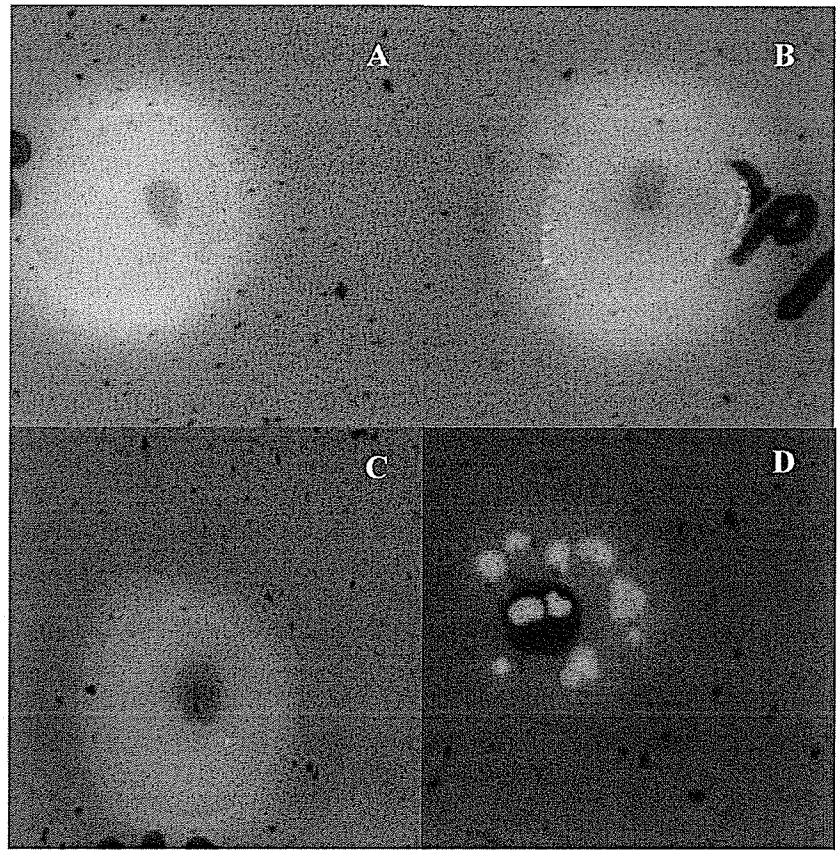

Figure 5.3. Siderophore production on Chrome Azurol S Agar. The formation of the orange halo around the bacterial colony, in an otherwise blue agar, indicates the production of siderophores. A- strain DF190; B – strain PA23; C – strain DFE4; and D – strain DFE16.

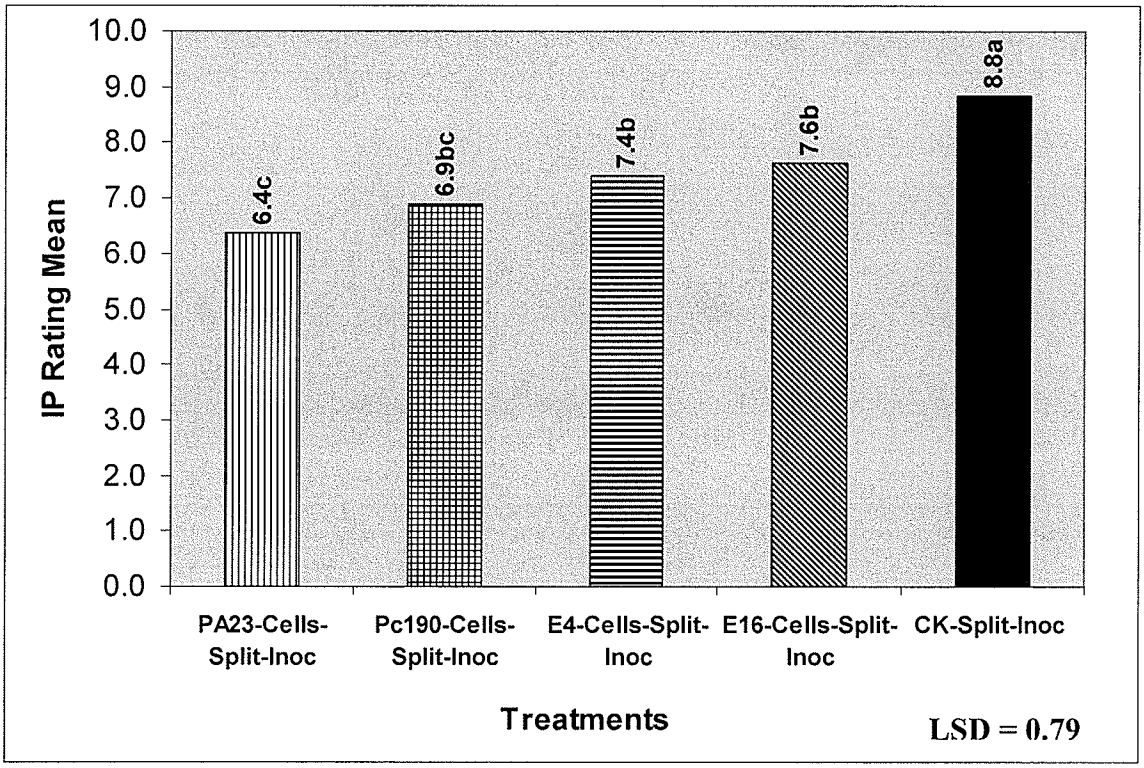

Figure 5.4. Effect of split inoculation of bacterial cells on the suppression of pycnidiospores of *Leptosphaeria maculans* on cotyledon leaves of Westar.

Bacterial cells were inoculated on one cotyledon leaf and 24 h later the pathogen pycnidiospores were inoculated on the other cotyledon leaf (split inoculation). Bacterial isolates Pseudomonas *chlororaphis* strain DF190, *Bacillus cereus* strain DFE4 and *B. amyloliquefaciens* strain DFE16 were used. Disease was scored 10-12 days post pathogen inoculation. Analysis of variance and a mean separation test (Fisher's Least Significant Difference), at $P = 0.05$, were performed.

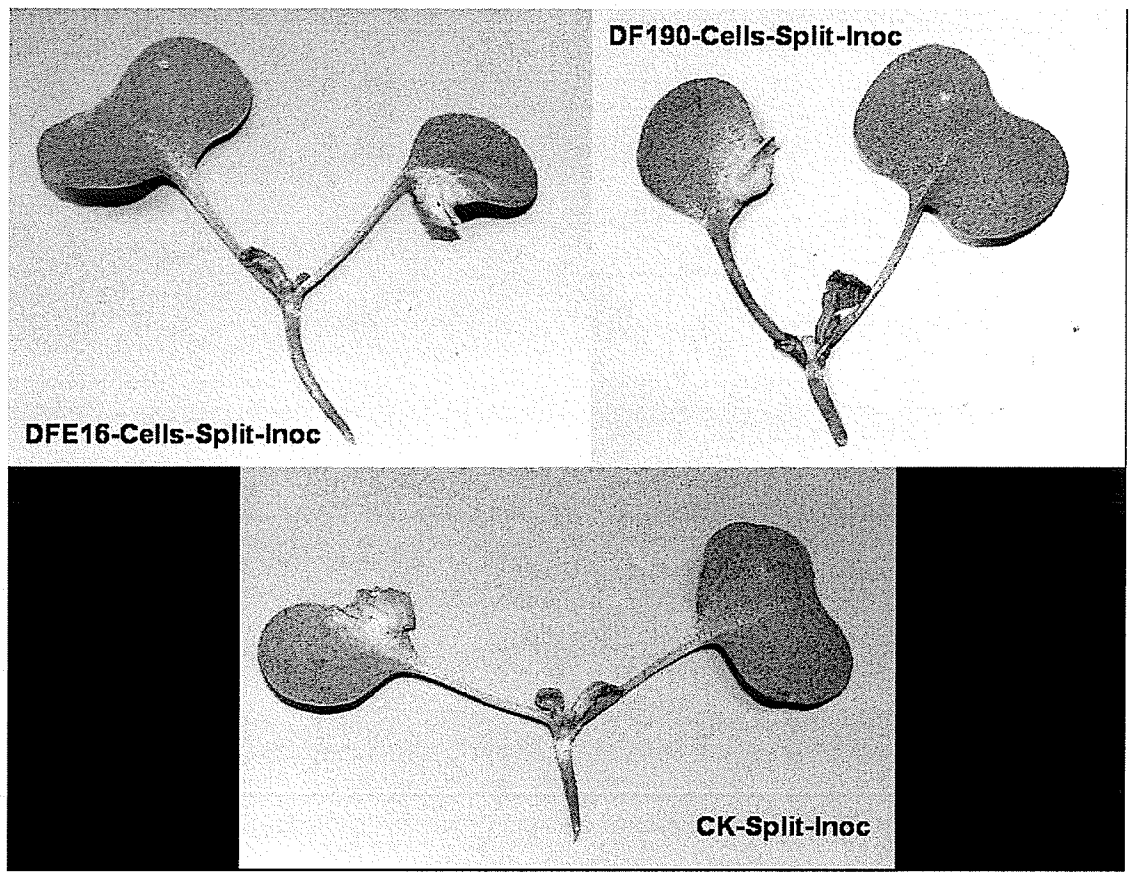

Figure 5.5. Effect of split inoculation of bacterial cells on the suppression of pycnidiospores of *Leptosphaeria maculans* on cotyledon leaves of Westar.

A – Split inoculation of strain DFE16 on the cotyledon on the left and pathogen pycnidiospore on the cotyledon on the right; B - Split inoculation of strain DF190 on the cotyledon on the right and pathogen pycnidiospore on the cotyledon on the left; C - Split inoculation of water on the cotyledon on the right and pathogen pycnidiospore on the cotyledon on the left (pathogen control). Disease suppression was not observed in the bacterial treatments.

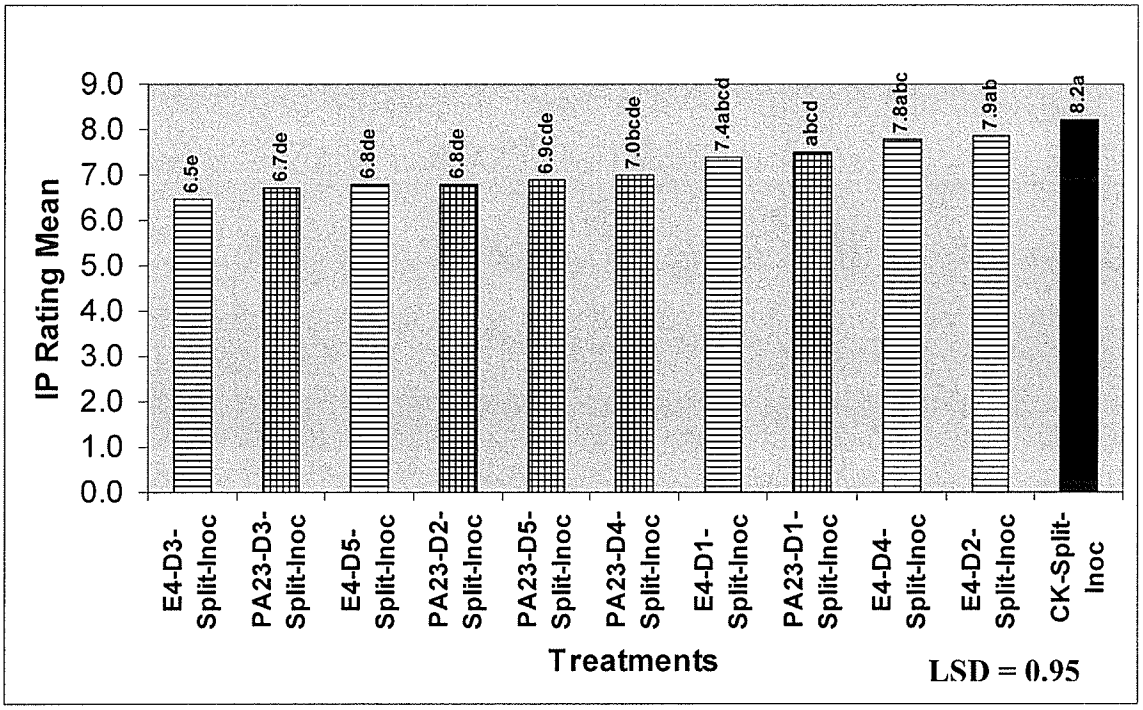

Figure 5.6. Effect of split inoculation of culture broth extracts on the suppression of pycnidiospores of *Leptosphaeria maculans* on Westar cotyledons.

Bacterial broths extracts of *Pseudomonas chlororaphis* strain PA23 and *Bacillus cereus* strain DFE4 were inoculated on one cotyledon and 24 h later pathogen pycnidiospores were inoculated on the other cotyledon (split inoculation). Bacterial broths were 1, 2, 3, 4 and 5 days old. Disease was scored 10-12 days after pathogen inoculation. Analysis of variance and a mean separation test (Fisher's Least Significant Difference), at $P = 0.05$, were performed.

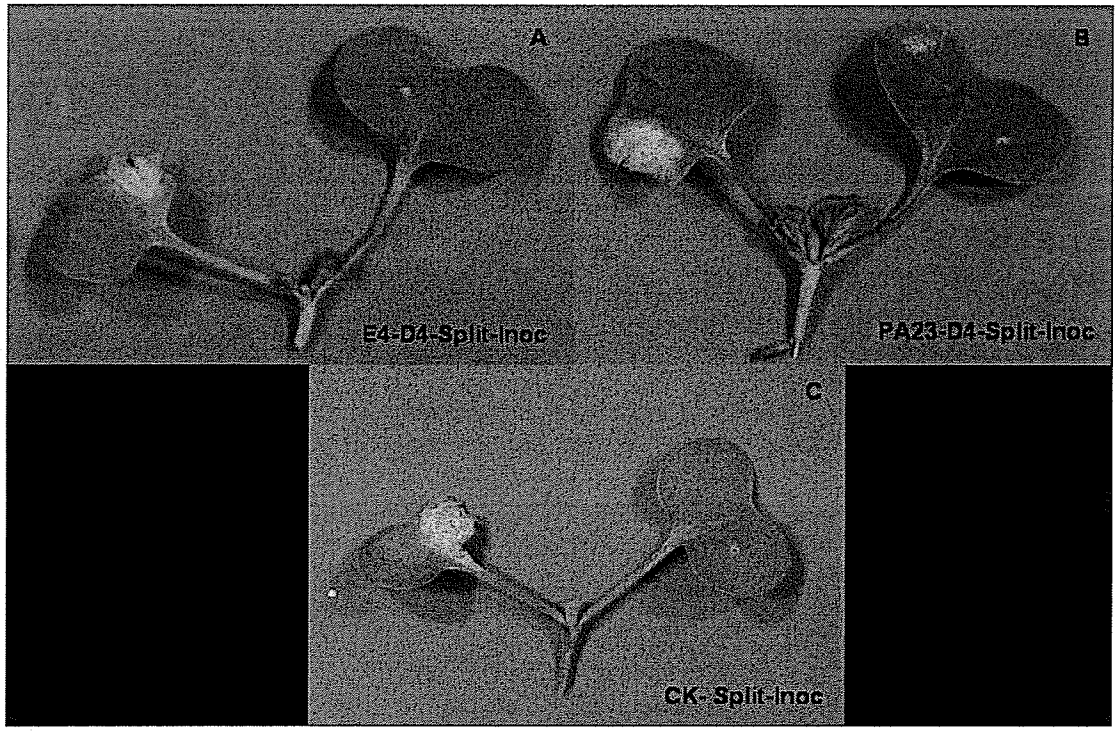

Figure 5.7. Effect of split inoculation of culture broth extracts on the suppression of pycnidiospores of *Leptosphaeria maculans* on Westar cotyledons.

A – Day 4 broth extract of *Bacillus cereus* strain DFE4 was inoculated on the right cotyledon leaf 24 h prior to the inoculation of the pathogen pycnidiospore on the left cotyledon leaf; B – Day 4 broth extract of *Pseudomonas chlororaphis* strain PA23 was inoculated on the right cotyledon leaf 24 h prior to the inoculation of the pathogen pycnidiospore on the left cotyledon leaf; C – Plain Luria Bertani broth extract inoculated on the right cotyledon leaf 24 h prior to the inoculation of the pathogen pycnidiospore on the left cotyledon leaf (pathogen control). None of the bacterial broth treatments, when split inoculated suppressed the blackleg lesion development on the cotyledons.

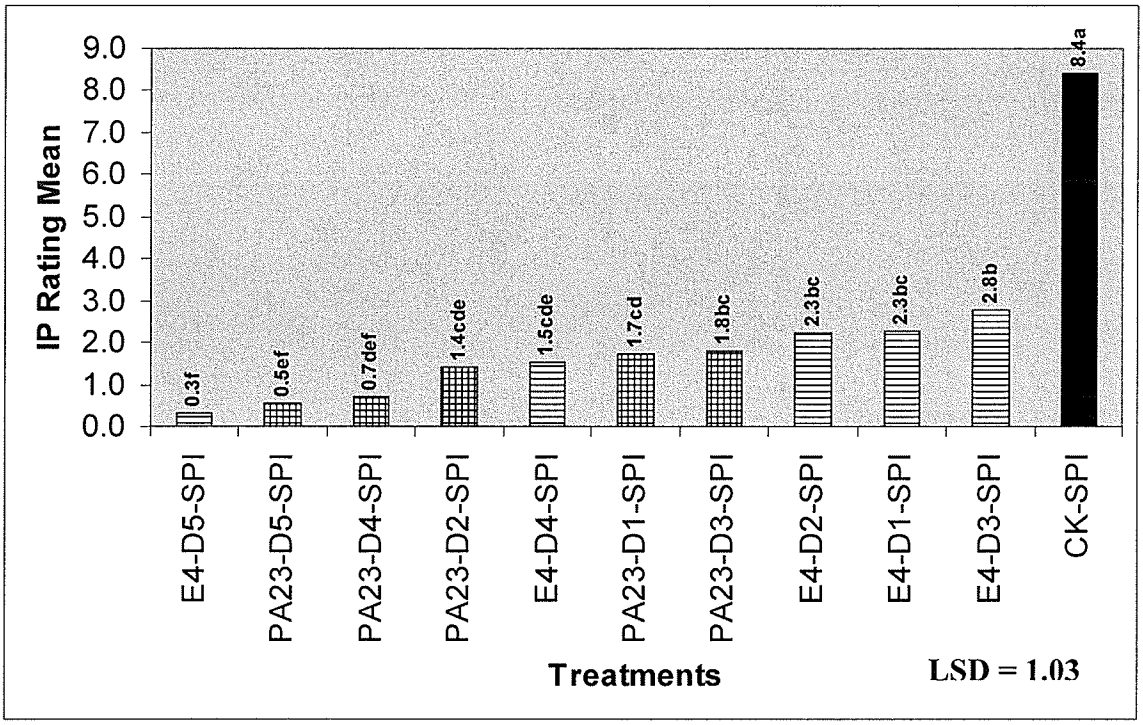

Figure 5.8. Effect of single point inoculation of culture broth extracts on the suppression of pycnidiospores of *Leptosphaeria maculans* on Westar cotyledons.

Bacterial broth extracts of *Pseudomonas chlororaphis* strain PA23 and *Bacillus cereus* strain DFE4 were locally inoculated on same cotyledon 24 h prior to pathogen pycnidiospores inoculation. Bacterial broths were 1, 2, 3, 4 and 5 days old. Disease was scored 10-12 days after pathogen inoculation. Analysis of variance and a mean separation test (Fisher's Least Significant Difference), at $P = 0.05$, were performed.

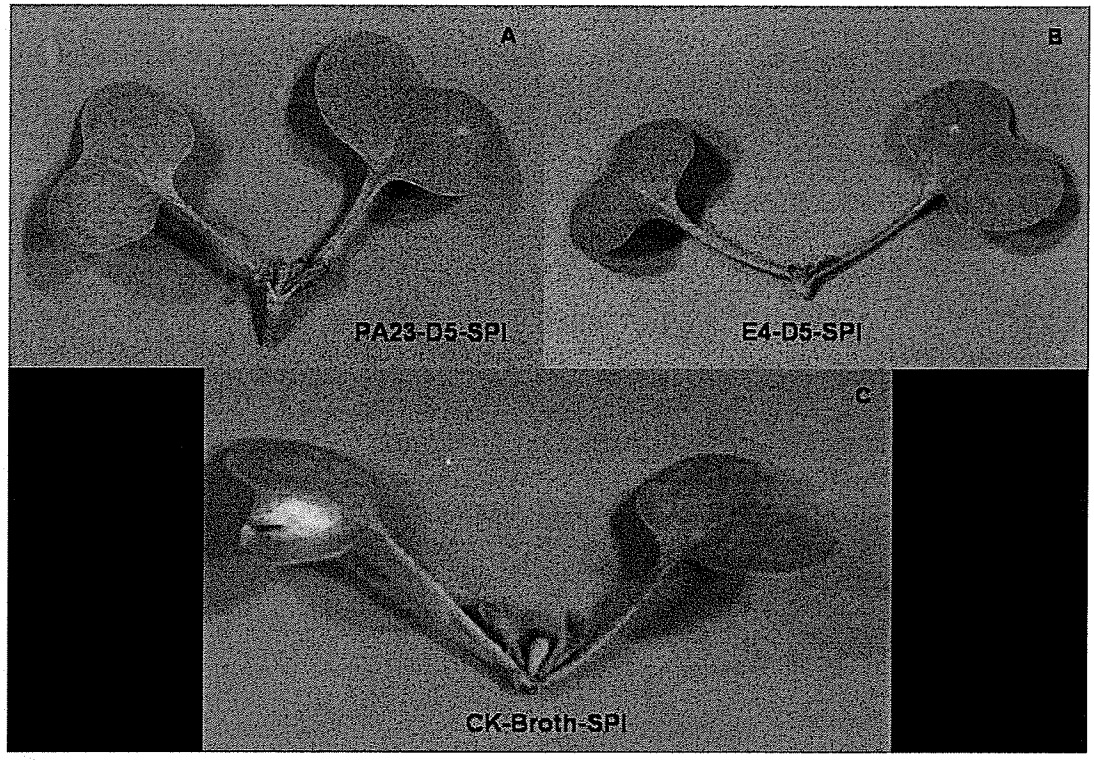

Figure 5.9. Effect of single point inoculation of culture broth extracts on the suppression of pycnidiospores of *Leptosphaeria maculans* on Westar cotyledons.

A – Day 5 broth extract of *Pseudomonas chlororaphis* strain PA23 was inoculated in the same spot as the pathogen pycnidiospores, but 24 h earlier; B – Day 5 broth extract of *Bacillus cereus* strain DFE4 was inoculated in the same spot as the pathogen pycnidiospore, but 24 h earlier; C – Plain Luria Bertani broth extract was inoculated in the same spot as the pathogen pycnidiospores, but 24 h earlier (pathogen control). All bacterial broth extracts when locally inoculated with the pycnidiospores, inhibited pycnidiospore germination and significantly suppressed the disease on the cotyledon leaves.

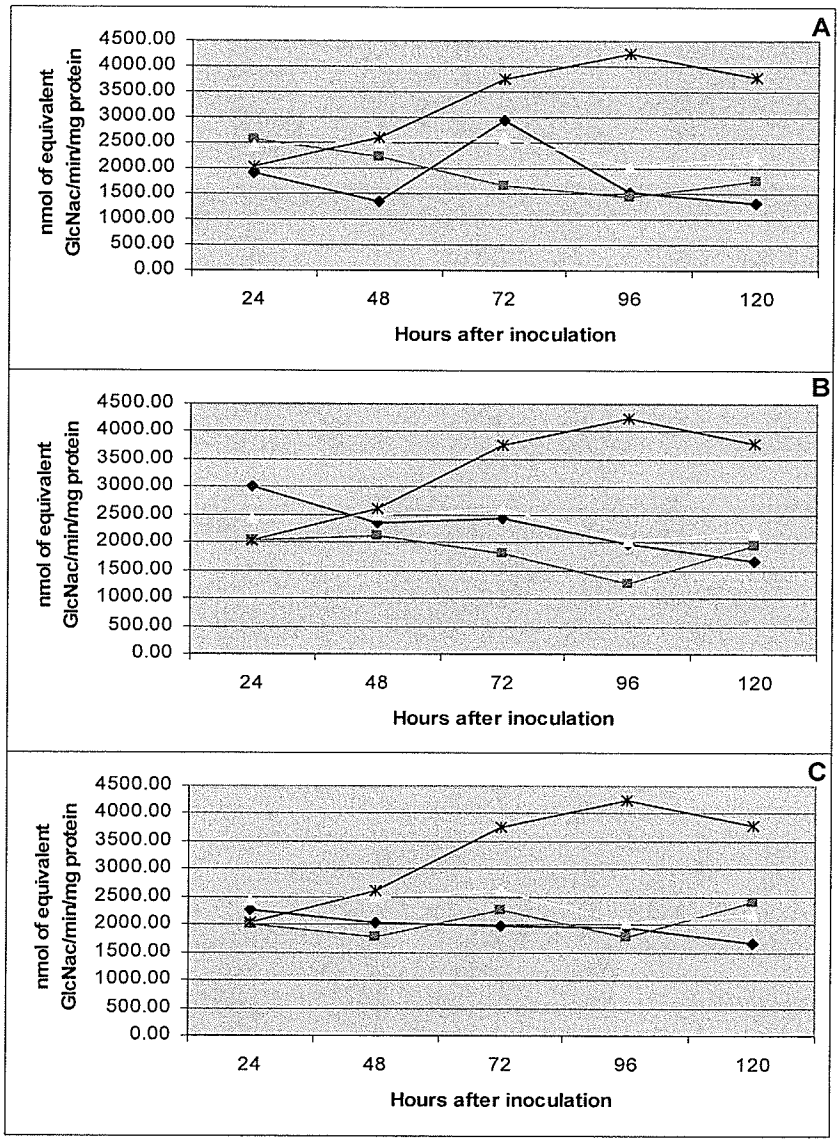

Figure 5.10. Colorimetric estimation of chitinase activity upon single point inoculation of bacterial cells and pycnidiospores of *Leptosphaeria maculans.*

Bacterial cells were inoculated 24 h prior to pycnidiospores at the same wound site. The treatments included – Bacteria inoculated alone (—◆—); Bacteria challenge inoculated with the pathogen (—■—); Pathogen inoculated alone (—▲—); and water control (—x—). A – Chitinase activity for *Pseudomonas chlororaphis* strain DF190; B – Chitinase activity for *Bacillus cereus* strain DFE4; C – Chitinase activity for *B. amyloliquefaciens* strain DFE16.

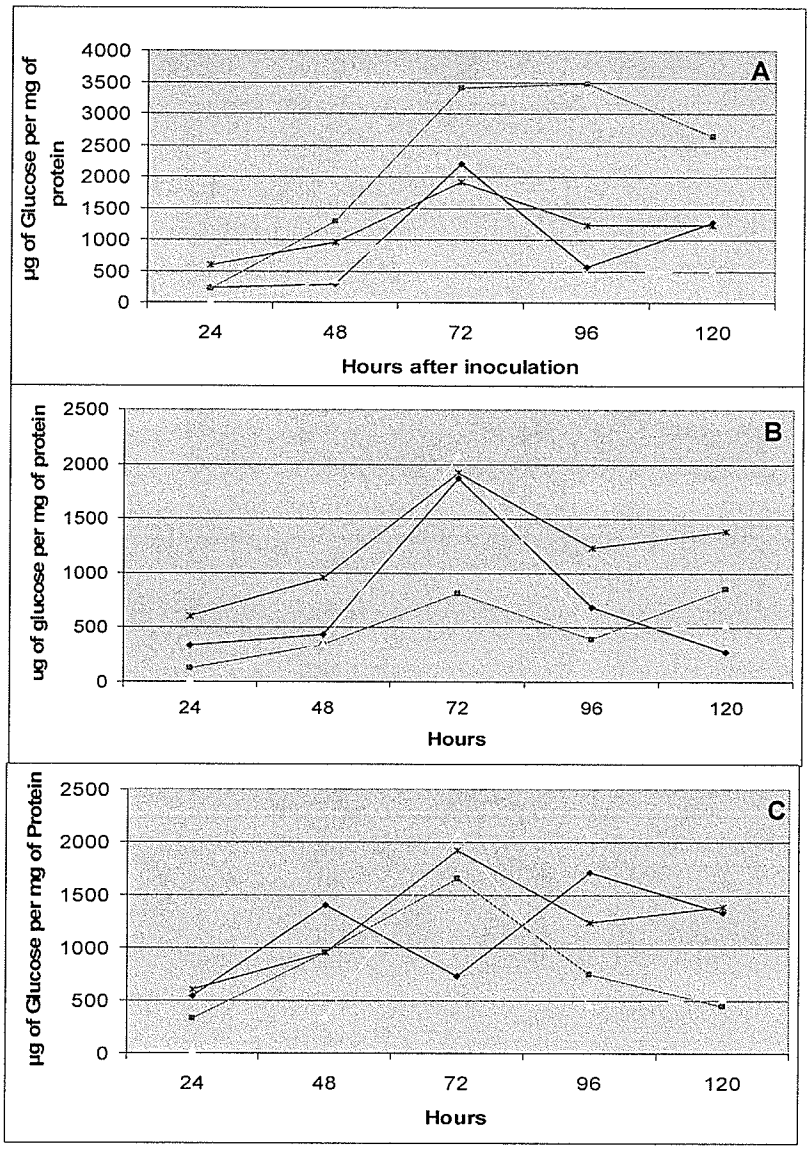

Figure 5.11. Colorimetric estimation of β-1,3 glucanase activity upon single point inoculation of bacterial cells and pycnidiospores of *Leptosphaeria maculans.*

Bacterial cells were inoculated 24 h prior to pycnidiospores at the same wound site. The treatments included – Bacteria inoculated alone (-◆-); Bacteria challenge inoculated with the pathogen (-■-); Pathogen inoculated alone (-▲-); and water control (-x-). A – β-1,3 Glucanase activity for *Pseudomonas chlororaphis* strain DF190; B – β-1,3 Glucanase activity for *Bacillus cereus* strain DFE4; C – β-1,3 Glucanase activity for *B. amyloliquefaciens* strain DFE16.

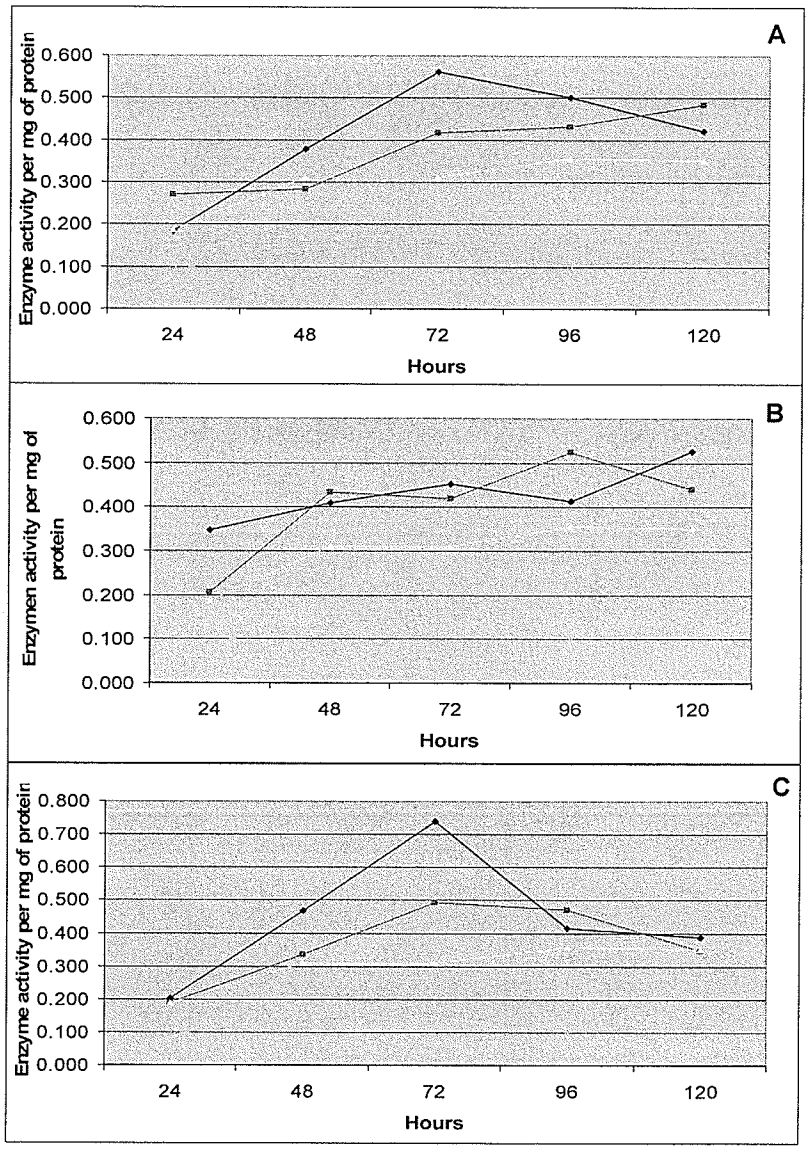

Figure 5.12. Colorimetric estimation of peroxidase activity upon single point inoculation of bacterial cells and pycnidiospores of *Leptosphaeria maculans.*

Bacterial cells were inoculated 24 h prior to pycnidiospores at the same wound site. The treatments included – Bacteria inoculated alone (—◆—); Bacteria challenge inoculated with the pathogen (—▲—); Pathogen inoculated alone (—■—); and water control (—x—). A – Peroxidase activity for *Pseudomonas chlororaphis* strain DF190; B – Peroxidase activity for *Bacillus cereus* strain DFE4; C – Peroxidase activity for *B. amyloliquefaciens* strain DFE16.

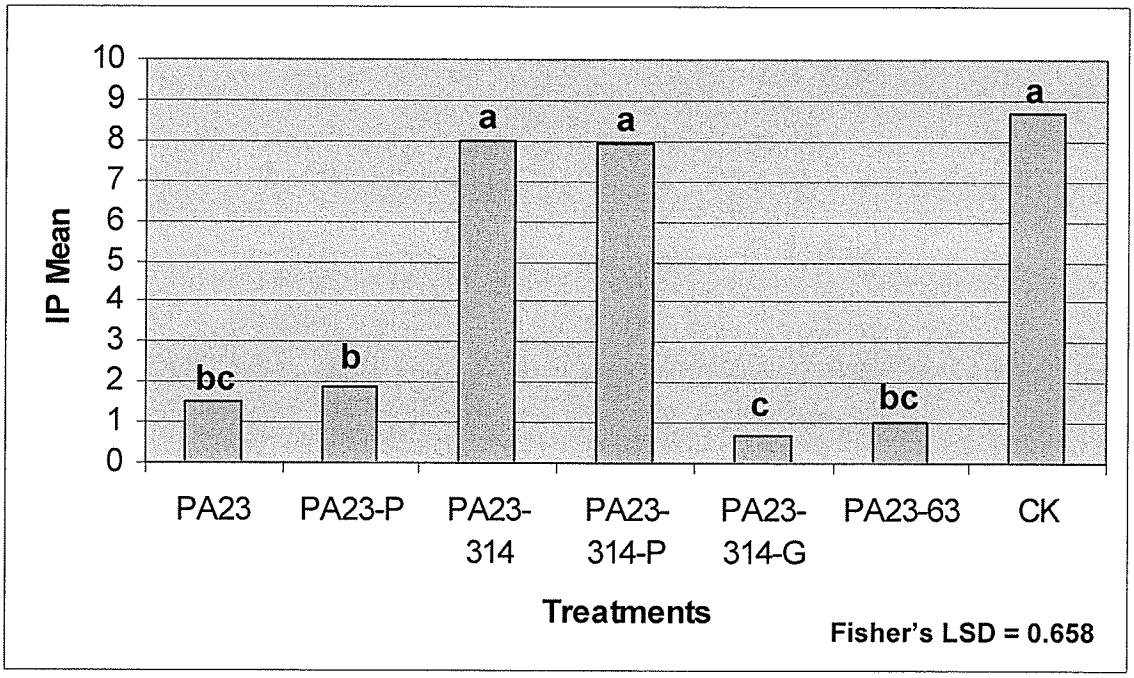

Figure 5.13. Blackleg suppression assay with Tn5 mutants of *Pseudomonas chlororaphis* strain PA23 on canola cv. Westar cotyledons.

Bacteria were inoculated 24 h prior to pycnidiospores of *Leptosphaeria maculans* on the same wound site. The bacteria that were assayed include; *P. chlororaphis* strain PA23 (wild type), *P. chlororaphiis* PA23-pUCP23 (wild type with the plasmid vector); PA23-314 (*gacS* mutant), PA23-414-pUCP23 (*gacS* mutant with the plasmid vector), PA23-314G (complemented *gacS* mutant), PA23-63 (*phzE* mutant). The cotyledons were scored on a scale of 0 – 9, 10-12 d post pycnidiospore inoculation. Analysis of variance (ANOVA) and a mean separation test (Fisher's Least Significant Difference), at P = 0.05, were performed.

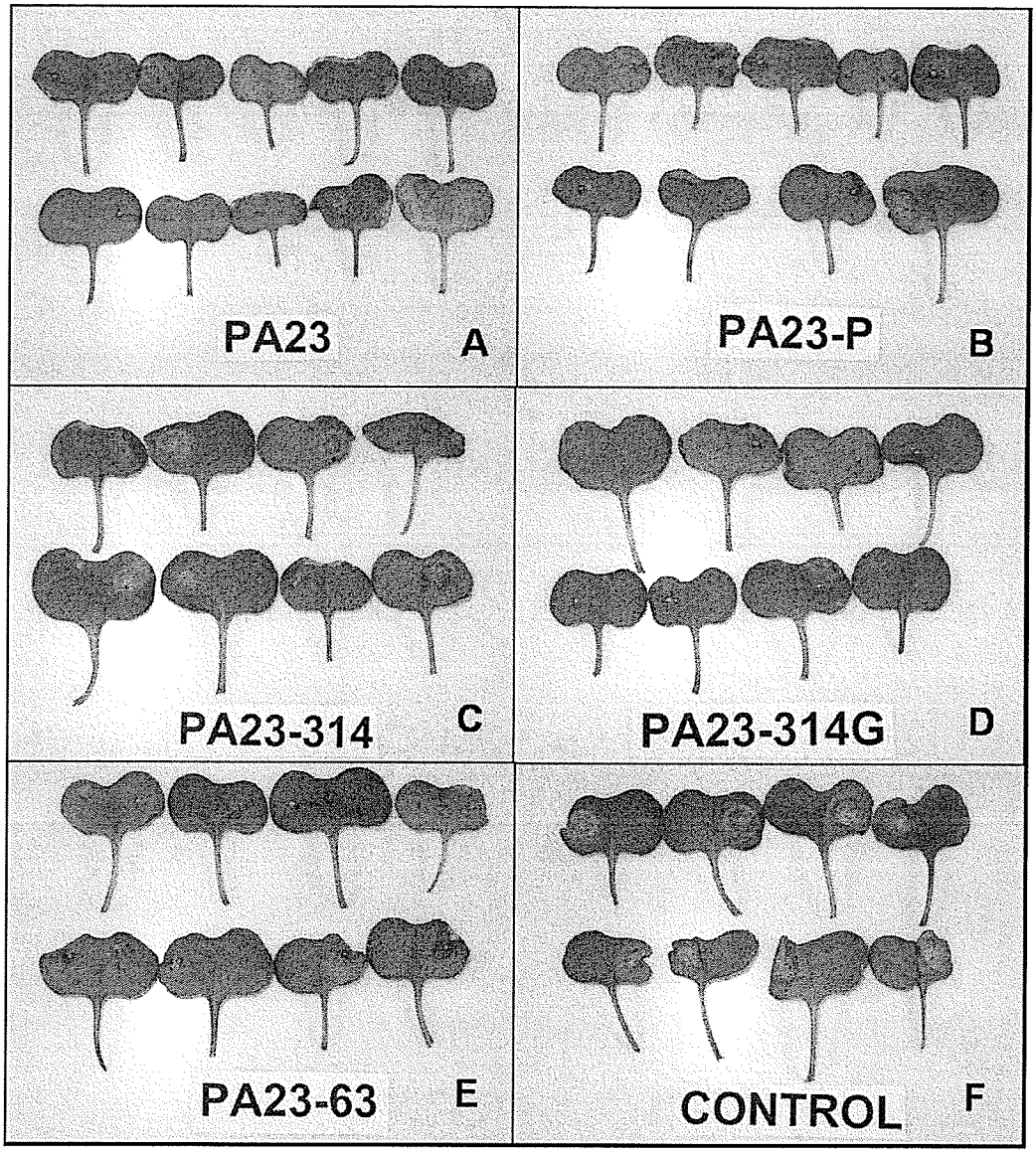

Figure 5.14. Blackleg suppression assay with Tn5 mutants of *P. chlororaphis* strain PA23 on Westar cotyledons.

A – *P. chlororaphis* strain PA23- wild type; B Strain PA23-pUCP23; C – Strain PA23-314 (*gacS* mutant); D – Strain PA23-314 *gacS* (re-complemented with *gacS* gene from the wild type); E – Strain PA23-63 (*phzE* mutant); F – Pathogen control.

6.0 GENERAL DISCUSSION

Canola/rapeseed (*Brassica napus* L.) is an economically important crop, with Canada leading the way as one of the largest producer in the world. The large scale cultivation of the crop is associated with pests and diseases, of which the most economically important threat to canola is a fungal stubble-borne disease known as blackleg, caused by *Leptosphaeria maculans* (Desm.) Ces & De Not (anamorph, *Phoma lingam* (Tode:Fr./Desm.)). The disease, so far, has been managed by tillage, 3-4 year crop rotation and use of resistant cultivars. In Western Canada, the agronomic shift towards conservation tillage and farmers tendency for a shorter rotation seem to favor the stubble-borne disease, which was evident with the shift in the population of the pathogen from the predominant PG2 to PG3 and PG4. This has led to the breakdown in the resistance in the cultivars, bred for PG2 resistance. The breakdown in the cultivar resistance is most likely going to lead to increase in fungicide usage. Increase in fungicide use not only favors change in pathogen population structure towards fungicide-resistant races but also leads to environmental hazards. To prevent the excessive use of fungicides, an integrated approach is needed for the control of blackleg, which would involve new control measures, such as biological control of the pathogen, along with other cultural control measures and limited fungicide use. Biological control with non-pathogenic antagonistic bacteria has been successfully used in other cropping systems, such as wheat, cotton etc. Bacteria mediate biocontrol through various mechanisms such as competition, siderophore production, antibiosis and induction of systemic resistance in the host, of which antibiosis mediated biocontrol is the most researched and established.

Our study has brought to the fore several findings, towards the biocontrol of *L. maculans* using antagonistic bacterial agents, which have not been previously reported. They are as follows:

1) Biocontrol of *L. maculans* in *in vitro* (plate), and *in vivo* (greenhouse and field) experiments with bacteria from canola stubble and leaf endophytes.

2) Development of specific PCR-primers for the detection of genes involved in the biosynthesis of antibiotics zwittermicin A and lipopeptides iturin A, bacillomycin D and surfactin in *Bacillus spp.*, isolated as endophytes from canola leaves.

3) The PCR detection of genes involved in the biosynthesis of phenazine, iturin A, bacillomycin D and surfactin was backed up with the biochemical detection of these compounds.

4) Production of 2-acetamidophenol by a *Pseudomonas chlororaphis* strain.

5) The detection of the genes involved in the biosynthesis, and confirmation of the production of lipopeptide antibiotics iturin A, bacillomycin D and surfactin by a *B. cereus* isolate.

6) Absence of systemic and local induction of resistance by bacteria or their cell-free extracts towards suppression of *L. maculans*. Inhibition of the pathogen is through the localized direct antifungal activity of the bacteria and their cell-free culture extracts, which points towards activity of antibiotics as the potential mechanism of control.

7) Mutagenesis experiments with *Pseudomonas chlororaphis* strain PA23 indicate the potential role of pyrrolnitrin, and not that of phenazine, as the antibiotic involved in biocontrol of *L. maculans*.

Earlier reports of biocontrol of *L. maculans* by the fungi *Cyathus striatus* and *C. olla* (Tewari et al 1997), and bacterium *Paenibacillus polymyxa* PKB1 (Kharbanda et al 1999) were only carried out at *in vitro* levels. Our study established the ability of the bacteria to control *L. maculans in planta*. Also, we established the significance of the time of inoculation of the bacteria. The application of the bacteria prior to the pathogen, offers time to multiply and establish populations on the leaf surface. Competence of the BCA and synchronization of its activity, in time and space, with the pathogen are key factors that determine the efficiency of a BCA (Folman et al. 2003). It is the early seedling infection, and in particular the infection of cotyledons, that is of overriding importance in causing the most severe disease epidemics (Bokor et al., 1975; Barbetti and Khangura, 1999). Timing of bacterial application at the right susceptible stage of the crop, as established in our field study, where bacteria (DF192 and DFE16) applied at the cotyledon stage suppressed the disease as efficiently as the fungicide. The bacteria prevented the early infection of the cotyledon leaves, which could have led to systemic infection of the stem and cankering of the stem.

Our study identified bacterial antagonists, which mediated biocontrol of blackleg in the phyllosphere of canola. The practice of no-till systems leaves the canola stubble on the soil surface, which helps in the longevity of the stubble and the survival of the pathogen, thus creating a suitable environment for the release of the wind-borne ascospores and the rain-splashed pycnidiospores. The bacterial application on the leaves

seems to prevent the germination of the spores and their entry into the host tissue at the site of infection. Phyllosphere bacteria could act as potential resource for biocontrol on leaves, as they have naturally evolved to compete and survive in the harsh conditions that prevail in the phyllosphere. Among the phyllosphere bacteria, *Bacillus spp.*, which in our study were isolated as leaf endophytes from canola, are known for their antifungal antibiotics (Edwards et al. 1994) and environmental stress-resistant endospores (Sadoff, 1972), and have high potential to be implemented as aerial leaf spray disease control agents. Our bacterial biocontrol agents *Bacillus amyloliquefaciens* strain DFE16 and *B. cereus* strain DFE4 performed well under field conditions, when sprayed on the leaf surface. *L. maculans* is incapable of producing cutinase (Annis & Goodwin (1996) and has to penetrate the host through stomata or wounds. In the rhizosphere and leaves, nutrients are not dispersed uniformly but rather, are localized in and around plant debris, stomata, wounds, lesions, and fungal propagules (Thomashow et al 1997). The quantity and quality of nutrients available and the ability to compete successfully for them are major determinants of microbial population size and metabolic activity, both of which are integrally linked to the regulation of antibiotic synthesis. When antibiotics have been detected in nature it has been in samples enriched in these microhabitats, which are localized regions of intense microbial interaction (Weller and Thomashow, 1990 and 1993).

Our study used PCR-based screening for the detection of genes involved in the biosynthesis of antibiotics and the identification of potential antibiotic producing bacterial biocontrol agents of *L. maculans* isolated from canola. This is the first study that has identified bacteria harboring genes involved in the biosynthesis of antibiotics

phenazine, pyrrolnitrin, zwittermicin A, iturin A, bacillomycin D and surfactin, and also exhibiting antagonistic activity towards the blackleg pathogen. The PCR-based screening has been a useful approach for the identification of potential antibiotic producing bacteria, especially when the antibiotic of interest is known and primers designed to detect genes involved in the biosynthesis, as compared to the conventional screening procedures, which are both time-consuming and laborious (Raaijmakers et al 2002). PCR-based screening has been more successful than the Fatty Acid Methyl Ester analysis in identifying zwittermicin A producers (Raffel et al 1996). To broaden its use, PCR-based screening, along with southern hybridization, can be used to screen for and identify how conserved these antibiotic biosynthetic genes are in bacteria isolated from canola and non-canola members that are part of a crop rotation in the control of blackleg. Similar, studies could be implemented to understand the effect of tillage and no-tillage systems on antibiotic-producing bacteria. This would enable us to understand better the distribution and function of the indigenous antibiotic producing bacteria, which would result in identifying and implementing better biocontrol agents of *L. maculans* in an integrated disease management system.

This study followed up the detection of antibiotic biosynthetic genes with the establishment of the role of antibiosis mediated direct antagonism of the bacteria and their broth extract towards the control of *L. maculans*. The biochemical detection of the lipopeptides iturin A, bacillomycin D and surfactin in *B. amyloliquefaciens* strain DFE16 and *B. cereus* strain DFE4 not only is a confirmation of the production of these antibiotics, but also an indication of their potential role in the phyllosphere biocontrol of the blackleg pathogen. These multiple antibiotic producing bacteria seem to offer great

potential as biocontrol agents of *L. maculans* through their wide-spectrum antifungal activity with different modes of action, which would create multiple hurdles for the pathogen in developing resistance to these antibiotics. The different *Pseudomonas* and *Bacillus* biocontrol agents can be applied as a mixture, which would more closely mimic the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of control, and allow the combination of various mechanisms without the need for genetic engineering (Raupach and Kloepper 1998). In our study, *P. chlororaphis* strain PA23 and *B. cereus* strain DFE4 exhibited mutual resistance with unaltered growth and cell number when co-inoculated, thereby creating the capacity to be applied as a mixture for the biocontrol of blackleg. This needs to be investigated further.

Follow-up studies would enhance our knowledge and understanding of the role of individual bacterial antibiotics and their mechanism of action in the biocontrol of *L. maculans*. A research associate in our lab is investigating the role of pyrrolnitrin in biocontrol. *P. chlororaphis* strain PA23 has been used to generate mutants of pyrrolnitrin, targeting the pyrrolnitrin biosynthetic gene cluster, using Tn5 mutagenesis, followed by complementation studies. This would establish the role of pyrrolnitrin in the antifungal activity of the strain. Also, as demonstrated earlier with the endochitinase gene of *Enterobacter agglomerans* (Chernin et al 1997), the pyrrolnitrin and the phenazine biosynthetic clusters have been cloned and transformed into an *Escherichia coli* background to understand the exclusive role of each antibiotic in the biocontrol of *L. maculans* (Habibian et al, unpublished personal communication). Similarly, for *B. amyloliquefaciens* strain DFE16 and *B. cereus* strain DFE4, producers of lipopeptide antibiotics iturin A, bacillomycin D and surfactin, Tn5 mutagenesis and re-

complementation experiments need to be carried out to understand the role of each antibiotic towards the antifungal activity of the bacteria. Also, assaying for the production and antifungal activity of extracellular chitinase (Chernin et al 1997) and protease (Dunne et al 1997) by these bacterial strains would help us understand if other mechanisms of control are present or if antibiotic production is the sole mechanism of control of blackleg mediated by these bacteria. Detection and quantification of *in situ* antibiotic production (Raaijmakers et al 1999) would help us understand the role played by these antibiotics at the site of infection. Further, the use of reporter gene systems, such as green fluorescence protein (Bloemberg et al 2000), act as powerful tools that allow us to visualize both the pathogen and the biocontrol agent, and also to determine whether the spatial colonization pattern of the pathogen coincide with spatial colonization and antibiotic production pattern of the biocontrol agent.

Heterologous chromosomal expression (Timms-Wilson et al 2000) of antibiotic biosynthetic cluster, which is more stable and less likely to affect the fitness of the heterologous strain, also acts as a powerful tool in improving the efficacy of bacterial biocontrol agents. Several transgenic strategies are also being developed for the control of blackleg, which involve the transformation and expression of foreign genes in canola that offer resistance to the pathogen. Canola cultivars expressing a pea protein (DRR206) decreased severity of stem cankers (Wang and Fristensky 2001) and cultivars expressing a small cysteine-rich protein (MiAMPi) derived from macadamia nut show reduced lesion development on cotyledons (Kazan et al. 2002). Henin et al (2001) observed the increase in resistance to *L. maculans* in *B. napus,* when the plants were transformed with a two-component system involving the interaction of resistance gene product (*Cf9* from

tomato) and the corresponding avirulence gene product (*Avr9* from *Cladosporium fulvum* Cke.). All these results demonstrate that canola is flexible to genetic transformations and offers room for the transformation of the bacterial antibiotic biosynthetic genes, and their subsequent expression and antifungal activity at the site of infection of the pathogen. The robustness of these transgenic plants has to be tested under field conditions. Also, single gene resistance may not be the right approach for blackleg resistance, since the pathogen populations change rapidly under selection pressure and readily be able to overcome such resistances (Howlett 2004). The bacterial antibiotics also serve as good models for the development of new environmentally safe fungicides. For example, the substitution of the chlorine group in the pyrrole ring of pyrrolnitrin with a cyano-group helped in the development of two derivatives, which are now marketed as commercial fungicides by the name of fepiclonil and fludioxonil (Ligon et al 2000). These fungicides are more stable than the original compound, and have good applicator, consumer and environmental safety. Similarly, the lipopeptide antibiotics iturin A and bacillomycin D, owing to their novel mode of action of membrane pore formation leading to cell leakage, are excellent targets for design and development of novel fungicides. Further, the *Bacillus* species which are natural producers of these lipopeptides could act as a rich genetic source for the construction of large biocombinatorial libraries of genetically engineered peptide synthetases for novel peptide design. The multienzyme modules, involved in the biosynthesis of lipopetides such as iturin A, can be used as model systems to study the principles of genetic recombination in nonribosomal peptide biosynthesis and to create new peptide products with desired properties (Vater et al 2003), such as enhanced antifungal activity.

In summary, from a commercial point of view, the technology from the lab has to be transferred to the field. Some simple, practical farming solutions for blackleg disease control that can be brought about, from the findings of this research, through further studies are: 1) Use of the bacteria at the phyllosphere to control infection; 2) Use of antibiotic producing antagonistic bacteria, isolated from the stubble, to control the over wintering pathogen; 3) Use of endophytes, owing to their multiple mechanisms of disease control and plant growth promotion; 4) Potential for the lipopeptide antibiotics as source of new fungicides; and 5) Use of biotechnology to isolate and express the beneficial antifungal traits in canola for the production for new disease resistant cultivars beneficial to the growers.

7.0 LITERATURE CITED

Adolphe, D., Daun, J., Fitzpatrick, K., Gruener, L., Macyk, D., Mayko, J., Peters, L., and Wilkins, D. 2002. Canola. Available from http://www.canola-council.org. (accessed Dec. 12. 2002)

Agriculture and Agri-Food Canada. 2001. Citing online sources: Did you know? Available from http:// aceis.agr.ca/fact_e.phtml (accessed 17 Oct. 2001).

Ainsworth, G. C. 1973. Introduction and Keys to higher taxa. *In* The Fungi: An Advanced Treatise. Edited By G.C. Ainsworth, F.K. Sparrow, and A.S. Sussman. New York and London. IVB, pp 1-7.

Altman, J., and Lawlor, S. 1966. The effect of some chlorinated hydrocarbons on some soil bacteria. Journal of Applied Bacteriology 29:260-265.

Andersen, R. A., Hamilton-Kemp, T. R., Hildebrand, D. F., McCraken, C. T. Jr., Collins, R. W., and Fleming, P. D.1994. Structure-antifungal activity relationships among C_6 and C_9 aliphatic aldehydes, ketones and alcohols. Journal of Agricultural and Food Chemistry 42:1563-1568.

Anjaiah, V., Koedam, N., Nowak-Thompson, B., Loper, J. E., Hofte, M., Tambong, J. T., and Cornelis, P. 1998. Involvement of phenazines and anthranilate in the antagonism of *Pseudomonas aeruginosa* PNA1 and Tn5 derivatives toward *Fusarium* spp. and *Pythium* spp. Molecular Plant Microbe Interactions 11:847-854.

Annis, S. L., and Goodwin, P. H. 1996. Comparison of cell wall degrading enzymes produced by highly and weakly virulent isolates of *Leptosphaeria maculans* in culture. Microbiological Research 151, 401-406.

Ansan-Melayah, D., Balesdent, M. H., Delourme, R., Pilet, M. L., Tanguy, X., Renard, M., and Rouxel, T. 1998. Genes for race-specific resistance against blackleg disease in *Brassica napus* L. Plant Breeding 117:373-378.

Archibold, D. D., Hamilton-Kemp, T. R., Barth, M. M., and Langlois, B. E. 1997. Identifying natural volatile compounds that control gray mold (*Botrytis cineria*) during postharvest storage of strawberry, blackberry and grape. Journal of Agricultural and Food Chemistry 45:4032-4037.

Arima, K., Imanaka, H., Kousaka, M., Fukuda, A. and Tamura, C. 1964. Pyrrolnitrin, a new antibiotic substance, produced by *Pseudomonas*. Agricultural and Biological Chemistry 28: 575-576.

Atlas, R., and Bartha, R, (*Editors*). 1993. Microbial Ecology. The Benjamin/Cummings Publishing Co, Inc., USA. 455pp.

Audenaert, K., Pattery, T., Cornelis, P., and Hofte, M. 2002. Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. Molecular Plant Microbe Interactions 15:1147–1156.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, k. (eds.). 1995. Short Protocols in Molecular Biology. 3rd ed. John Wiley & Sons. New York.

Bais, H. P., Fall, R., and Vivanco, J. M. 2004. Biocontrol of *Bacillus subtilis* against infection of *Arabidopsis* roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. Journal of Plant Physiology 134:307–319.

Baker, K. F. 1987. Evolving concepts of biological control of plant pathogens. Annual Review of Phytopathology 25:67-85.

Baker, K. F., and, Cook, R.J. (*Editors*) 1974. Biological control of plant pathogens. W.H.Freeman and Company, San Francisco. 433pp.

Bakker P. A. H. M., Ran, L. X., Pieterse, C. M. J., and VanLoon, L.C. 2003. Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. Canadian Journal of Plant Pathology 25:5–9.

Balesdent, M. H., Attard, A., Ansan-Melayah, D., Delourme, R., Renard, M., and Rouxel, T. 2001. Genetic control and host range of avirulence toward *Brassica napus* cultivars Quinta and Jet Neuf in *Leptosphaeria maculans*. Phytopathology 91:70-76.

Balesdent, M. H., Gall, C., Robin, P., and Rouxel, T. 1992. Intraspecific variation in soluble mycelial protein and esterase patterns of *Leptosphaeria maculans* French isolates. Mycological Research 96:677-684.

Balesdent, M. H., Jedryczka, M., Jain, L., Mendes-Pereira, E., Bertrandy, J., and Rouxel, T.1998. Conidia as a substrate for internal transcribed spacer-based PCR identification of members of the *Leptosphaeria maculans* species complex. Phytopathology 88:1210-1217.

Bangera, M. G., and Thomashow, L. S. 1999. Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from *Pseudomonas fluorescens* Q2-87. Journal of Bacteriology 181:3155-3163.

Barbetti, M. J., and Khangura, R. K. 1999. Managing blackleg in the disease-prone environment of Western Australia. Procedings of the 10th International Rapeseed Congress, Canberra, Australia. http://www.regional.org.au/papers/index/htm.

Barbetti, M. J., and Khangura, R. K. 2000. Fungal diseases of canola in Western Australia. Perth, WA: Agriculture Western Australia, Bulletin no. 4406.

Bargabus, R. L., Zidack, N. K., Sherwood, J. W., and Jacobsen, B. J. 2002. Characterization of systemic resistance in sugar beet elicited by a non-pathogenic, phyllosphere-colonizing *Bacillus mycoides*, biological control agent. Physiological and Molecular Plant Pathology 61:289-298.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zockand, A., and Smalla, K. 2002. Plant-dependant genotypic and phenotypic diversity of antagonistic rhizobacteria obtained from different *Verticillium* host plants. Applied and Environmental Microbiology 68: 3328-338.

Benbrook, C. M., Groth III, E., Halloran, J. M., Hansen, M. K., and Marquardt, S. 1996. Pest management at the crossroads. Consumers Union, New York.

Bender, C. L., Rangaswamy, V., and Loper, J. 1999. Polyketide production by plant-associated pseudomonads. Annual Review of Phytopathology 37:175-196.

Bloemberg, G. V, Wijfjes, A. H. M., Lamers, G .E. M., Stuurman, N., and Lugtenberg, B. J. J. 2000. Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. Molecular Plant Microbe Interaction 13: 1170–1176.

Bokor, A., Barbetti, M. J., Brown, A. G. P., MacNish, G. C., Poole, M. L., and Wood, P. M. C. R. 1975. Blackleg—a major hazard to the rapeseed industry. Journal of Agriculture, Western Australia 16: 7–10.

Bokor, A., Barbetti, M.J., Brown, A. G. P. and Wood, P.M. 1975. Blackleg of rapeseed. Journal of Agriculture, Western Australia 16:7-10.

Boller, T., and Mauch, F. 1988. Colorimetric assay for chitinase. Methodology of Enzymology 161:430-435.

Bostock, R. M. 2005. Signal crosstalk and induced resistance: Straddling the line between cost and benefit. Annual Review of Phytopathology 43:545-580.

Bradley, C., Parks, P. S., Chen, Y., and Fernando, W. G. D. 2005. First Report of Pathogenicity Groups 3 and 4 of *Leptosphaeria maculans* on Canola in North Dakota. Plant Disease 89:776.

Broderick, N. A., Goodman, R. M., Handelsman, J. and Raffa, K. F. 2003. Effect of host diet and insect source on synergy of gypsy moth (Lepidoptera: Lymantriidae) mortality to *Bacillus thuringiensis subsp. kurstaki* by zwittermicin A. Environmental Entomology. 32:387–391.

Broderick, N. A., Goodman, R. M., Raffa, K. F., and Handelsman, J. 2000. Synergy between zwittermicin A and *Bacillus thuringiensis subsp. Kurstaki* against gypsy moth (Lepidoptera: Lymantriidae). Environmental Entomology 29:101–107.

Brown, A. G. P., Barbetti, M. J., and Wood, P. M. 1976. Effect of benomyl on 'blackleg' disease of rape in Western Australia. Australian Journal of Experimental Agriculture and Animal Husbandry 16:276-279.

Bull, C. T., Weller, D. M., and Thomashow, L. S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. Phytopathology 81:954-959.

Bunting, E. S. 1986. Oilseed rape in perspective. In Oilseed rape. Edited by D.H. Scarisbrick and R.W. Daniels. Mackays of Chantham, Great Britan. pp. 1-31

.

Burkhead, K. D., Schisler, D. A. and Slininger, P.J. 1994. Pyrrolnitrin production by biological-control agent *Pseudomonas cepacia* B37w in culture and in colonized wounds of potatoes. Applied and Environmental Microbiology 60: 2031–2039.

Conrath, U., Pieterse, C.M.J., and Mauch-Mani, B. 2002. Priming in plant pathogen interactions. Trends in Plant Science 7:210-216.

Campbell, R. 1989. Biological Control of Microbial Plant Pathogens. Cambridge University Press, Cambridge.

Canola Council of Canada. 2001. Citing online sources: Canadian Canola Industry. Available from http://www.canola-council.org/ind_overview.html (accessed 15 May. 2006)

Canola Council of Canada. 2006. Citing online sources: Crop Production. Available from http://www.canola-council.org/blacklegcultural.aspx (accessed 15 May 2006)

Canola Council of Canada. 2006. Citing online sources: Crop Production. Available from http://www.canola-council.org/cropproduction9/cropproduction9_1.html?gInitialPosX=10px&gInitialPosY=10px&gZoomValue=100 (accessed 15 May 2006)

Canola Council of Canada. 2007. Citing online sources: Overview of Canada's Canola Industry. Available from http://www.canola-council.org/ind_overview.html (accessed 13 December 2007)

Challis, G. L., and Hopwood, D. A. 2003. Synergy and contingency as driving forces for the evolution of multiple secondary metabolite production by *Streptomyces* species. Proceedings of the National Academy of Science 100:14555-14561.

Chancey, S. T., Wood, D. W., Pierson, E. A., and Pierson, L. S. 2002. Survival of GacS/GacA mutants of the biological control bacterium *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere. Applied and Environmental Microbiology 68:3308–3314

Chang, C. J., Floss, H. G., Hook, D. G., Mabe, J. A., Manni, P. E., Martin, L. L., Schroder, K., and Shieh, T. L. 1981. The biosynthesis of the antibiotic pyrrolnitrin by *Pseudomonas aureofaciens*. Journal of Antibiotics 24:555-566.

Chapon, A., Guillerm, A. Y., Delalande, L., Lebreton, L., and Sarniguet, A. 2002. Dominant colonization of wheat roots by Pseudomonas fluorescens Pf29A and selection of the indigenous microflora in the presence of take-all fungus. European Journal of Plant Pathology 108:449-459.

Chen, C., Bauske, E.M., Musson, G., Rodríguez-Kábana, R., and Kloepper, J.W. 1995. Biological control of Fusarium wilt of cotton by use of endophytic bacteria. Biological Control 5: 83-91.

Chen, C. Y., and Howlett, B. J. 1996. Rapid necrosis of guard cells is associated with the arrest of fungal growth in leaves of Indian mustard (*Brassica juncea*) inoculated with avirulent isolates of *Leptosphaeria maculans*. Physiological and Molecular Plant Pathology 48:73-81.

Chen, Y. 2006. Molecular and phenotypic characterization of population variation of *Leptosphaeria maculans* and induced resistance to blackleg disease of canola caused by a weakly virulent isolate of *Leptosphaeria biglosa.* PhD thesis dissertation. University of Manitoba.

Chen, Y., and Fernando, W.G. D. 2005. First Report of Canola Blackleg Caused by Pathogenicity Group 4 of *Leptosphaeria maculans* in Manitoba. Plant Disease 89: 339.

Chen, Y., and Fernando, W. G. D. 2006. Induced resistance to blackleg (*Leptosphaeria maculans*) disease of canola (*Brassica napus*) caused by a weakly virulent isolate of *Leptosphaeria biglobosa*. Plant Disease 90:1059-1064.

Chernin, L., Brandis, A., Ismailov, Z. and Chet, I. 1996. Pyrrolnitrin production by an *Enterobacter agglomerans* strain with broad spectrum activity towards fungal and bacterial phytopathogens. Current Microbiology 32: 208–212.

Chernin, L. S., de la Fuenta, L., Sobolev, V., Haran, S., Vorgias, C. E., Oppenheim, A. B., and Chet, I. 1997. Molecular cloning, structural analysis, and expression in *Escherichia coli* of a chitinase gene from *Enterobacter agglomerans*. Applied and Environmental Microbiology 63:834-839.

Chernin, L., Ismailov, Z., Haran, S., and Chet, I. 1995. Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. Applied and Environmental Microbiology 61:1720-1726.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., and Lugtenberg, B. J. J. 2003. Phenazine and their role in biocontrol by *Pseudomonas* bacteria. New Phytologist 503-523.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., Mulders, I. H. M., Dekkers, L. C., and Lugtenberg, B J. J. 2000. Root colonization by phenazine-1-carboxamide- producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Molecular Plant Microbe Interactions 12:1340-1345.

Chin-A-Woeng, T. F. C., Bloemberg, G. V., van der Bij, A J., van der Drift, K M. G. M., Schripsema, J., Kroon, B., Scheffer, R. J., Keel, C., Bakker, P. A. H. M., Tichy, H. V., de Bruijn, F. J., Thomas-Oates, J. E., and Lugtenberg, B. J. J. 1998. Biocontrol by phenazine-1-carboxamide-producing *Pseudomonas chlororaphis* PCL1391 of tomato root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici.* Molecular Plant Microbe Interactions 11:1069-1077.

Chiou, A.bL., and Wu, W.bS. 2003. Formulation of *Bacillus amyloliquefaciens* B190 for control of lily grey mould (*Botrytis elliptica).* Journal of Phytopathology 151:13-18

Cho, S. J., Lee, S. K., Cha, B. J., Kim, Y. H., and Shin, K. S. 2003. Detection and characterization of the *Gloeosporium gloeosporioides* growth inhibitory compound iturin A from *Bacillus subtilis* strain KS03. FEMS Microbiology Letters 223:47-51.

Conrath, U., Pieterse, C. M. J. & Mauch-Mani, B. 2002. Priming in plant–pathogen interactions. Trends in Plant Science. 7, 1360–1385.

Cook, R. J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. Phytopathology 31:5340.

Cook, R. J. and Baker, K .F. 1983. The nature and practice of biological control of plant pathogens. American Phytopathological Society. St. Paul, MN.

Cornelis, P. and Matthijs, S. 2002. Diversity of siderophore mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. Environmental Microbiology 4:787–798.

Cosmina, P., Rodriguez, F., de Ferra, F., Grandi, G., Perego, M., Venema, G., and van Sinderen, D. (1993) Sequence analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. Molecular MIcrobiology 8: 821-831.

Coventry, E., Noble, R., Mead, A., and Whipps, J. M. 2005. Suppression of Allium white rot (*Sclerotium cepivorum*) in different soils using vegetable wastes. European Journal of Plant Pathology 111: 101–112.

De Bellis, P., and Ercolani, G. L. 2001. Growth interactions during bacterial colonization of seedling rootlets. Applied and Environental Microbiology 67: 1945–1948.
de Boer, M., van der Sluis, I., van Loon, L. C. and Bakker, P. A. H. M. 1999. Combining fluorescent *Pseudomonas* spp. strains to enhance suppression of fusarium wilt of radish. European Journal of Plant Pathology 105:201–210.

De Souza, J. T., and Raaijmakers, J. M. 2003. Polymorphisms within the prnD and pltC genes from pyrrolnitrin and pyoluteorin-producing *Pseudomonas* and *Burkholderia* spp. FEMS Microbiology Ecology 43:21-34.

Delaney, S. M., Mavrodi, D. V., Bonsall, R. F., and Thomashow, L. S. 2001. *phzO*, a gene for biosynthesis of 2- hydroxylated phenazine compounds in *Pseudomonas aureofaciens* 30-84. Journal of Bacteriology 181:318-327.

Delourme, R., Pilet-Nayel, M. L., Archipiano, M., Horvais, R., Tanguy, X., Rouxel, T., Brun, H., Renard, M., and Balesdent, M. H. 2004. A cluster of major specific resistance genes to *Leptosphaeria maculans* in *Brassica napus*. Phytopathology 94:578-583.

Dhingra, O. D., Coelho-Netto, R. A., Rodrigues, F. A., Silva, G. J and Maia, C. B. 2006. Selection of endemic nonpathogenic endophytic Fusarium oxysporum from bean roots and rhizosphere competent fluorescent *Pseudomonas* species to suppress Fusarium-yellow of beans. Biological control 39: 75 – 84.

Dobranic, J.K., Johnson, J.A., and Alikhan, Q.R. 1995. Isolation of endophytic fungi from eastern larch (*Larix laricina*) leaves from New Brunswick, Canada. Canadian ournal of Microbiology 41: 194-198.

Domsch, K. H. 1959. Die wirkung von bodenfungiciden III. Quantitative varenderungen der bodenflora. Z. Pflkrankh 66:17-26.

Downey, R. K., and Röbbelen, G. 1989. *Brassica* species. *In* Oil Crops of the World (G. Röbbelen, R. K. Downey, A, Ashri eds.) McGraw-Hill Publishing Company, New York. pp. 339-362.

Dubery, J. A., Teodorczuk, L. G., and Louw, A. E. 2000. Early responses in methyl jasmonate-preconditioned cells towards pathogen derived elicitors. Molecular Cell Biology Research Communications 3:105-110.

Duffy, B. K., and Défago, G. 2000. Controlling instability in *gacS-gacA* regulatory genes during inoculant production of *Pseudomonas fluorescens* biocontrol strains. Applied and Environmental Microbiologyl. 66:3142-3150.

Duijff, B. J., Meijer, J. W., Bakker, P. A. H. M., and Schippers. B.1993. Siderophore-mediated competition for iron and induced resistance in the suppression of fusarium wilt of carnation by fluorescent *Pseudomonas* spp. Netherlands Journal of Plant Pathology 99:277-289.

Duijff, B. J., Recorbet, G., Bakker, P. A. H. M., Loper, J. E., and Lemanceau, P. 1999. Microbial antagonism at the root level is involved in the suppression of fusarium wilt by the combination of nonpathogenic *Fusarium oxysporum* Fo47 and *Pseudomonas putida* WCS358. Phytopathology 89: 1073–1079.

Duitman, E. H., Hamoen, L. W., Rembold, M., Venema, G., Seitz, H., Saenger, W., Bernhard, F., Reinhardt, R., Schmidt, M., Ullrich, C., Stein, T., Leenders, F. and Vater, J. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proceedings of the National Academy of Science 96: 13294–13299.

Dunlap, C., Delaney, J., Fenton, A., Lohrke, S., Moënne-Loccoz, Y and O'Gara, F. 1996. The biotechnology and application of Pseudomonas inoculants in the biocontrol of phytopathogens. *In*: Stacey, G., Mullin, B., and Gresshoff, P.M (*Editors*) Biology of Plant Microbe Interactions. International Society for Molecular Plant Microbe Interactions. St. Paul, MN, USA. pp:441-448

Dunne, C., Crowley, J. J., Moenne-Loccoz, Y., Dowling, D. N., de Bruijn, F. J., and O'Gara, F.1997. Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W18 is mediated by an extracellular proteolytic activity. Microbiology 143:3921-3931.

Edwards, S. G., Mckay, T., and Seddon, B. 1994. Interaction of *Bacillus* species with phytopathogenic fungi – Methods of analysis and manipulation for biocontrol purposes. In Ecology of Plant Pathogens ed. Blakeman, J. P., and Williamson, B. Wallingford: CAB International. pp. 101-118.

Elad, Y. and Stewart, A. 2004. Microbial control of *Botrytis* spp. In: Elad Y, Williamson B, Tudzynski P, Delan N, eds. *Botrytis: Biology, Pathology and Control.* Dordrecht, The Netherlands: Kluwer Academic, 223–41.

El Banna, N., and Winkelmann, G. 1998. Pyrrolnitrin from *Burkholderia cepacia*: antibiotic activity against fungi and novel activities against streptomycetes. Journal of Applied Microbiology 85:69-78.

El Tarabily, K. A., Hardy, G. E. S. J., Sivasithamparam, K., Hussein, A. M., and Kurtboke, D. I. 1997. The potential for the biological control of cavity-spot disease of carrots, caused by *Pythium coloratum*, by streptomycete and non-streptomycete actinomycetes. New Phytologist 137:495-507.

Ellis, C., Karafyllidis, L., and Turner, J. G. 2002. Constitutive activation of jasmonate signaling in *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae* and *Myzus persicae*. Molecular Plant Microbe Interaction 15:1025-1030.

Elmholt, S. 1991. Side effects of propiconazole Tilt-250EC on non-target soil fungi in a field trial compared with natural stress effects. Microbial Ecology 22:99-108.

Emmert, E. A. B., and Handelsman, J. 1999. Biocontrol of plant disease: a (Gram-) positive perspective. FEMS Microbiology Letters 171:1-9.

Emmert, E. A. B., Klimowicz, A. K., Thomas, M. G., and Handelsman, J. 2004. Genetics of zwittermicin A production by *Bacillus cereus*. Applied and environmental Microbiology 70:104-113.

Felton, G. W., and Dahlman, D.L. 1984. Non-target effect of fungicide toxicity of Maneb to the parasitoid *Microplitis croceipes* : Hymenoptera Braconidae. Journal of Economic Entomology 77:847-850.

Férézou, J. P., Quesneau-Thierry, A., Servy, C., Zissmann, E., and Barbier, M. 1980. Sirodesmin PL biosynthesis in *Phoma lingam* Tode. Journal of the Chemical Society, Perkin Transactions 11739-1746.

Fernando, W. G. D., and Chen, Y. 2003. First Report on the presence of *Leptosphaeria maculans* Pathogenicity Group-3, causal agent of Blackleg of Canola in Manitoba. Plant Disease 87:1268.

Fernando, W. G. D., Nakkeeran, S., Zhang, Y. and Savchuk, S. 2007. Biological control of *Sclerotinia sclerotiorum* (Lib.) de Bary by *Pseudomonas* and *Bacillus* species on canola petals. Crop Protection 26: 100–107.

Fernando, W. G. D., Parks, P. S., Tomm, G., Viau, L. V. and Jurke, C. 2003. First report of the Blackleg disease caused by *Leptosphaeria maculans* on canola in Brazil. Plant Disease 87:314 on-line D-2003-0117-01N

Fernando, W. G. D., and Pierson, L. S. 1999. The effect of increased phenazine antibiotic production on the inhibition of economically important soil-borne plant pathogens by *Pseudomonas aureofaciens* 30-84. Archives of Phytopathology and Plant Protection 32:491-502.

Fernando, W. G. D., Ramarathnam, R., Krishnamoorthy, A. S., and Savchuk, S. 2005. Identification and use of potential bacterial organic volatiles in biological control. Soil Biol. Biochemistry 37: 955-964.

Fitt, B. D. L., Gladders, P., Turner, J. A., Sutherland, K. G., Welham, S. J., and Davies, J. M. L. 1997. Prospects for developing a forecasting scheme to optimise use of fungicides for disease control on winter oilseed rape in the UK. Aspects of Applied Biology 135-142.

Folman, L. B., Postma, J., and van Veen, J. A. 2003. Inability to find consistent bacterial biocontrol agents of *Pythium aphanidermatum* in cucumber using screens based on ecophysiological traits. FEMS Microbiology Ecology 45: 72-87.

Fordos J. 1859. Rec. Trav. Soc. Emul. Sci. Pharm. 3:30.

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

Fravel, D. R. 2005. Commercialization and implementation of biocontrol. Annual Review of Phytopathology 43: 337–59.

Friedrich, L., Lawton, K., Reuss, W., Masne,r P., Specker, N., Gut Rella ,M., Meier, B., Dincher, S., Staub, T., and Uknes, S.1996. A benzothiadiazole induces systemic acquired resistance in tobacco. Plant Journal 10:61-70.

Fry, W. E., Goodwin, S. B., Dyer, A. T., Matuszak, J. M., Drenth, A., Tooley, P. W., Sujkowski L. S, Koh, Y. J., Cohen, B. A., Spielman, L. J., Deahl, K. L., Ingli, D. A., and Sandlan, K. P. 1993. Historical and recent migrations of *Phytophthora infestans*: chronology, pathways, and implications. Plant Disease 77: 653–661.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. 1993. Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261:754-756.

Gall, C., Balesdent, M. H., Desthieux, I., Robin, P., and Rouxel, T. 1995. Polymorphism of Tox0 *Leptosphaeria maculans* isolates as revealed by soluble protein and isozyme electrophoresis. Mycological Research 99:221-229.

Garbeva, P., van Veen, J. A., and van Elsas, J. D. 2004. Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology 42: 243 – 270.

Garbeva, P., Voesenek, K. and van Elsas, J. D. 2004. Quantitative detection and diversity of the pyrrolnitrin biosynthetic locus in soil under different treatments. Soil Biology and Biochemistry 36: 1453–1463.

Gardiner, D. M., Jarvis, R. S., and Howlett, B. J. 2005. The ABC transporter gene in the sirodesmin biosynthetic gene cluster of *Leptosphaeria maculans* is not essential for sirodesmin production but facilitates self-protection. Fungal Genetics and Biology 42:257–263.

Gershon, H., Clarke, D. D., and Gershon, M. 1993. Reexamination of the thermolytic rearrangement of 4-halophenyl azides to 2-aminophenols and other products. Monatshefte für Chemie 124:367-379.

Gilbert, G. S, Parke, J, Clayton, M. K., and Handelsman, J. 1993. Effect of an introduced bacterium on bacterial communities of roots. Ecology 74:840-854.

Govrin, E. M., and Levine, A. 2000. The hypersensitive reaction facilitates plant infection by the necrotrophic fungus *Botrytis cinerea*. Current Biology 10:751-757.

Greer, M. A. 1950. Nutrition and goiter. Physiological Reviews 30: 513-538.

Gugel, R.K. and Petrie, G.A. 1992. History, occurrence, impact, and control of blackleg of rapeseed. Canadian Journal of Plant Pathology 14:36-45.

Guo, X. W., Fernando, W. G. D., and Entz., M. H. 2005. Effects of crop rotation and tillage on the blackleg disease of canola. Canadian Journal of Plant Pathology. 27:53-57.

Guo, X. W., Fernando, W. G. D., and Entz, M. 2006. Dynamics of Infection by *Leptosphaeria maculans* on Canola (*Brassica napus*) as Influenced by Crop Rotation and Tillage. Archives of Phytopathology and Plant Protection (*in press*).

Haas, D., and Keel, C. 2003. Regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease. Annual Review of Phytopathology 41:117-153.

Haas, D., Blumer, C. and Keel, C. 2000. Biocontrol ability of fluorescent pseudomonads genetically dissected: importance of positive feedback regulation. Current Opinion in Biotechnology. 11: 290–297.

Hall, R. 1992. Epidemiology of blackleg of oilseed rape. Canadian Journal of Plant Pathology, 14:46-55.

Hammer, P. E., Burd, W., Hill, D. S., Ligon, J. M.,and van Pee, K. H. 1999. Conservation of the pyrrolnitrin gene cluster among six pyrrolnitrin-producing strains. FEMS Microbiology Letters 180:39-44.

Hammer, P. E., Hill, D. S., Lam, S. T., van Pee, K. H. and Ligon, J. M. 1997. Four genes from *Pseudomonas fluorescens* that encode the biosynthesis of pyrrolnitrin. Applied and Environmental Microbiology 63: 2147–2154.

Hammerschmidt, R. 1999. Induced disease resistance: How do induced plants stop pathogens? Physiological and Molecular Plant Pathology 55:77-84.

Hammerschmidt, R., Nuckles, E. M., and Kuc, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotricum lagenarium*. Physiological and Molecular Pathology 20:73-82.

Hammond, K. E., and Lewis, B.G. 1987. The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans*. Plant Pathology 36:135-147.

Hammond, K. E., Lewis, B. G., and Musa, T.M. 1985. A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans*. Plant Pathology 34:557-565.

Handelsman, J., and Stabb, E. V. 1996. Biocontrol of soilborne plant pathogens. Plant Cell 8:1855-1869.

Hase, S., Van Pelt, J. A., Van Loon, L. C., and Pieterse, C. M. J. 2003. Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. Physiological and Molecular Plant Pathology 62:219-226.

Hassan, H. M. and Fridovich, I. 1980. Mechanism of the antibiotic action of pyocyanine. Journal of Bacteriology 141:156–163.

He, H., Silo-Suh, L. A., Clardy, J., and Handelsman, J. 1994. Zwittermicin A, an antifungal and plant protection agent from Bacillus cereus. Tetrahedron Letters 35:2499-2502.

Heeb, S., and Haas, D. 2001. Regulatory roles of the GacS/GacA two-component system in plant-associated and other gram-negative bacteria. Molecular Plant Microbe Interactions 14:1351–1363.

Henkel, and Cie, M.B.H. 1967. Brit. Patent 1, 132, 792.

Hennin, C., Hofte, M., and Diederichsen, E. 2001. Functional expression of *Cf9* and *Avr9* genes in *Brassica napus* induces enhanced resistance to *Leptosphaeria maculans*. Molecular Plant Microbe Interaction 14: 1075–1085.

Heungens, K., and Parke, J. L. 2000. Zoospore homing and infection events: Effects of the biocontrol bacterium *Burkholderia cepacia* AMMDR1 on two oomycete pathogens of pea (*Pisum sativum* L.). Applied and Environmental Microbiology 66:5192-5200.

Hiradate, S., Yoshida, S., Sugie, H., Yada, H., and Fujii, Y. 2002. Mulberry anthracnose antagonists (iturins) produced by *Bacillus amyloliquefaciens* RC-2. Phytochemistry 61:693-698.

Hodgetts, W. J. 1917. On the forcible discharge of spores of *Leptosphaeria acuta*. New Phytologist 16:139-146.

Hohlneicher, U., Hartmann, R., Taraz, K., and Budzikiewicz, H. 1995. Pyoverdin, Ferribactin, Azotobactin-a New Triade of Siderophores from *Pseudomonas chlororaphis* ATCC 9446 and Its Relation to *Pseudomonas fluorescens* ATCC 13525. Zeitschrift-fuer-Naturforschung-Section-C-Biosciences. 50: 337-344.

Hoitink, H. A. J., and Boehm, M. J. 1999. Biocontrol within the context of soil microbial communities: a substrate-dependent phenomenon Annual Review of Phytopathology 37:427-446.

Howell, C. R., Beier, R. C., and Stipanovic, R. D. 1988. Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of Pythium preemergence damping-off by the bacterium. Phytopathology. 78:1075-1078.

Howlett, B. J. 2004. Current knowledge of the interaction between *Brassica napus* and *Leptosphaeria maculans*. Canadian Journal of Plant Pathology 26:245-252.

Howlett, B. J., Idnurm, A., and Pedras, M. S. C. 2001. *Leptosphaeria maculans*, the causal agent of blackleg disease of *Brassicas*. Fungal Genetics and Biology. 33:1-14.

Hsieh, F. C., Li, M. C., Lin, T. C., and Kao, S. S. 2004. Rapid detection and characterization of surfactin-producing *Bacillus subtilis* and closely related species based on PCR. Current Microbiology 49:186-191.

Huang, H., Huang, J. W., Saidon, G., and Erickson, R. 1997. Effect of allyl alcohol and fermented agricultural wastes on carpogenic germination of sclerotia of *Sclerotinia sclerotiorum* and colonization of *Trichoderma* spp. Canadian Journal of Plant Pathology 9:43-46.

Huang, C. J., Wang, T. K., Chung, S. C. and Chen, C. Y. 2005. Identification of an antifungal chitinase from a potential biocontrol agent, *Bacillus cereus* 28-9. Journal of Biochemistry and Molecular Biology 38: 82-88.

Hwang, J., Chiton, W. S., and Benson, D. M. 2002. Pyrrolnitrin production by *Burkholderia cepacia* and biocontrol of Rhizoctonia stem rot of poinsettia. Biological Control 25:56–63.

Iavicoli, A., Boutet, E., Buchala, A., and Metraux, J. P. 2003. Induced systemic resistance in *Arabidopsis thaliana* in response to root inoculation with *Pseudomonas fluorescens* CHA0. Molecular Plant Microbe Interactions 16:851-858.

Ishii, H. 2006. Impact of fungicide resistance in plant pathogens on crop disease control and agricultural environment. Japan Agricultural Research Quarterly 40: 205 – 211.

Jacobsen, B. J., and Backman, P. A. 1993. Biological and cultural disease controls: Alternatives and supplements to chemicals in IPM systems. Plant Disease 77: 311-315.

Janisiewicz, W. J., and Korsten, L. 2002. Biological control of post-harvest diseases of fruits. Annual Review of Phytopathology 40:411–441.

Jensen, L. B. 1994. First record of ascomata of *Leptosphaeria maculans* on winter-grown oilseed rape in Denmark. Plant Pathology 43:759-761.

Johansson, P. M., and Wright, S. A. 2003. Low-temperature isolation of disease-suppressive bacteria and characterization of a distinctive group of Pseudomonads. Applied and Environmental Microbiology 69: 6464-6474.

Jones, H., Carmody, P., Stubbs, P., Walton, G., Brennan, R., Khangura, R., Berlandier, F., Michael, P., Piper, T., Mangano, P., Lemon, J., Herbert, A., Phelps, D., Minchin, B., and Stanley, M. 2001. Canola growing rgion of Western Australia. Printed by: Information and Media Services: Printing, South Perth, AGWEST. Agriculture Western Australia's website: http://www.agric.wa.goc.au/ento.

Kalbe, C., Marten, P. and Berg, G. 1996. Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. Microbiological Research 151: 433–439.

Kang, Y., Carlson, R., Tharpe, W., and Schell, M. A. 1998. Characterization of genes involved in biosynthesis of a novel antibiotic from *Burkholderia cepacia* BC11 and their role in biological control of *Rhizoctonia solani*. Applied and Environmental Microbiology 64:3939-3947.

Kavitha, K., Mathiyazhagan, S., Sendhilvel, A., Nakkeeran, S., Chandrasekar, G., and Fernando, W. G. D. 2005. Broad spectrum action of phenazine against active and dormant structures of fungal pathogens and root knot nematode. Archives of Phytopathology and Plant Protection 38:69-76.

Kazan, K., Rusu, A., Marcus, J. P., Goulter, K. C., and Manners, J. M. 2002. Enhanced quantitative resistance to *Leptosphaeria maculans* conferred by expression of a novel antimicrobial peptide in canola (*Brassica napus* L.). Molecular Breeding 10: 63–70.

Keel, C., Schider, U., Maurhofer, M., Voisard, C., Laville, J., Burger, P., Wirthner, P., Hass, D., and Defago, G. 1992. Suppression of root diseases by *Pseudomonas fluorescens* CHA0: importance of the secondary metabolite 2, 4- diacetylphloroglucinol. Molecular Plant Microbe Interactions 5: 4-13.

Keel, C., Weller, D. M., Natsch, A., Défago, G., Cook, R. J., and Thomashow, L. S. 1996. Conservation of the 2, 4-diacetylphloroglucinol biosynthesis locus among fluorescent *Pseudomonas* strains from diverse geographic locations. Applied and Environmental Microbiology 62:552-563.

Keel, C., Wirthner, P., Oberhansli, T., Voisard, C., Burger-, Haas, D., and Defago, G. 1990. Pseudomonads as antagonists of plant pathogens in the rhizosphere: role of the antibiotic 2,4-diacetylphloroglucinol in the suppression of black root rot of tobacco. Symbiosis Rehovot 9:327-341.

Keri, M. 2000. Genetic studies of host-pathogen interaction between Brassica napus and *Leptosphaeria maculans*. PhD Thesis Dissertation. University of Manitoba.

Kharbanda, P.D., and Tewari, J.P. 1996. Integrated management of canola diseases using cultural methods. Canadian Journal of Plant Pathology 18:168-175.

Kharbanda, P. D., Yang, J., Beatty, P., Jensen, S., and Tewari, J. P. 1999. Biocontrol of *Leptosphaeria maculans* and other pathogens of canola with *Paenibacillus polymyxa* PKB1. Proceedings of the 10th International Rapeseed Congress, Canberra, Australia. http://www.regional.org.au/papers/index/htm.

Kirner, S., Hammer, P. E., Hill, D. S., Altmann, A., Fischer, I., Weislo, L. J., Lanhan, M., van Pee, K.H., Ligon, J.M., 1998. Functions encoded by pyrrolnitrin biosynthetic genes from *Pseudomonas fluorescens*. Journal of Bacteriology 180: 1939–1943.

Kishore, K.G., Pande, S. and Podile, A. R. 2005. Biological control of late leaf spot of peanut (*Arachis hypogaea*) with chitinolytic bacteria. Phytopathology 95: 1157-1165.

Kloepper, J. W., Leong, J., Teintze, M., and Schroth, M. N. 1980. Pseudomonas siderophores: a mechanism explaining disease suppressive soils. Current Microbiology 4:317-320.

Kloepper, J. W., Ryu, C. M., and Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology 94:1259-1266.

Kloepper, J. W., Wei, G., and Tuzun, S. 1992. Rhizosphere population dynamics and internal colonization of cucumber by plant growth-promoting rhizobacteria which induce systemic resistance to *Colletotricum orbiculare.* In "Biological Contol of Plant Diseases", ed. New York: Tjamos, E. S., Plenum Press, p: 185-191.

Koch, E., Song, K., Osborn, T. C., and Williams, P. H. 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Molecular Plant Microbe Interactions 4:341-349.

Kolte, S. J. 1985. Diseases of annual edible oilseed crops. Vol. II. Rapeseed, mustard and sesame diseases. CRC Press, Inc, Boca Raton, FL, USA. pp.135.

Koumoutsi, A., Chenm X. H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J., and Borriss, R. 2004. Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. Journal of Bacteriology 186:1084-1096.

Kurle, J. E., Grau, C. R., Oplinger, E. S., and Mengistu, A. 2001. Tillage, crop sequence and cultivar effects on Sclerotinia stem rot incidence and yield in soybean. Agronomy Journal 93: 973–982.

Kuswinanti, T., Sock, J., and Hoppe, H. H. 1995. Variation in virulence of aggressive isolates of *Leptosphaeria maculans* based on cotyledon reactions on an extended differential set. Proceedings of the 9th International Rapeseed Congress., 4-7 July 1995, Cambridge, UK. pp. 1248-1250.

Lamey, H. A. 1995. Survey of blackleg and Sclerotinia stem rot of canola in North Dakota in 1991 and 1993. Plant Disease 79:322-324.

Lamey, H. A., and Hershman, D. E. 1993. Black leg of canola (*Brassica napus*) caused by *Leptosphaeria maculans* in North Dakota. Plant Disease 77:1263.

Leeman, M., Den Ouden, F. M., Van Pelt, J. A.., Dirkx, F. P. M., Steijl, H., Bakker, P. A. H. M. and Schippers, B. 1996. Iron availability affects induction of systemic resistance to fusarium wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86:149-155.

Leeman, M., Van Pelt, J. A., Den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M., and Schippers, B. 1995a. Induction of systemic resistance against fusarium wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. Phytopathology 85:1021–1027.

Leeman, M., VanPelt, J.A., Den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M., and Schippers, B.1995b. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. European Journal of Plant Pathology 101:655–664.

Leeman, M., den Ouden, F. M., van Pelt, J. A., Cornelissen, C., Matamala-Garros, A., Bakker, P. A. H. M., and Schippers, N. 1996. Suppression of Fusarium wilt of raddish by co-inoculation of fluorescent *Pseudomonas* spp. and root-colonizing fungi. European Journal of Plant Pathology 102:21-31.

LePage, R., and Penaud, A. 1995. Tout se joue avec le premier pic d' ascospores. CETIOM – Oleoscope 28: 23-27 (in French) (referred from West et 2001).

Liao, X., Vining, L. C., and Doull. 1995. Physiological control of trophopahse-ididophase separation in streptomycete cultures producing secondary metabolites. Canadian Journal of Microbiology 41:309-315.

Lidert, Z. 2001. Biopesticides: Is there a path to commercial success? In *Enhancing Biocontrol Agents and Handling Risks*, ed. MVurro, J Gressel, T Butt, GE Harman, A Pilegram, et al., p. 283. Amsterdam: IOS Press. 295 pp.

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann, D., Kempf, H. J., and Van Pee, K. H. 2000. Natural products with antifungal activity from Pseudomonas biocontrol bacteria. Pest Management Science.56:688-695.

Ligon, J. M., Hill, D. S., Hammer, P. E., Torkewitz, N. R., Hofmann, D., Kempf, H.J. and van Pee, K. H. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. Pesticide Management Science 56: 688–695.

Lin, T. S., Chen, C. L., Chang, L. K., Tschen, J. S. and Liu, S. T. 1999. Functional and transcriptional analyses of a fengycin synthetase gene, *fenC*, from *Bacillus subtilis*. Journal of Bacteriology 181: 5060–5067.

Lindow, S. E. 1987. Competitive exclusion of epiphytic bacteria by ice-negative *Pseudomonas syringae* mutants. Applied and Environmental Microbiology 53:2520-2527

Loper, J. E., and Buyer, J. S. 1991. Siderophores in microbial interactions on plant surfaces. Molecular Plant Microbe Interaction 4: 5-13.

Loper, J. E., and Henkels, M. D. 1999. Utilization of heterologous siderophores enhances levels of iron available to *Pseudomonas putida* in the rhizosphere. Applied and Environmental Microbiology 65:5357-5363.

Lugtenberg, B.J.J., Dekkers, L.C., Bansraj, M., Bloemberg, G.V., Camacho, M., Chin-A-Woeng, T.F.C., van den Hondel, C., Kravchenko, L., Kuiper, I., Lagopodi, A.L., Mulders, I., Phoelich, C., Ram, A., Tikhonovich, I., Tuinman, S., Wijffelman, C., and Wijfjes, A. 1999. *Pseudomonas* genes and traits involved in tomato root colonization. *In*:

De Wit PJGM, Bisseling T, Stiekema WJ, (*Editors*). 1999 IC-MPMI Congress Proceedings: biology of plant–microbe interactions, Vol. 2. St Paul, MN, USA: International Society for Molecular Plant–Microbe Interactions, pp:324–330.

Mackie, A. E., and Wheatley, R. E. 1999. Effects of the incidence of volatile organic compound interactions between soil bacterial and fungal isolates. Soil Biology and Biochemistry 31:375-385.

Maget-Dana, R., and Peypoux, F. 1994. Iturins, a special class of pore-forming lipopeptides: Biological and physicochemical properties. Toxicology 87:151-174

Mahuku, G. S., Hall, R., and Goodwin, P. H. 1996. Co-infection and induction of systemic acquired resistance by weakly and highly virulent isolates of *Leptosphaeria maculans* in oilseed rape. Physiological and Molecular Plant Pathology 49:61-72.

Malamy, J., Carr, J. P., Klessig, D. F., and Raskin, I.1990. Salicylic acid: a likely endogenous signal in the resistance response of tobacco to viral infection. Science 250:1002-1004.

Manitoba Agriculture Food and Rural Initiatives. 2006. Citinig online sources: Manitoba's Canola Industry Profile. Available from: http://www.gov.mb.ca/agriculture/statistics/pdf/aac04s06.pdf (accessed 15 May 2006)

Marcroft, S. J., Sprague, S. J., Pymer, S .J., Salisbury, P. A. and Howlett, B. J. 2004. Crop isolation, not extended rotation length, reduces blackleg (*Leptosphaeria maculans*) severity of canola (*Brassica napus*) in south-eastern Australia. Australian Journal of Experimental Agriculture 44:601-606.

Matthijs, S., Tehrani, K. A., Laus, G., Jackson, R. W., Cooper, R. M., and Cornelis, P. 2007. Thioquinolobactin, a *Pseudomonas* siderophore with antifungal and anti-*Pythium* activity. Environmental Microbiology 9: 425–434.

Maurhofer, M, Keel, C, Schnider, U, Voisard, C, Haas D., and Defago, G. 1992. Influence of enhanced antibiotic production in Pseudomonas fluorescens strain CHA0 on its disease suppressive capacity. Phytopathology 82:90-195.

Mavrodi, D. V., Blankenfeldt, W., and Thomashow, L. S. 2006. Phenazine Compounds in Fluorescent *Pseudomonas* Spp. Biosynthesis and Regulation. Annual Review of Phytopathology. 44:417-445.

Mavrodi D. V., Ksenzenko, V. N., Bonsall, R. F., Cook, R. J., Boronin, A. M., and Thomashow, L. S. 1998. A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. Journal of Bacteriology 180:2541-2548.

Mavrodi, O.V., McSpadden Gardener, B.B., Mavrodi, D.V., Bonsall, R.F., Weller, D.M., and Thomashow, L.S 2001. Genetic diversity of *phlD* from 2,4-diacetylphloroglucinol-producing fluorescent *Pseudomonas* spp. Phytopathology 90: 35-43.

Mayerhofer, R., Bansal, V. K., Thiagarajah, M. R., Stringam, G. R., and Good, A. G. 1997. Molecular mapping of resistance to *Leptosphaeria maculans* in Australian cultivars of *Brassica napus*. Genome 40: 294-301.

Mazzola, M., Cook, R. J., Thomashow, L. S., Weller, D. M., and Pierson, L. S. 1992. Contribution of phenazine antibiotic biosynthesis to the ecological competence of fluorescent pseudomonads in soil habitats. Applied and Environmental Microbiology 58:2616–2624.

McCleary, B, V., and Shameer, I. 1987. Assay of malt beta-glucanse using azo-barley glucan – an improved precipitant. Journal of the Institute of Brewing 93:87-90.

McDowell, J. M., and Dang, J. L. 2000. Signal transduction in the plant immune response. Trends in Biochemical Sciences 25-79-82.

McGee, D. C. 1977. Blackleg (*Leptosphaeria maculans* (Desm.) Ces. & de Not.) of rapeseed in Victoria: sources of infection and relationships between inoculum, environmental factors and disease severity. Australian Journal of Agricultural Research 28:53-62.

McGee, D. C. and Petrie, G. A. 1978. Variability *of Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology 68:625-630.

McSpadden Gardener, B. B. 2007. Diversity and ecology of biocontrol *Pseudomonas* spp. in agricultural systems. Phytopathology 97:221-226.

McSpadden Gardener, B. B., and Fravel, D. R. 2002. Biological control of plant pathogens: Research, commercialization, and application in the USA. Online. Plant Health Progress doi:10.1094/PHP-2002-0510-01-RV.

McSpadden Gardener, B. B., Mavrodi, D. V., Thomashow, L. S., and Weller, D. M. 2001. A rapid polymerase chain reaction-based assay characterizing rhizosphere populations of 2,4-diacetylphloroglucinol-producing bacteria. Phytopathology 91: 44-54.

McSpadden-Garderner, B. B., Schroeder, K. L., Kalloger, S. E., Raaijmakers, J, M., Thomashow, L. S., and Weller, D. M. 2000. Genotypic and phenotypic diversity of *phlD*-containing Pseudomonas strains isolated from the rhizosphere of wheat. Applied and Environmental Microbiology 66:1939-1946.

Menkhaus, M., Ullrich, C., Kluge, B., Vater, J., Vollenbroich, D., and Kamp, R. M. 1993. Structural and functional organization of the surfactin synthetase multienzyme system, Journal of Biological Chemistry 268: 7678-7684.

Metraux, J. P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. 1991. Induced resistance in cucumber in response to 2, 6-dichloroisonicotinic acid and pathogens. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol 1. Edited by Hennecke H, Verma DPS. DPS: Kluwer Academic Publishers;:432-439.

Métraux, J. P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. 1990. Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science 250:1004–1006.

Meziane, H., Sluis, I. V. D., Van Loon, L. C., Hofte, M., and Bakker, P. A. H. M. 2005. Determinants of *Pseudomonas putida* WCS358 involved in inducing systemic resistance in plants. Molecular Plant Pathology 6:177-185.

Milner, J. L., Stohl, E. A., and Handelsman, J. 1996. Zwittermicin A resistance gene from *Bacillus cereus*. Journal of Bacteriology 178:4266-4272.

Mitchell, R., and Hurwitz, E. 1965. Suppression of *Pythium debaryanum* by lytic rhizosphere bacteria. Phytopathology 55:156-158.

Moyne, A. L., Cleveland, T. E., and Tuzub, S. 2004. Molecular characterization and analysis of the operon encoding the antifungal lipopeptide bacillomycin D. FEMS Microbiology Letters 34:43-49.

Moyne, A. L., Shelby, R., Cleveland, T. E., and Tuzun, S. 2001. Bacillomycin D: An iturin with antifungal activity against *Aspergillus flavus*. Journal of Applied Microbiology 90:622-629.

Mulligan, C. N. 2005. Environmental applications for biosurfactants. Environmental Pollution 133:183–198.

Nakano, M. M., Magnuson, R., Myers, A., Curry, J., Grossman, A. D., and Zuber, P. 1991. SrfA is an operon required for surfactin production, competence development and efficient sporulation in *Bacillus subtilis* .Journal of Bacteriology 173: 1770-1778.

Nakayama, T., Homma, Y., Hashidoko, Y., Mitzutani, J., and Tahara. S. 1999. Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. Applied and Environmental Microbiology 65: 4334–4339.

Natsch, A., Keel, C., Pfirter, H. A., Haas, D., and Defago, G. 1994. Contribution of the global regulator gene *gacA* to persistence and dissemination of *Pseudomonas fluorescens* biocontrol strain CHA0 introduced into soil microcosms. Applied and Environmental Microbiology 60:2553–2560.

Neeno-Eckwall, E. C., and Schottel, J. L. 1999. Occurrence of antibiotic resistance in the biological control of potato scab disease. Biological Control 16:199-208.

Nelson, E. B. 2004. Microbial dynamics and interactions in the spermosphere. Annual Review of Phytopathology 42:271–309.

Nelson, E. B., Chao, W. L., Norton, J. M., Nash, G. T., and Harman, G. E. 1986. Attachment of *Enterobacter cloacae* to hyphae of *Pythium ultimum* possible role in the biological control of pythium preemergence damping-off. Phytopathology 76:327-335.

Nishikori, T., Naganawa, H., Muraoka, Y., Aoyagi, T., and Umezawa, H. 1986. Plispastins; new inhibitors of phospholopase A2, produced by Bacillus cereus BMG302-fF67.III. Structural elucidation of plispastins. Journal of Antibiotics 39:755-761.

Ohlson, R. 1972. Production and trade in rapeseed. *In* Rapeseed cultivation, composition, processing and utilization. *Edited by* L.A. Appleqvist. American Elsevier Publishing Company, Inc, New York, NY. pp. 9-35.

Okada, A., Banno, S., Ichiishi, A., Kimura, M., Yamaguchi, I., and Fujimura, M. 2005. Pyrrolnitrin Interferes with Osmotic Signal Transduction in *Neurospora crassa.* Journal of Pesticide Science 30:378–383.

Olivain, C., Alabouvette, C. and Steinberg, C. 2004. Production of a mixed inoculum of *Fusarium oxysporum* Fo47 and *Pseudomonas fluorescens* C7 to control *Fusarium* diseases. Biocontrol Control Science and Technology 14:227–238.

Ongena, M., Jacques, P., Touré, Y., Destain, J., Jabrane, A., and Thonart, P. 2005. Involvement of fengycin-type lipopeptides in the multifaceted biocontrol potential of *Bacillus subtilis* Applied Microbiology and Biotechnology 69:29–38.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J. L. and Thonart, P. 2007. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environmental Microbiology 9:1084–1090.

Orson , J. 1995. *In* Brassica Oilseeds: Production and utilization. *Edited By* D. Kimber and D.I. McGregor. CAB International, Wallington, UK. pp. 93-109.

Osburn, R.M., Milner, J. L., Oplinger, E.S., Smith, R. S., and Handelsman, J. 1995. Effect of *Bacillus cereus* UW85 on the yield of soybean at two field sites in Wisconsin. Plant Disease 79:551-556.

O'Sullivan, D. J., and O'Gara, F. 1992. Traits of Fluorescent Pseudomonas spp. Involved in Suppression of Plant Root Pathogens. Microbiological Reviews 56:662-676.

Parbery, D. G. 1996. Trophism and the ecology of fungi associated with plants. Biological Reviews of the Cambridge Philosophical Society 71:473-527.

Parke, J. L. 1990. Population dynamics of *Pseudomonas cepacia* in the pea spermosphere in relation to biocontrol of *Pythium*. Phytopathology 80:1307-1311.

Park, R. Y., Choi, M. H., Sun, H. Y., and Shin, S. H. 2005. Production of Catechol-Siderophore and Utilization of Transferrin-Bound Iron in *Bacillus cereus.* Biological & Pharmaceutical Bulletin 28:1132 -1135.

Pedras, M. S. C. 1998a. Towards an understanding and control of plant fungal diseases in Brassicaceae. Recent Research Developments in Agricultural and Food Chemistry 2:513–532.

Pedras, M. S. C. 1998b. Phytoalexins from crucifers: Mimicking or overcoming plant chemical defenses? Recent Research Developments in Phytochemistry 2:259–267.

Pedras, M. S. C., and Biesenthal, C. J. 2000. HPLC analyses of cultures of *Phoma* spp.: Differentiation among groups and species through secondary metabolite profiles. Canadian Journal of Microbiology 46:685–691.

Pedras, M. S. C., Nycholat, C. M., Montaut, S., Xu, Y. M., and Khan, A. Q. 2002. Chemical defenses of crucifers: elicitation and metabolism of phytoalexins and indole-3-acetonitrile in brown mustard and turnip. Phytochemistry 59:611-625.

Pedras, M. S. C., and Okanga, F. I. 1999. Strategies of cruciferous pathogenic fungi: Detoxification of the phytoalexin cyclobrassinin by mimicry. Journal of Agricultural and Food Chemistry 47:1196–1202.

Pedras, M. S. C., and Séguin-Swartz, G. 1992. The "blackleg" fungus: Phytotoxins and phytoalexins. Canadian Journal of Plant Pathology 14:67–75.

Pedras, M. S. C., and Taylor, J. L. 1993. Metabolism of the phytoalexin brassinin by the blackleg fungus. Journal of Natural Products 56:731–738.

Pedras, M .S. C., Okanga, F. I., Zaharia, I. L, and Khan, A. Q. 2000. Phytoalexins from crucifers: Synthesis, biosynthesis, and biotransformation. Phytochemistry 53:161–176.

Pedras, M. S. C., Séguin-Swartz, G., and Abrams, S. R. 1990. Minor phytotoxins from the blackleg fungus *Phoma lingam.* Phytochemistry 29: 777–782.

Pedras, M. S. C., Smith, K. C., and Taylor, J. L. 1998. Production of 2,5-dioxopiperazine by a new isolate type of the blackleg fungus *Phoma lingam.* Phytochemistry 49:1575–1577.

Pedras, M. S. C., Taylor, J. L., and Nakashima, T. T. 1993. A novel chemical signal from the blackleg fungus: Beyond phytotoxins and phytoalexins. Journal of Organic Chemistry 58:4778–4780.

Pepper, J. L. and Dowd, S. E. 2002. PCR application for plant and soil microbes. *In*: Manual of environmental Microbiology. *Edited by* Hurst, C.J., Crawford, R. L., Knusden, G.R., McInerney, M.J., and Stetzenbach, L.D. ASM Press, Wahington, DC, USA. pp. 573-582.

Petrie, G. A. 1978. Occurrence of a highly virulent strain of blackleg (*Leptosphaeria maculans*) on rape in Saskatchewan (1975-77). Canadian Plant Disease Survey 58:21-25.

Petrie, G.A. 1979. Prevalence of a highly virulent strain of *Leptosphaeria maculans* (blackleg) in seed samples of rape and turnip rape produced in Western Canada in 1976 and 1977. Canadian Journal of Plant Science 59:899-901.

Petrie, G. A., Mortensen, K., and Dueck, J. 1985. Blackleg and other diseases of rapeseed in Saskatchewan, 1978 to 1981. Canadian Plant Disease Survey 65:35-41.

Petrie, G. A., Mortensen, K., and Dueck, J. 1985. Blackleg and other diseases of rapeseed in Saskatchewan, 1978 to 1981. Canadian Plant Disease Survey 65:35-41.

Peypoux, F., Besson, F., Michel, G. and Delcambe, L. 1981. Structure of Bacillomycin D, a New Antibiotic of the Iturin Group. European Journal of Biochemistry 118: 323-327.

Peypoux, F., Bonmatin, J. M., and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. Applied Microbiology and Biotechnology 51:553-563.

Picó, Y., Font, G., Ruiz, M. J., and Fernández, M. 2006. Control of pesticide residues by liquid chromatography-mass spectrometry to ensure food safety. Mass Spectrometry Reviews 25: 917– 960.

Pierson, L. S. and Thomashow, L. S. 1992. Cloning and heterologous expression of the phenazine biosynthetic locus from *Pseudomonas aureofaciens* 30-84. Molecular Plant Microbe Interaction 5:330–339.

Pieterse, C. M. J., Van Wees, S. C. M., Hoffland, E., Van Pelt, J. A., and Van Loon, L. C. 1996. Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell 8:1225-1237.

Pieterse, C. M. J., Van Wees, S. C. M., Ton, J., Van Pelt, J. A., and van Loon, L. C. 2002. Signalling in rhizobacteria-induced systemic resistance in *Arabidopsis thaliana*. Plant Biology 4:535-544.

Pieterse, C. M. J., van Wees, S. C. M., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, N., Weisbeek, P. J., and van Loon, L. C. 1998. A novel signaling pathway controlling induced systemic resistance in Arabidopsis. Plant Cell 10:1571-1580.

Pieterse, C. M. J., Van Pelt, J. A., Van Wees, S. C. C., Ton, J., Kloosterziel, K. M. L., Keurentjes, J. J. B., Verhagen, V. W. M., Knoester, M., Sluis, I. V. D., Bakker, P. A. H. M and Van Loon L. C. 2001. Rhizobacteria-mediated induced systemic resistance: triggering, signaling and expression. European Journal of Plant Pathology 107: 51-61.

Pleban, S., Chernin, L., and Chet, I. 1997. Chitinolytic enzymes of an endophytic strain of *Bacillus cereus*. Letters in Applied Microbiology 25:284-288.

Plummer, K. M., Dunse, K., and Howlett, B. J. 1994. Non-aggressive strains of the blackleg fungus, *Leptosphaeria maculans*, are present in Australia and can be distinguished from aggressive strains by molecular analysis. Australian Journal of Botany 42:1-8.

Poritsanos, N. J. 2005. Molecular mechanisms involved in the secondary metabolite production and biocontrol of *Pseudomonas chlororaphis* PA23. Masters Thesis Desertation, University of Manitoba.

Pozo, M. J., van Loon, L. C., and Pieterse, C. M. J. 2005. Jasmonates- Signals in plant-microbe interactions. Journal of Plant Growth Regulation 23:211-222.

Press, C. M., Wilson, M., Tuzun, S., and Kloepper, J. W. 1997. Salicylic acid produced by *Serratia marcescens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. Molecular Plant Microbe Interactions 10:761-768.

Purwantara, A., Barrins, J. M., Cozijnsen, A. J., Ades, P. K., and Howlett, B. J. 2000. Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. Mycological Research 104:772-781.

Raaijmakers, J. M., Bonsall, R. F., and Weller, D. M. 1999. Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. Phytopathology 89:470-475.

Raaijmakers, J. M., Vlami, M., and de Souza, J. T. 2002. Antibiotic production by bacterial biocontrol agents. Antonie van Leeuwenhoek 81:537-547.

Raaijmakers, J. M., and Weller, D. M. 1998. Natural plant protection by 2,4-diacetylphlorogluconol producing *Pseudomonas* spp. in take-all decline soils. Molecular Plant Microbe Interaction 11:144-152.

Raaijmakers, J. M., and Weller, D. M. 2001. Exploiting genotypic diversity of 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp.: characterization of superior root-colonizing *P. fluorescens* strain Q8r1-96. Applied and Environmental Microbiology 67:2545-2554.

Raaijmakers, J. M., Weller, D. M., and Thomashow, L. S. 1997. Frequency of antibiotic producing Pseudomonas spp. in natural environments. Applied and Environemntal Microbiology 63:881-887.

Raffel, S. J., Stabb, E. V., Milner, J. L., and Handelsman, J. 1996. Genotypic and phenotypic analysis of zwittermicin Aproducing strains of *Bacillus cereus*. Microbiology 142:3425-3436.

Ramamoorthy, V., Viswanathan, R., Raguchander, T., Prakasam, V., and Samiyappan, R., 2001. Induction of systemic resistance by plant growth promoting rhizobacteria in crop plants against pests and diseases. Crop Protection 20, 1–11.

Ramarathnam, R., and Fernando, W. G. D. 2006. Preliminary phenotypic and molecular screening for potential bacterial biocontrol agents of *Leptosphaeria maculans,* the blackleg pathogen of canola. Biocont Sci Technol "in press"

Ran, L. X., Li, Z. N., Wu, G. J., van Loon, L. C., and P.A.H.M. Bakker. 2005. Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp. European Journal of Plant Pathology 113:59–70.

Raupach, G. S., and Kloepper, J. W. 1998. Mixtures of plant growth-promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology 88:1158–1164.

Ravnskov, S., Larsen, J., and Jakobsen, I. 2002. Phosphorus uptake of an arbuscular mycorrhizal fungus is not effected by the biocontrol bacterium *Burkholderia cepacia.* Soil Biology & Biochemistry 34: 1875–1881.

Redekop, B. 2002. Plant Breeder started new farm industry. Winnipeg Free Press, Thursday, Jan. 10, 2002.

Rempel, C. B., and Hall. R. 1993. Dynamics of production of ascospores of *Leptosphaeria maculans* in autumn on stubble of the current year's crop of spring rapeseed. Canadian Journal of Plant Pathology 15:182-184.

Rimmer, S. R. 2006. Resistance genes to *Leptosphaeria maculans* in *Brassica napus.* Canadian Journal of Plant Pathology 28: S288-S297.

Rimmer, S. R., and Berg, C. G. J. 1992. Resistance of oilseed Brassica spp. to blackleg caused by *Leptosphaeria maculans*. Canadian Journal of Plant Pathology 14:56-66.
Rimmer, S. R., and Buchwaldt, L. 1995. Diseases. *In* Brassica Oilseeds: Production and utilization. *Edited By* D. Kimber and D.I. McGregor. CAB International, Wallington, UK. pp. 111-140.

Rogalsky, J. R., McNair, V., Mace, P., and Adolp, D. 1980. Lesson 1. In "Rapeseed '80". January 14-March, 1980. pp. 101-144.

Roongsawang, N., Thaniyavarn J., Thaniyavarn, S., Kameyama, T.; Haruki, M., Imanaka, T., Morikawa M., and Kanaya, S. 2002. Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: Bacillomycin L, plipastatin, and surfactin. Extremophiles 6:499-506

Roos, I. M. M. and Hattingh, M. J. 1986. Resident populations of *Pseudomonas syringae* on stone fruit tree leaves in South Africa. Phytophylactica 18: 55-58.

Rosales, A. M., Thomashow, L., Cook, R. J., and Mew, T. W. 1995. Isolation and identification of antifungal metabolites produced by rice-associated antagonistic *Pseudomonas* spp. Phytopathology 85: 1028-1032.

Rouxel, T., Kollmann, A., Boulidard, L., and Mithen, R. 1991. Abiotic elicitation of indole phytoalexins and resistance to *Leptosphaeria maculans* within *Brassiceae*. Planta 184:271–278.

Rouxel, T., Sarniguet, A., Kollmann, A., and Bosquet, J. F. 1989. Accumulation of a phytoalexin in *Brassica* spp. in relation to a hypersensitive response to *Leptosphaeria maculans.* Physiological and Molecular Plant Pathology 34:507–517.

Rozen, S., and Skaletsky, H. J. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology.* Humana Press, Totowa, NJ, pp 365-386.

Russel, P. E. 1995. Fungicide resistance: occurrence and management. Journal of Agricultural Science 124:317-323.

Ryan, A. D., and Kinkel, L. L. 1997. Inoculum density and population dynamics of suppressive and pathogenic *Streptomyces* strains and their relationship to potato scab biocontrol. Biological Control 10:180-186.

Ryu, C. M., Hu, C. H., Reddy, M. S., and Kloepper, J. W. 2003. Different signaling pathways of induced resistance by rhizobacteria in *Arabidopsis thaliana* against two pathovars of *Pseudomonas syringae*. New Phytologist 160:413-420.

Ryu, C. M., Farag, M. A., Hu, C. H., Reddy, M. S., Kloepper, J. W., and Pare, P. W. 2004. Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology 134:1017-1026.

Sadoff, H. L. 1972. Sporulation antibiotics of *Bacillus* species. In Spores V ed. Halvorson, H. O., Hanson, R., and Campbell, L. L. Bethesda: American Society of Microbiology. pp:157-166.

Salisbury, P. A., Ballinger, D. J., Wratten, N., Plummer, K. M., and Howlet, B. J. 1995. Blackleg disease on oilseed Brassica in Australia: a review. Australian Journal of Experimental Agriculture 35:665-672.

Sansford, C. 1995. Oilseed rape: development of stem canker (*Phoma lingam*) and its effect on yield. Proceedings of the 9th International Rapeseed Congress., 4-7 July 1995, Cambridge, UK. pp. 577-579.

Sascha, D., Eppelmann, K. and Marahiel, M. A. 2002. Heterologous expression of nonribosomal peptide synthetasese in *B. subtilis*: construction of a bi-directional *B. subtilis/E. coli* shuttle vector system. FEMS Microbiology Letters 216:185–191.

Savchuk, S. 2002. Evaluation of biological control of *Sclerotinia sclerotiorum* on canola (*Brassica napus L.*) in the laboratory, in the greenhouse and in the field. M.Sc. Thesis Dissertation, University of Manitoba.

Schmidli-Sacherer, P., Keel, C., and Défago, G. 1997. The global regulator GacA of *Pseudomonas fluorescens* CHA0 is required for suppression of root diseases in dicotyledons but not in gramineae. Plant Pathol. 46:80-90.

Schmidt-Eisenlohr, H., Gast, A., and Baron, C. 2003. Inactivation of *gacS* gene does not affect the competitiveness of *P. chlororaphis* in the *Arabidopsis thaliana* rhizosphere. Applied and Environmental Microbiology 69:1817-1826.

Schoonbeek, H. J., Raaijmakers, J. M., and Waard, M. A. D. 2002. Fungal ABC transporters and microbial interactions in natural environments. Molecular Plant Microbe Interactions 15: 1165–1172.

Schumann, G. (*Editor*) 1991. Plant Diseases: Their Biology and Social Impact. APS Press, USA.

Seo, H. S., Song, J. T., Cheong, J. J., Lee, Y. H., Hwang, I., Lee, J. S., and Chol, Y. D. 2001. Jasmonic acid carboxyl methyltransferase: A key enzyme for jasmonate-regulated plant responses. Proceedings of the National Academy of Science 98:4788-4793.

Sharifi-Tehrani, A., Zala, M., Natsh, A., Moënne-Loccoz, Y., and Defago, G. 1998. Biocontrol of soil-borne fungal diseases by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp., with different restriction profiles of amplified 16S rDNA. European Journal of Plant Pathology 104:631-643.

Shoda, M. 2000. Bacterial control of plant diseases. Journal of Bioscience and Bioengineering 89:515–521..

Shoemaker, R. A., and Brun, H. 2001. The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans*. Canadian Journal of Botany 79:412-419.

Shojania, S., McComb, M. E., Oleschuk, R. D., Perreault, H., Gesser, H. D., Chow, A., 1999. Qualitative analysis of complex mixtures of VOCs using the inside needle capillary adsorption trap. Canadian Journal of Chemistry 77:1716-1727.

Silo-Suh, L. A., Lethbridge, B .J., Raffel, S. J., He, H., Clardy, J., and Handelsman, J. 1994. Biological activities of two fungistatic antibiotics produced by *Bacillus cereus* UW85. Applied and Environmental Microbiology 60:2023-2030.

Silo-Suh, L. A., Stabb, E. V., Raffel, S. J. and Handelsman, J. 1998. Target range of zwittermicin A, an aminopolyol antibiotic from *Bacillus cereus.* Current Microbiology 37:6–11.

Singh, D. 1958. Rape and mustard. Indian Central Oilseeds Committee., Bombay, pp.235.

Singh, J., and Faull, J. 1988. Antagonism and biological control. In Biological Control of Plant Diseases (Vol II). Edited by Mukerjii, K and Garg, K. CRC Press, Florida. Pp167-177.

Sjödin, C., Nyhammar, T., and Glimelius, K. 1988. Effects of toxic metabolites produced by *Phoma lingam* (Tode ex Fr.) Desm. on protoplasts, cells and plants of hosts and non-hosts of the pathogen. Physiological and Molecular Plant Pathology. 32:301–312.

Slininger, P. J., Burkhead, K. D., Schisler, D. A., and Bothast, R. J. 2000. Isolation, accumulation and identification of 2-acetamidophenol in the liquid cultures of wheat take all biocontrol agent *Pseudomonas fluorescens* 2-79. Applied Microbiology and Biotechnology 54:376-381.

Sock, J., and Hoppe, H.H. 1999. Pathogenicity of sirodesmin-deficient mutants of *Phoma lingam.* Journal of Phytopathology 147:169–173.

Somda, I. and Brun, H. 1995. Occurrence of *Leptosphaeria maculans* on mustards in Brittany, France. Plant Disease 79:754.

Somda, I., Harkous, S., and Brun, H. 1997. Bipolar heterothallism in B-group isolates of *Leptosphaeria maculans*. Plant Pathology 46:890-896.

Somda, I., Renard, M., and Brun. H. 1996. Morphology, pathogenicity and isozyme variation amongst French isolates of *Leptosphaeria maculans* recovered from *Brassica juncea* cv. Picra. Plant Pathology 45:1090-1098.

Sovero, M. 1993. Rapeseed, a new oilseed crop for the United States. *In* New Crops. *Edited by* J. Janick and J.E. Simon. Wiley, New York. Available from http://www.hort.purdue/edu/newcrop/proceedings1993/v2-302.html (accessed 23 March 2001)

Special crops, Publication 1944. Manitoba Department of Agriculture and Immigration, Winnipg. pp. 3.

Stachelhaus, T., Schneider, A., and Marahiel, M. A. 1995. Rational design of peptide antibiotics by targeted replacement of bacterial and fungal domains. Science (Washington D C) 269:69-72.

Statistics Canada. 2003. Extraction system of agricultural statistics.

Staswick, P.E., Yuen, G.Y, and Lehman, G.C. 1998. Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. Plant Journal 15:747-754.

Steller, S., Sokoll, A., Wilde, C., Bernhard, F., Franke, P. and Vater, J. 2004. Initiation of Surfactin Biosynthesis and the Role of the SrfD-Thioesterase Protein. Biochemistry 43:11331-11343.

Sticher, L., Mauch-Mani, B., and Metraux, J. P.1997. Systemic acquired resistance. Annual Review of Phytopathology 35:235-270.

Stohl, E. A., Milner, J. L., and Handelsman, J. 1999. Zwittermicin A biosynthetic cluster. Gene 237:403-411.

Strange, R. N. 1993. Plant Disease Control: Towards Environmentally Acceptable Methods. Chapman & Hall, NY.

Stutz, E.W., Defago, G., and Kern, H. 1986. Naturally occurring fluorescent pseudomonads involved in the suppression of black root rot of tobacco. Phytopathology 76:181-185.

Szlávik, T., Barasits., and Fernando. W. G. D. 2006. First Report of Pathogenicity Group-3 of *Leptosphaeria maculans* on winter rape in Hungary. Plant Disease 90:684 published on-line as DOI: 10.1094/PD-90-0684C.

Tekauz, A., and Howard, R. J. 1988. Conservation tillage – Effect on crop diseases. *In* Land Degradation and Conservation Tillage. Partial Proceedings of the 34 Annual CSSS/AIC meeting, 21-24 Aug, Calgary, Alberta. pp. 215-226b.

Tewari, J. P., Shinners, T. C., and Briggs, K. G. 1997. Production of calcium oxalate crystals by two species of Cyathus in culture and in infested plant debris. Verlag der Zeitschrift fuÈ r Naturforschung 52:42-425.

Thomashow, L. S., Bonsall, R. F., and Weller, D. M. 2002. Antibiotic production by soil and rhizosphere microbes in situ. *In*: Manual of environmental Microbiology. *Edited by* Hurst, C.J., Crawford, R. L., Knusden, G.R., McInerney, M.J., and Stetzenbach, L.D. ASM Press, Wahington, DC, USA. pp. 638-647.

Thomashow, L. S., Bonsall, R. F., and Weller, D. M. 1997. Antibiotic production by soil and rhizosphere microbes in situ. *In*: Manual of environmental Microbiology. *Edited by* Hurst, C.J., Knusden, G.R., McInerney, M.J., Stetzenbach, L.D and Walter M.D. ASM Press, Wahington, DC, USA. pp. 493-499.

Thomashow, L. S., and Weller, D. M. 1988. Role of phenazine antibiotic from P*seudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici.* Journal of Bacteriology 170:3499-3508.

Thomashow, L. S., and Weller, D. M. 1996. Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. *In*: Plant-Microbe Interactions, Vol. 1. *Edited by.* Stacey, G. and Keen, N. Chapman & Hall, New York, USA pp.187-235.

Thomashow, L.S., Weller, S.D., Bonsall, R.F., and Pierson, L.S. 1990. Production of antibiotic phenazine-1-carboxylic acid by fluorescent *Pseudomonas* in the rhizosphere of wheat. Applied and Environmental Microbiology 56: 908-912.

Thomma, B. P. H. J., Pennickx, I. A. M. A., Cammue, B. P. A., and Broekaert, W. F. 2001. The complexity of disease signaling in *Arabidopsis*. Current Opinion in Immunology 13:63-68.

Timms-Wilson, T. M., Ellis, R. J., Renwick, A., Rhodes, D. J., Mavrodi, D. V., Weller, D. M., Thomashow, L.S., and Bailey, M.J. 2000. Chromosomal insertion of phenazine-1-carboxylic acid biosynthetic pathway enhances efficacy of damping-off disease control by *Pseudomonas fluorescens*. Molecular Plant Microbe Interactions 13:1293-1300.

Tjamos, E. C., Tsitsigiannis, D. I., Tjamos S. E., Antoniou, P. P., and Katinakis, P. 2004. Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. European Journal of Plant Pathology 110:35-44.

Trauger, J. W., Kohll, R. M., Mootz, H. D., Mahariel, M. A. and Walsh, C.T. 2000. Peptide cyclization catalyzed by the thioesterase domain of tyrocidine synthetase. Nature 407: 215–218.

Tsuge, K., Akiyama, T., and Shoda, M. 2001. Cloning, sequencing and characterization of the iturin A operon. Journal of Bacteriology 183:6265-6273.

Turkington, T. K., Clayton, G. W. Klein-Gebbinck, H., and Woods, D. L. 2000. Residue decomposition and blackleg of canola: influence of tillage practices. Canadian Journal of Plant Pathology 22:150-154.

Ullrich, C., Kluge, B., Palacz, Z., and Vater, J. 1991. Cell-free biosynthesis of surfactin, a cyclic lipopeptide produced by *Bacillus subtilis.* Biochemistry 30: 6503-6508.

Umezawa, H., Aoyagi, T., Nishikiori, T., Okuyama, A., Yamagishi, Y., Hamada, M. and Takeuchi, T. 1986. Plipastatins: new inhibitors of phospholipase A2, produced by *Bacillus cereus* BMG202-fF67. I. Taxonomy, production, isolation and preliminary characterization. Journal of Antibiotics 39: 737–744.

Valois, D., Fayad, K., Barbasubiye, T., Garon, M., Dery, C., Brzezinski, R., and Beaulieu, C. 1996. Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi,* the causal agent of raspberry root rot. Applied and Environmental Biology 62:1630-1635.

Van Dijk, K., and Nelson, E. B. 2000. Fatty acid competition as a mechanism by which *Enterobacter cloacae* suppresses *Pythium ultimum* sporangium germination and damping-off. Applied and Environmental Microbiology 66:5340-5347.

Van Loon, L. C. 1997. Induced resistance in plants and the role of pathogenesis-related proteins. European Journal of Plant Pathology 103:753-765.

Van Loon, L. C. and Bakker, P. A. H. M. 2005. Induced systemic resistance as a mechanism of disease suppression by rhizobacteria. *In*: PGPR: Biocontrol and Biofertilization. *Edited by* Z.A. Siddiqui, Springer, Dordrecht, The Netherlands. pp.39-66.

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J.1998. Systemic resistance induced by rhizosphere bacteria. Annual Review of Phytopathology 36:453-483.

Van Loon, L. C. and Van Strien, E. A. 1999. The families of pathogenesis related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiological and Molecular Plant Pathology 55:85-97.

van Pee, K. H., Salcher, O. and Lingens, F. 1980. Formation of pyrrolnitrin and 3-(2-amino-3-chlorophenyl)pyrrole from 7- chlorotryptophan. Angewandte Chemie International Edition in English 19: 828.

Van Peer, R., and Schippers, B. 1992. Lipopolysaccharides of plant growth promoting *Pseudomonas* sp. strain WCS417r induce resistance in carnation to fusarium wilt. Netherlands Journal of Plant Pathology 98:129–139.

Van Sinderen, D., Galli, G., Cosmina, P., de Ferra, F., Withoff, S., Venema, G., and Grandi, G. 1993. Characterization of the srfA locus of *Bacillus subtilis*: only the valine-activating domain of *srfA* is involved in the establishment of genetic competence. Molecular MIcrobiology 8: 833-841.

Van Wees, S. C., Luijendijk, M., Smoorenburg, I., Van Loon, L. C. & Pieterse, C. M. J. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense related genes but stimulates the expression of the jasmonate inducible gene *Atvsp* upon challenge. Plant Molecular Biology 41:537–549.

Van Wees, S. C. M., Pieterse, C. M. J., Trijssenaar, A., 'T-Westende, Y. A. M., Hartog, F., and van Loon, L. C. 1997. Differential induction of systemic resistance in Arabidopsis by biocontrol bacteria. Molecular Plant Microbe Interactions 10:716-724.

Vanittanakom, N., Loeffler, W., Koch, U. and Jung, G. 1986. Fengycin—a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. Journal of Antibiotics 39: 888–901.

Vanterpool, T. C. 1961. Rape diseases in Saskatchewan in 1961. Canadian Plant Disese Survey 44:372-373.

Vanterpool, T. C. 1963. Blackleg (Phoma lingam). Canadian Plant Disease Survey 48:214.

Vater, J., Gao, X., Hitzeroth, G., Wilde, C., and Franke, P. 2002. "Whole Cell" – Matrix – Assisted Laser Desorption Ionization – Time of Flight Mass Spectrometry, an emerging technique for efficient screening of biocombinatorial libraries of natural compounds – present state of research. Combinatorial Chemistry and High Throughput Screening 6:557-667.

Verhagen, B. W. M., Glazebrook, J., Zhu, T., Chang, H. S., van Loon, L. C. and. Pieterse, C. M. J. 2004. The transcriptome of rhizobacteria-induced systemic resistance in *Arabidopsis.* Molecular Plant Microbe Interactions 17:895-908.

Vijayan, P., Shockey, J., Levesque, C. A., Cook, R. J., and Browse, J. 1998. A role for jasmonate in pathogen defense in *Arabidopsis*. Journal of Experimental Botany 53-747-756.

Vining, L. C. 1990. Functions of secondary metabolites. Annual Review of Microbiology 44:395-427

Volksch, B. and May, R. 2001. Biological control of *Pseudomonas syringae* pv. *glycinea* by epiphytic bacteria under field conditions. Microbiology Ecology 41:132-139.

Vollenbroich, D., Mehta, N., Zuber, P., Vater, J., and Kamp, R. M. 1994. Analysis of surfactin synthetase subunits in *srfA* mutants of *Bacillus subtilis* OKB 105.Journal of Bacteriology 176: 395-400.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Metraux J. P., and Ryals, J. A. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.

Webster, J. 1993. Introduction to fungi. Second edition. Cambrige University Press, Cambridge, UK. pp:390.

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

Weller, D. M. 2007. *Pseudomonas* biocontrol agents of soilborne pathogens: Looking back over 30 years. Phytopathology 97:250-256.

Weller, D. M., Howie, W. J., and Cook, R. J. 1988. Relationship between in-vitro inhibition of *Gaeumannomyces graminis* var *tritici* and suppression of take-all of wheat by fluorescent pseudomonads. Phytopathology 78:1094-1100.

Weller, D. M., Zhang, B. X., and Cook, R. J. 1985. Application of a rapid screening test for the selection of bacteria suppressive to take-all of wheat. Plant Disease 69:710-713.

West, J. S., Evans, N., Liu, S., Hu, B., and Peng L. 2000. *Leptosphaeria maculans* causing stem canker of oilseed rape in China. New Disease Reports http://www.bspp.org.uk/ndr/2000/2000-3.htm].

West, J. S., Kharbanda, P. D., Barbetti, M. J., and Fitt, B. D. L. 2001. Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. Plant Pathology 50:10-27.

Whipps, J. M. 1997. Development in the biological control of soil-borne plant pathogens. Advances in Botanical Research 26:1-134.

Whipps, J. M. 2001. Microbial interactions and biocontrol in the rhizosphere. Journal of Experimental Botany 52:487-511.

Whistler, C. A., Corbell, N. A., Sarniguet, A., Ream, W., and Loper, J. E. 1998. The two-component regulators of GacS and GacA influence accumulation of the stationary-phase sigma factor _s and the stress response in *Pseudomonas fluorescens* Pf-5. Journal of Bacteriology 180:6635–6641.

Williams, P. H. 1992. Biology of *Leptosphaeria maculans*. Canadian Journal of Plant Pathology 14:30-35.

Williams, R.H., and Fitt, B.D.L. 1999. Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. Plant Pathology 48:161-175.

Wilson, M., Campbell, H. L., Ji, P. Jones, J.B. and Cuppels, D. A. 2002. Biological control of bacterial speck of tomato under field conditions at several locations in North America. Phytopathology, 92: 1284–1292.

Wright, S. A. I., Zumoff, C. H., Schneider, L., and Beer, S. V. 2001. *Pantoea agglomerans* strain EH318 produces two antibiotics that inhibit *Erwinia amylovora* in vitro. Applied and Environmental Microbiology 67:284-292.

Yamaguchi, I. 1996. Pesticides of microbial origin and applications of molecular biology. In L. G. Cropping (ed.), Crop protection agents from nature: natural products and analogues. The Royal Society of Chemistry, London. United Kingdom. p 27-49.

Yan, A. 1990. Overview of rapeseed production and research in China. Proc. Int. Canola Conf. Potash and Phosphate Institute, Atlanta, pp. 29-35.

Yan, Z., Reddy, M. S., Ryu, C.M., McInroy, J. A., Wilson, M., and Kloepper, J. W. 2002. Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. Phytopathology 92:1329-1333.

Yao, S., Gao, X., Fuchsbauer, N., Hillen, Wolfgang., Vater, J., and Wang, J. 2003. Cloning, sequencing and characterization of the genetic region relevant to biosynthesis of

the lipopeptides iturin A and surfactin in *Bacillus subtilis*. Current Microbiology 47:272-277.

Yoshida, S., Shirata, A., and Hiradate, S. 2002. Ecological characteristics and biological control of mulberry anthracnose. Japan Agricultural Research Quarterly 36:89-95

Yu, G. Y., Sinclair, J. B., Hartman, G. L., and Bertagnolli, B. L. 2002. Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani*. Soil Biology and Biochemistry 34:955-963.

Yu, F., Lydiate, D. J., and Rimmer. S. R. 2005. Identification of two novel genes for blackleg resistance in *Brassica napus*. TAG Theoretical and Applied Genetics 110:969-979.

Zhang, Y. 2005. Biocontrol of sclerotinia stem rot of canola by bacterial antagonists and study of biocontrol mechanisms involved. M Sc Thesis Dissertation. Univerity of Manitoba.

Zhang, Y., Daayf, F., Fernando, W. G. D. 2004. Induced resistance against sclerotinia in canola mediated by bacterial biocontrol agents. In: Proceedings of the International Joint Workshop on PR-Proteins and Induced Resistance – Copenhagen, Denmark

Zhang, Y., Fernando, W. G. D., de Kievit, T., Berry, C., Daayf, F., and Paulitz, T. 2006. Detection of antibiotic-related genes from bacterial biocontrol agents with polymerase chain reaction. Canadian Journal of Microbiology 52: 476-481.

Zhang, S., Moyne, A. L., Reddy, M. S. and Kloepper, J. W. 2002. The role of salicylic acid in induced systemic resistance elicited by plant growth-promoting rhizobacteria against blue mold of tobacco. Biological Control 25:288-296.