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BIOLOGICAL CONTROL OF *LEPTOSPHAERIA MACULANS* BY USING A STRAIN OF *PAENIBACILLUS POLYMYXA*

By

Jian Yang 

A thesis submitted to the faculty of graduate studies and research

in partial fulfillment of the requirements for the degree of Doctor of Philosophy

In

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Biological Control of *Leptosphaeria maculans* by Using a Strain of *Paenibacillus polymyxa*** submitted by **Jian Yang** in partial fulfillment of the requirements for the degree of Doctor of Philosophy **in** Plant Science.

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ABSTRACT

A strain of the bacterium, *Paenibacillus polymyxa* PKB1, isolated from canola stubble was found to have an inhibitory effect against *Leptosphaeria maculans*, causal agent of the blackleg disease of canola. Research was conducted to evaluate the potential of *P. polymyxa* PKB1 as a biocontrol agent for the blackleg of canola.

P. polymyxa PKB1 significantly inhibited germination of the pycnidiospores of *L. maculans* and reduced the viability of this fungus and that of several other pathogenic fungi. None of the fungicides and herbicides tested was deleterious to the growth of the bacterium on nutrient agar. Results suggest that *P. polymyxa* PKB1 has a potential value as a biocontrol agent for managing the blackleg of canola.

In growth chamber experiments, Tilt® significantly reduced the number of pycnidia of *L. maculans*, whereas the bacterium significantly reduced viability of the fungus in the infected canola stubble. In field experiments, Tilt®, bacterium, and compost, in combination, significantly reduced the formation of ascospores, as well as survival of the fungus on infected canola stubble. There is a potential of developing an integrated control method for managing the blackleg disease of canola. Compost could be used as a carrier of the bacterium for field application.

Randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and digoxigenin-labelling techniques were used to develop DNA probes specific for *P. polymyxa* PKB1. Four species-specific probes were produced that hybridized with only the DNA of *P. polymyxa* strains but not with that of other *Bacillus* spp. Eight primers were designed, synthesized and tested with 13 strains of *P. polymyxa* and 10 strains of *Bacillus* spp. The designed primers specifically amplified only one band of *P. polymyxa* PKB1 and related strains of *P. polymyxa*.

A total of 152 isolates of *L. maculans* collected in western Canada were tested for genetic variability by using the RAPD-PCR technique. Virulent and weakly virulent isolates of *L. maculans* were clearly distinguishable and three major groups were identified within the virulent isolates. *P. polymyxa* PKB1 showed an inhibitory effect to the isolates from different groups. Results suggested that *P. polymyxa* PKB1 has a wide biocontrol spectrum and can inhibit isolates from different groups of *L. maculans* collected from western Canada.

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TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Chapter 1

Literature Review

Introduction

The crop rapeseed/canola (*Brassica napus* L.; *B. rapa* L.) is grown world-wide and is a major source of vegetable oil. Blackleg caused by *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. [conidial state: *Phoma lingam* (Tode: Fr.) Desmaz.] is one of the important diseases of this crop. Blackleg has been reported in 49 countries and is a serious yield-limiting factor all over the world where rapeseed is extensively grown (Garbrielson, 1983; Gugel and Petrie, 1972; Punithalingam and Holiday, 1972; Salisbury et al., 1995). It threatened the establishment of a rapeseed industry in Australia and caused a serious epidemic in the early 70's (Bokor et al., 1975). Blackleg is the major disease of rapeseed in parts of Europe (Gladders, 1995; Gladders and Musa, 1980; Jedryczka et al., 1999b; Philips et al., 1999). Severe blackleg in rapeseed has also occurred in several states in the U.S.A. (Hershman and Perkins, 1995; Lamey and Hershman, 1993; Lamey, 1995; Mengistu et al., 1990a). It was not reported in the United States until 1989 when a localized epidemic developed in south central Kentucky (Mengistu et al., 1990b), although the fungus was presentin the United States for many years, and was later reported in North Dakota in 1991 (Lamey and Hershman, 1993). The conidial stage (*P. lingam*) of the blackleg fungus was observed in Argentina in 1991 (Gaetan, 1995).

In Canada, the weakly virulent *P. lingam* strain was first found in seed samples in Saskatchewan in 1957 (Vanterpool, 1957), and superficial stem lesions were first observed on oil rape in 1961 (Vanterpool, 1961). These discoveries were considered to be of minor importance. A virulent strain was isolated from rapeseed residues in Saskatchewan in 1975 (Petrie, 1978). Since then, the disease has spread rapidly in western Canada (Evans et al., 1990, 1991, 1992, 1993, 1995; Gugel and Petrie, 1992; Hall, 1992; Harrison et al., 1995; Jesperson, 1989, 1990, 1994; Kharbanda, 1993; Kharbanda et al., 1988, 1989; Kirkham, 1992; Mathur and Platford, 1994; Petrie, 1978, 1985, 1993; Platford and van den Berg, 1989; van den Burg and Platford, 1991). In 1983 in Alberta, the virulent strain of *L. maculans* was found for the first time, in a field near Vermilion (Kharbanda and Stevens, 1983). In the 80's and early 90's, the disease spread to both spring and winter rapeseed in central Canada, especially in the winter-type cultivars of *B. napus* (Assabgui and Hall, 1990; Hall, 1992; Peters & Hall, 1987). Later, the disease was found in Peace River regions of British Columbia and Alberta (Harrison et al., 1995; Jesperson, 1994). A disease survey in Alberta showed

that the disease was occurring in most canola-growing areas (**Figure 1.1**). Crop losses caused by blackleg, in Canada alone, exceeded 50 million dollars annually before the introduction of resistant cultivars (Kharbanda, 1993). In Alberta, the virulent isolate of *L. maculans* was listed as a pest under the Agriculture Pest Act in 1984.

Host, Pathogen and Disease

Host: Brassicaceae is a large and varied family including many domesticated species. It consists of more than 200 genera, including *Brassica*. Brassica vegetables and oilseeds were domesticated whenever and wherever the economic value of the locally adapted weed was recognized. Because of their ability to survive and grow at relatively low temperatures, they are well adapted to cultivation at higher elevations and as winter crops in the temperate regions.

Three *Brassica* species, *B. napus* L., *B. rapa* L. (syn. *B. campestris* L.) and *B. juncea* Czern., commonly known as rapeseed, are the world's major vegetable-oil producers. Brassica oil crops annually occupy millions of acres of the world's agricultural lands and provide over 8% of the world's edible vegetable oil (Downey, 1983). In Canada, Brassica oil crops are the major source of edible oil that accounts for 63% of the vegetable oil and 82% of the salad-oils consumption (Bergh, 1991).

The seeds of the rapeseed crops contain over 40% oil and over 40% protein in the dry meal after oil extraction (Downey, 1983). Canola is a genetic variation of rapeseed developed by Canadian plant breeders specifically for its nutritional qualities, particularly for its low level of saturated fat. In the 50's, the nutritional aspects of rapeseed oil, especially the eicosenoic and erucic fatty acid contents were questioned. Also, the high glucosinolates were found to affect the meal quality of rapeseed. In the 70's and early 80's, the development of low erucic acid in rapeseed oil and low glucosinolate in the meal created an entirely new natural vegetable oil with physical and nutritional properties. To distinguish this new oil from all previously known vegetable oils, the term "canola" was registered in Canada. It describes rapeseed containing less than 2% erucic acid as a percentage of the total fatty acids in the oil and less than 30 μmoles g^{-1} of glucosinolates in the oil-free meal (Bergh, 1991; Downey, 1983). The first "double low" variety, Tower, was produced in 1974. Now, all rapeseed varieties grown in Canada are canola standard. It

is one of the major cash crops, and approximately 13.5 million acres were planted in 1998 in Canada. It produced almost 7.6 million tonnes of canola seed valued at almost $3 billion (Statistics Canada, 1999).

Pathogen: *Leptosphaeria maculans* is an ascomycetous fungus of the order Pleosporales. Its anamorph is *Phoma lingam*, a deuteromycete of the order Sphaeropsidales (Agrios, 1997). The first description of the fungus was made by Tode (1791) as a saprophyte of cabbage (*B. oleracea rubra*) and was named *Sphaeria lingam* (Tode ex Fr.). According to Henderson (1918), the parasitic relationship of the fungus with a living plant of the genus *Brassica* was first recorded in France in 1849 by Desmaziere, who later transferred the fungus to the genus *Phoma*. The fungus has also been named *Phoma brassica* (Thum.), *P. oleracea*, *P. napobrassicae* (Rostrup), *Plenodomus lingam* (Tode *ex* Fr.) Hohn, *Phyllosticta brassica* Westend (Ndimande, 1976; Punithalingam and Holiday, 1972; Williams, 1992). In addition, the non-pathogenic *P. herbarum* Westend and the weakly parasitic *P. exigua* have also been reported on crucifers (Boerema, 1976).

Smith (1956) first discovered the perfect stage of *P. lingam* and he named it *Leptosphaeria napi* (Fuckel) Sacc. Later Smith and Sutton (1964) and Boerema and van Kesteren (1964) changed the name to *L. maculans*. Synonyms for the perfect state are *Sphaeria maculans* (Desm.), *S. napi* Fuck. and *Pleospora maculans* (Desm.) Tul., *P. napi* Fuck. (Boerema and van Kesteren, 1964; Punithalingam and Holiday, 1972). Ndimande (1976) has thoroughly reviewed the history of the pathogen.

In the anamorph stage, the fungus produces an asexual fruiting body pycnidium that contains pycnidiospores. Pycnidia on stems and leaves are immersed, erupment, gregarious, variable in shape and with narrow ostioles. Ndimande (1976) described two pycnidial phenotypes: type I was dark brown to black in color, and variable in shape, size and intensity of color, and type II was black, dense, and variable in shape, and formed singly or confluently. Pycnidiospores are single-celled, hyaline, cylindrical, with a guttule at each end of the spore (Ndimende, 1976).

The ascocarps (pseudothecia) of the perfect stage of *L. maculans* are formed on the debris of host plants in the presence of two compatible mating types (A, a or +, -). Pseudothecia are globose, black, and with protruding ostioles. Asci are cylindrical to clavate, sessile, and contain eight ascospores. The ascospores are 5-septate, yellowish-tan in color, fusiform and cylindrical, and with slight or no constriction at the central septum. The pseudoparaphysis are filiform, hyaline, and septate (Punithalingam and Holiday, 1972).

The fungus can also produce pseudothecia *in vitro* under special conditions (Hill and Williams, 1988; Mengistu et al., 1990a, 1993, 1995; Petrie and Lewis, 1985; Somda et al., 1997; Xu and Williams, 1987). Gall (1994) reported that only Tox^+ isolates (highly virulent, group A) were able to produce the sexual stage *in vitro*. Tox^+ isolates produce phytotoxins, sirodesmin PL and phomenoic acid. Differences among isolates in their ability to produce phytotoxins are discussed under pathogen-host interactions.

In culture, it has been sometimes observed that spontaneous sectors developed from colonies of *L. maculans*, and they have been proved to be stable when transferred several times to new media (Lange, 1993; Ndimande, 1976). It was believed that this probably resulted from mutation in the original mycelium (Ndimande, 1976).

Disease: The disease cycle of blackleg of canola is shown in **Figure 1.2**. The fungus is seed- and stubble-borne (Wood and Barbetti, 1977). It can survive long periods in the seed coats of *Brassica*. The pathogen overwinters on infected canola stubble and produces the sexual fruiting bodies, pseudothecia, with asci and ascospores in them. Rain-splashed pycnidiospores and airborne ascospores serve as important sources of primary inocula that are dispersed to new crops (Bokor et al., 1975; Kharbanda, 1993; McGee, 1974; Ndimande, 1976) and cause disease. Spores landing on the host cotyledons and leaves germinate and produce germ tubes that enter the host tissues through stomata or wounds (Hammend and Lewis, 1986, 1987). Hyphae grow intercellularly (symptomless) and later cause cell degradation and death, resulting in a necrotic lesion on the host. The infection hyphae grow further into the stem through the petiole and induce a stem canker (Hammond and Lewis, 1987). Pycnidia are produced in lesions. In nature, the fungus persists in a saprophytic mode, colonizing dead residual tissues. Pseudothecia are formed continuously on the host stubble and discharge ascospores (Gladders and Musa, 1980; McGee and Petrie, 1979; Petrie, 1995; Rempel and Hall, 1993). The production of ascospores is greatly affected by temperature, moisture, light, and nutrients (Petrie, 1994). Ascospores are formed in the same year on winter canola stems in Ontario (Rempel and Hall, 1993). In western Canada, ascospores are formed on the overwintering canola stubble and discharged in the next spring and early summer (McGee and Petrie, 1979) and continue to discharge from the stubble for 3 – 5 years (Petrie, 1995). Pseudothecia of *L. maculans* on canola stubble discharge ascospores within one week of harvest in Kentucky, U.S.A. (Hershman and Perkins, 1995). Secondary inoculum consists mainly of pycnidiospores

produced on infected canola plants and ascospores from infected stubble of previous years. Ascospores are effectively dispersed by wind over a few kilometers (Hall, 1992), whereas pycnidiospores are distributed primarily by rain-splash within short distances and cause secondary infection under suitable conditions. Lesions develop on leaves, stems, and pods and produce more pycnidia. Ascospores appear to be more infective than pycnidiospores (Wood and Barbetti, 1977). Infection by ascospores is affected by temperature and wetness duration (Biddulph et al., 1999). Primary infection of seedlings from the ascospore inoculum results in latent infection on both susceptible and resistant cultivars although the period of latent infection is much shorter on a resistant cultivar (Xi et al., 1991b). It was also observed that latent infection was present in commercial canola cultivars (*B. napus*) as well as in wild crucifers such as stinkweed (*Thlaspi arvense*) infected by different *L. maculans* strains (Petrie et al., 1995). *L. maculans* isolates were also found in symptomless *T. arvense*, *Descurainia sophia*, and *Capsella bursa-pastoris*. In the absence of a canola crop, weed species may also act as a reservoir of inoculum. Up to three isolates occurred simultaneously in individual stems of *T. arvense* and *B. napus*. The potential for exchange of genetic material among closely related strains could lead to the generation of new pathotypes.

The disease affects all plant parts. However, stem and crown canker are the most destructive manifestations of the disease on *Brassica* plants. Virulent isolates cause severe infections that result in girdling, stem splitting, wilting and lodging, whereas weakly virulent isolates cause only superficial infection on the stems without destructive damage. Cankers restrict the flow of nutrients and moisture within the conductive tissues in the stem, causing premature ripening. Stems of infected plants are often blackened internally. Crown cankers may extend below the soil surface (Gugel and Petrie, 1992; Hall, 1992).

Pathogen Variability

L. maculans isolates exhibit considerable variability in cultural morphological characteristics, pathogenicity and virulence. This variability was observed as early as in 1918 (Henderson, 1918). Based on the examination of 400 isolates of *P. lingam*, Cunningham (1927) classified these isolates into two types according to their cultural and pathogenic characteristics: (1) relatively slow-growing and strongly pathogenic on *Brassica* sp. and (2) faster growing and weakly pathogenic on *Brassica* sp. Further evidence proved that blackleg isolates were broadly divisible into two groups, virulent and weakly virulent isolates, based on their ability to attack *Brassica* (Bonman et al., 1981; Humpherson-Jones, 1983; Koch et al. 1989;

McGee and Petrie, 1978). The virulent isolates of *L. maculans* can cause infections on cotyledons, leaves, roots, stems, pods and seeds. On the stem, usually on the stem base, these isolates cause cankers that can kill the plant. Numerous pycnidia are present at the center of lesion, and pycnidiospores ooze out when the conditions are suitable. The weakly virulent isolates generally cause only superficial lesions on plants late in the growing season and are not of economic significance. Also, the sporulation of weakly virulent isolates is relatively slow, less amount and late than virulent isolates (Gugel and Petrie, 1992). Kharbanda (1993) evaluated 350 isolates collected in Alberta and found considerable variations in their morphological characteristics, amount of aerial mycelium, production of pycnidia, color of pigmentation in the medium, and overall colony appearance. The isolates were classified into 15 groups according to their cultural characteristics. Of the 67 isolates collected in Alberta, Saskatchewan and Manitoba, 67% were classed as being "aggressive" and the rest as being "non-aggressive" (Lange, 1993). The two groups of isolates differed in cultural morphology and pathogenicity (Hacziwskyj and Drysdale, 1984; Hill et al., 1984; Johnson and Lewis, 1994; Kutcher, 1990), soluble protein and isozyme patterns (Balesdent et al., 1992; Gall et al., 1995; Sippll et al., 1988), polygalacturonase zymograms (Annis and Goodwin, 1997a), production of sirodesmin (Koch et al., 1989), glucose phosphate isomerase (Brun et al., 1997; Sippll et al., 1995), cell wall degradation enzymes (Annis and Goodwin, 1996), extracellular enzymes and fungal surface proteins (Hassan et al., 1991). The variability could be detected based on seedling and adult plant evaluation on *Brassica* species (Ballinger and Salisbury, 1996). Immunochemical techniques were also used to differentiate virulent and weakly virulent isolates (Dahiya, 1988; Stace-Smith et al., 1993). Different terms were used to describe virulent isolates, such as 'highly virulent', 'aggressive', 'A', 'Tox^{+}' or '$sirodesmin^{+}$', while terms 'non-aggressive', 'avirulent', 'B', 'NA', 'Tox^{0}' (0 means no toxin) or '$sirodesmin^{0}$' were used for weakly virulent isolates.

DNA analysis showed considerable genetic variations between the virulent and weakly virulent isolates. DNA polymorphism in *L. maculans* was first described in the repetitive DNA sequences (Johnson and Lewis, 1990). Since then, extensive investigations have been conducted to distinguish these two groups of isolates, using the analysis of restriction fragment length polymorphism (RFLP) analysis (Koch et al., 1991; Patterson and Kapoor, 1995), karyotype analysis (Taylor et al., 1991), random amplified polymorphic DNA -polymerase chain reactions (RAPD-PCR) (Goodwin and Annis, 1991; Hassan et al.,

1991; Keri and Rimmer, 1997; Meyer et al., 1992; Plummer et al., 1994; Schafer and Wostemeyer, 1992, 1994), amplified fragment length polymorphism (AFLP) analysis (Pongam et al., 1999), repetitive element based (rep)-PCR (Jedryczka et al., 1999a), and ribosomal DNA sequences (Balesdent et al., 1998; Morales et al., 1993a, 1995; Xue et al., 1992). Due to the great differences between these two groups of isolates, it has been proposed that they belong to different species (Pedras et al., 1995). It was found that the weakly virulent isolates could produce a yellow pigment. The secondary metabolite profiles and the molecular genetic characteristic studies indicated that the weakly virulent isolate was closely related *to Phoma wasabiae* (Pedras et al., 1995). Jedryczka et al. (1999a) used the Rep-PCR technique to characterize 90 isolates of *L. maculans* collected from Poland and found that 93.3% belonged to the "non-aggressive" species. The blackleg disease completely devastated some oilseed rape crops in north-western Poland in the middle 1980s. It was suggested that the world-wide *L. maculans* population is actually comprised of at least two species and that the role of non-aggressive isolates in blackleg disease needs further investigation.

An unusual repetitive element (LMR1) from the highly virulent isolates of *L. maculans* was identified, cloned and sequenced (Taylor and Borgmann, 1994). This element could be transferred to the weakly virulent isolate naturally (rare case) and caused the isolate became a virulent type. It was suggested that some gene(s) encoding pathogenicity factors of the virulent isolates were coincidentally attached to the transposable element that was transferred.

In recent years, a number of 'weakly virulent' isolates of *L. maculans* of rapeseed were found to be virulent on wasabi (*Wasabia japonica*, Japanese horseradish); others were found to produce toxins. Jedryczka et al. (1999a, b) reported that 'sirodesmin^{0}' (no sirodesmin produced) isolates caused disease on *B. napus* in Poland. It is possible that sirodesmin production is not related to the virulence of *L. maculans* isolates. Weakly virulent isolates could mate in vitro. All the new findings support that these two groups of isolates represent different taxons. The taxonomic re-evaluation of the strains has been carried out by French and Canadian scientists (Seguin-Swartz, 2000).

Within the virulent group, isolates can be further divided into three pathogenicity groups according to the differential interaction phenotypes expressed on cotyledons, hypocotyls and stems of *Brassica* species (Koch et al., 1991; McGee and Petrie, 1978; Newman, 1984). Koch et al. (1991) developed a set of four differential *B. napus* cultivars and classified *L. maculans* isolates into four pathogenicity groups (PG1

– PG4). Weakly virulent strains fell into PG1, based on the cotyledon reaction of the susceptible cultivar Westar. Virulent strains were divided into three groups (PG2 – PG4) on the basis of their reaction on the cultivars Westar, Glacier and Quinta. By using three additional *B. napus* lines, isolates of *L. maculans* from Europe and Australia were divided into five pathogenicity groups based on host-pathogen interactions (Keri and Rimmer, 1997). Recently, virulent isolates from various regions of Germany were differentiated into six pathogenicity groups based on an expanded set of *Brassica napus* differentials, Lirabon, Quinta, Glacier and Jet Neuf (Kuswinanti et al., 1999). Western Canadian isolates fell into PG2 only (Kutcher et al., 1993; Mengistu et al., 1991; Rimmer and van den Berg, 1992), and PG4 was predominant in Ontario, Canada (Mahuku et al., 1997). Isolates collected from south central Kentucky were mostly in PG4; a small number were in PG3 but none in PG2 (Hershman and Perkins, 1995), whereas virulent isolates from North Dakota belonged to PG2 only (Lamey and Hershman, 1993). Kharbanda (1993) tested 350 isolates including representative isolates from the University of Wisconsin and found that the representative isolates did not give the reaction as reported. It was suggested that the cultivars used in their study did not prove to be truly differential hosts. To determine the pathogenicity group of an isolate, methodology was a limiting factor for conclusion of virulence (Williams, 1992). Purification of differential hosts, temperature and the amount of inoculum used may affect the results of the test.

As more molecular tools become available, more genetic variations among *L. maculans* isolates are being detected. DNA analysis gave variable results regarding the relationship between DNA patterns and pathogenicity groups. No relationship between RAPD patterns and PGs was found in 93 highly virulent isolates of *L. maculans* (Mahuku et al., 1997), whereas a consistent match of RAPD pattern and PGs was observed in another study by Keri and Rimmer (1997). A partial relationship between the pathogenic phynotype and RFLP data was demonstrated (Koch et al., 1991). A total of 49 virulent isolates representing three pathogenicity groups (PG2, 3 and 4) from different geographical areas was tested for the variation, using AFLP analysis (Pongam et al., 1999). It was found that isolates from western Canada, North Dakota and Georgia belonged to one group that were relatively less variable, whereas those from Ontario, Australia, Germany, and France and most isolates from United Kingdom formed another group which exhibited greater variation. The variation has significant effects on breeding for disease resistance and on disease control. Disease resistance could be lost through race change at a single virulent locus.

Knowledge of the fungus's virulence characteristics could lead to development of better disease management strategies.

Genetics

The life cycle of *L. maculans* is illustrated in **Figure 1.3**. The fungus forms the asexual fruiting body pycnidium and the sexual fruiting body pseudothecium. Ascogenous dikaryotic hyphae are formed shortly before ascus development. Morphologically distinct pycnidiospores emanating from multilocular pycnidia act as spermatia since the presumed spermagonia occur only in close proximity to young pseudothecia (Ndimande, 1976). Multinucleate hyphal cells appear through hyphal fusion (Clavert and Pound, 1949).

Control of the sexual compatibility of *L. maculans* is heterothallic. The fungus forms pseudothecia in the presence of two compatible mating types (A, a or +, -). Ndimande (1976) first proved that the pseudothecia in *L. maculans* resulted from cross fertilization. He observed four phenotypically different biotypes from the same ascus. It was believed that the primary diploid ascus nucleus is heterozygotic. He also observed that asci from the same pseudothecium produced similar four biotypes, whereas four biotypes from different pseudothecia had no similarity. This difference was due to genetic recombination during meiosis and could explain at least a part of the variability in *L. maculans*. Plummer et al. (1993) observed that eight ascospores from the same ascus had four pairs of distinct electrophoretic karyotypes and they proposed that these novel-sized chromosomes were generated during meiosis by parental homologs of unequal sizes recombining to produce homologs of novel sizes. Morales et al. (1993b) also reported the chromosomal length polymorphism in *L. maculans*. The effect of size changes of chromosomes could result in the variation of phenotypic characteristics of the fungus. Tetrad analysis by Gall (1994) demonstrated that mating type was controlled by a single gene. The fungus was heterothallic with two alleles controlling the mating type (Gall et al., 1994). Plummer and Howlett (1995) proposed that these novel chromosomal length polymorphisms were produced by reciprocal recombination between parental homologous chromosomes of unequal sizes.

Leclair et al. (1996) reported that following pulsed-field gel electrophoresis, all *L. maculans* field isolates displayed a minichromosome (MC) clearly separated from the overall electrokaryotype. MCs exhibited a length polymorphism ranging from 650 to 950 kb. These results suggested that the *L. maculans* MC behaved like a 'B' chromosome. Two linear DNA plasmids (pLm9 and pLm10, sized 9 and 10 kb,

respectively) were isolated from aggressive isolates of *L. maculans*, using pulsed-field gel electrophoresis (Lim and Howlett, 1994). They found that pLm9 and pLm10 copurify with a mitochondrially-enriched cell fraction and do not hybridize with chromosomal or mitochondrial DNA, or with each other. Results suggested that pLm9 and pLm10 encode their own replication and transcription enzymes.

The genetic basis of virulence of 24 isolates of *L. maculans* collected from various sites throughout south-eastern and south-western Australia were studied using five clone-lines of *B. napus*. The virulence of these isolates was found to be most likely controlled by a small number of genes. The broad-sense heritabilities (%) for virulence on two strains were estimated (Pang and Halloran, 1996b). Ansan-Melayah et al. (1995) identified the first avirulence gene of *L. maculans* AvrLm1. Williams et al. (1994) isolated the nitrate reductase (niaD) gene from *L. maculans* that was the first protein-encoding gene characterized from this fungus. The niaD gene was present as a single copy on a chromosome, which ranged in size from 2.6 to 2.8 Mb among the different *L. maculans* isolates examined.

Extrachromosomal plasmid-like DNA was found to be more commonly associated with the virulent isolates in Europe (Hassan et al., 1991). Electrophoretic karyotyping of Australian field isolates also revealed the presence of extrachromosomal DNA of about 8 and 9 kb, which migrated more rapidly than the mitochondrial DNA band (Plummer and Howlett, 1995). A single extrachromosomal DNA element and double-stranded RNA elements were detected in the Canadian virulent isolates (Patterson and Kapoor, 1996). The latter indicated the potential presence of mycoviruses in *L. maculans*.

Pathogen - Host Interactions

Pathogenicity: Once the spores of *L. maculans* land on the surface of a host plant, the interaction process begins. It was proposed that the pathogen entered the host tissue by an active or direct infection mode (mechanical or biochemical) (Ndimande, 1976; Rimmer and van den Berg, 1992) or by a passive mode (through stomata or wounds) (Abadie and Boudart, 1982; Hammond and Lewis, 1987; Nathaniels and Taylor, 1983). It was later observed that pycnidiospores of *P. lingam* germinate and attack *Brassica* plants by passive penetration through stomata. Hyphae grow intercellularly to the substomal chamber, and gradually to the cortex, vascular parenchyma, and finally the conducting vessels. Cell walls of infected areas gradually hydrolyse (Abadie and Boudart, 1982). No direct penetration into the epidermal cells was observed (Abadie and Boudart, 1982; Hammond and Lewis, 1987). It is suggested that cell wall

degradation by fungal polysaccharide hydrolases and phytotoxin production are important features during infection of a susceptible host.

Phytotoxic heterocyclic amine compounds with a polysulfide bridge called "sirodesmins" are produced by the fungus *in vitro* (De March et al., 1986; Pedras et al., 1988, 1990; Sjodin et al., 1988). At least six sirodesmins and derivatives have been isolated (Pedras and Seguin-Swartz, 1992). Sirodesmin PL is the major phytotoxin produced by the fungus. Studies indicate that only the virulent isolates synthesize the toxins. Isolates are classified into two groups according to their ability to produce sirodesmin, Tox^0 and Tox^+(Gall et al., 1995). The phytotoxins produced by *L. maculans*, however, are not host-specific (De March et al., 1986; Pedras and Seguin-Swartz, 1992). The toxin can cause lesions on both host and non-host plants and can require a higher concentration to cause lesions than the host-specific toxins. Sirodesmin PL has no selective effect on protoplasts and cell aggregates of susceptible and resistant species of *Brassica* (Pedras and Seguin-Swartz, 1992). A host-selective phytotoxin, phomalide, has been found to be produced by isolates of *L. maculans* (Pedras and Biesenthal, 1998). Later, another host-selective phytotoxin, phomalairdenone, has been characterized from a virulent type of *P. lingam* (Pedras et al., 1999). The role of toxins in pathogenicity needs further investigation.

Cell wall degradation enzymes produced by the fungus play an important role in infection. Polygalacturonase is produced in culture, and this enzyme, as well as carboxymethyl cellulase and pectate lyase, is produced by the fungus in the host tissue (Easton and Rossall, 1985; Annis and Goodwin, 1997a). The characteristics of specific polygalacturonase isozymes may affect activity of enzymes in the plant and the virulence of *L. maculans* isolates to the host. Three genes encoding host cell wall degrading enzymes produced by *L. maculans* have been cloned and characterized (Sexton et al., 2000). These genes are located on different chromosomes and are not clustered in the genomes. Transcription of one of the genes can be detected during infection of *B. napus* and *B. juncea* cotyledons.

Both virulent and weakly virulent isolates can produce an enzyme cyanide hydratase that catalyses the breakdown of hydrogen cyanide to a less toxic compound, and *L. maculans* can utilize the reaction product, formamide, as a sole source of nitrogen (Sexton and Howlett, 2000). A gene encoding the enzyme, cyanide hydratase, has been cloned (Sexton and Howlett, 2000).

Host defence response: Research has shown that the level of glucosinolates in *Brassica* leaves is positively correlated with disease resistance (Mithen, 1992; Mithen and Magrath, 1992). The secondary metabolites of glucosinolate hydrolysis are toxic or inhibitory to the fungal growth of *Alternaria* spp. (Milford et al., 1989), *L. maculans* (Mithen et al., 1986), and *Peronospora parasitica* (Greenhalgh and Mitchell, 1976). Glucosinolates are degraded in a reaction catalyzed by thioglucosidases, denoted as myrosinases when plant tissue is damaged. Then, toxic compounds such as isothiocyanates, epithionitriles and thiocyanates are released. It is assumed that these compounds are an important part of the defence mechanism of glucosinolate-containing plants against fungal pathogens and insects (Rask et al., 2000). However, contrasting results have been obtained by Giamoustaris and Mithen (1997). They found that there is no simple positive relationship between the glucosinolate content and resistance to *L. maculans*. It is possible that *L. maculans* and other pathogens can detoxify or are tolerant to glucosinolates and their hydrolytic products. This detoxification may be an important pathogenicity factor in *L. maculans*.

Responses of host plants to the infection by *L. maculans* have been studied by transmission electron microscopy, scanning electron microscopy, X-ray microanalysis, and flame spectrophotometry (Hammond and Lewis, 1986). Lignification of cell walls, development of a callose-impregnated cambium, the formation of a curiously convoluted wall heavily impregnated with callose and lignin, and rapid accumulation of calcium were observed in infected plant tissues. Calcium specifically accumulated at the surface of secondary cell walls within and around the perimeter of lesions in association with previously induced lignin co-polymers. These reactions reflect the mechanisms of host resistance to the pathogen (Hammond and Lewis, 1987). Another response of the host to the fungus is production of inhibitory materials in plant tissues. Extracts of leaves from spring canola cultivars inhibit the activity of polygalacturonase produced by the highly virulent isolates of *L. maculans* (Annis and Goodwin, 1997b). The inhibitor is a low-molecular-weight, heat-stable compound(s). Annis and Goodwin (1997a) also found that calcium levels in leaf extracts from different cultivars were significantly positively correlated to the level of polygalacturonase inhibitory activity and the resistance of the cultivars to *L. maculans*. Dixelius (1994) used two isolates of *L. maculans*, the fungal toxin sirodesmin PL and salicylic acid, to induce stress in plantlets of *B. napus* (susceptible) and *B. nigra* (resistant). The result showed that resistant *B. nigra*

produced three proteins (proteins 2, Q and S) in a very rapid response to the treatments while these three proteins were absent in susceptible *B. napus*.

Phytoalexins are secondary metabolites synthesized by plants in response to various forms of stress such as pathogen infection. Elicitation of phytoalexins has been studied in *B. napus* (Pedras and Seguin-Swartz, 1992) and wasabi (*Wasabia japonica*), which is resistant to *L. maculans*. Three phytoalexins have been identified in *B. napus – L. maculans* interactions, and two from wasabi (Pedras et al., 1999). Both resistant and susceptible plants can synthesize phytoalexins, and the protective effects of phytoalexins appear to be restricted to the area of infection. It was suggested that the production of phytoalexins is not a necessary indication of disease resistance or susceptibility.

It was found that the blackleg fungus has enzymes to carry out the biotransformation in a different way from those involved in the biotransformation of the brassica phytoalexin brassinin. The biotransformation of phytoalexin brassicanal A by *L. maculans* was investigated, and three main products were detected (Pedras and Khan, 1996). Pedras and Okanga (2000) reported that *P. lingam* could detoxify the cruciferous phytoalexin brassinin. The biotransformation by virulent and avirulent isolates of *P. lingam* proceeded to yield different final products that showed no inhibitory activity against the pathogen.

High resistance to blackleg has been found in *B. juncea* (Sacristan and Gerdemann, 1986). There is a spectrum of responses at different times after inoculation. Rapid necrosis of guard cells is associated with the arrest of fungal growth in leaves. Resistance responses in resistant *Brassica* spp. and wild crucifers include deformation of hyphae, accumulation of dark coloured material and callose in the mesophyll layer (Chen and Howlett, 1996), rapid cell death, tissue browning, and lignin deposition (Chen and Seguin-Swartz, 1999).

Disease Diagnosis and Pathogen Detection

Rapid and accurate disease diagnosis can provide valuable information for disease management. Since the traditional methods are time-consuming (Petrie, 1988), molecular detection techniques have been developed to solve this problem (Voigt et al., 1997). Polyclonal and monoclonal antibodies to the virulent isolates of *L. maculans* were produced (Dahiya, 1988; Stace-Smith et al., 1993). Monoclonal antibody based enzyme-linked immunosorbent assay (ELISA) was developed for detecting the virulent blackleg isolates (Stace-Smith et al., 1993). Quantification of *L. maculans*-growth in cotyledons of *B. napus* was

conducted using the ELISA technique (Balesdent et al., 1995). It was found that the polyclonal antisera produced by injecting rabbits with fractions of *L. maculans* were insufficiently specific in use for detection and identification of the blackleg strains either in culture or in plant tissues. The Blackleg Alert® testing kit based on the monoclonal antibody developed by Brooks Diagnostics Ltd. in cooperation with the Alberta Research Council is commercially available in Canada (Williams and Fitt, 1999). This technique allows a fast and specific detection of virulent blackleg isolates in infected leaves, stems and stubble in 45 min. Isolates from cultures can also be identified for their virulent pathotype.

Seed testing and seed certification programs have been developed to prevent the disease spreading to blackleg-free areas through infected canola seeds (Gugel and Petrie, 1992; Kharbanda, 1989a). In Alberta, Alberta Agriculture regulations limit the seed infection to less than 0.1% ( ). That is, any seed lot that has more than 0.1% *P. lingam* infection cannot be sold for seeding purposes. Correlations between severity and incidence of crown canker, and between severity of pod lesions and incidence of plants with pod lesions were obtained, and the seed infection could be estimated according to these disease parameters (Chigogora and Hall, 1995). To detect seed contamination with the virulent *L. maculans*, a simple, sensitive and rapid method was developed based on the DNA amplification polymerase chain reaction assay (Taylor, 1993).

To detect the natural populations of *L. maculans* directly from leaf lesions, electrophoretic analysis was used (Brun et al., 1997). Four different electrophoretic patterns of allozymes of glucose phosphate isomerase (ET1-ET4) were characterized. This method is a useful and efficient way of identifying the pathogen directly in host lesions.

The internal transcribed spacer (ITS)-based PCR technique, a routine identification protocol requiring no DNA extraction, has been developed for large-scale identification of the *L. maculans* complex in France (Balesdent et al., 1998). Pycnidiospores were directly used for the PCR reaction. According to ITS size polymorphism of ribosomal DNA (rDNA), three groups were identified. The RFLP pattern also showed polymorphism among the different subgroups.

Non-radioactive DNA probes were developed using the RAPD-PCR technique (Schafer and Wostemeyer, 1994). The probes could be used for dot-blotting and Southern hybridization for rapid detection of virulent isolates of *L. maculans*. The advantages of using non-radioactive probes are the safety

to humans and to the environment, and the ease of handling. Quantification of the DNA of *L. maculans* in rapeseed by the PCR method was reported (Mahuku et al., 1995). The molecular techniques provide rapid and reliable differentiation of the two major pathotypes of *L. maculans*, but these techniques require expensive equipment and can be used only in disease diagnostic laboratories.

Disease Control

Cultural Control

Studies on the epidemiology of the blackleg of canola have shown that the pathogen is seed- and stubble-borne. It can survive under the seed coat as mycelium and produces ascospores, pycnidiospores, and mycelia on canola residues (Punithalingam and Holiday, 1972). Each of these structures can serve as primary inoculum. Ascospores are the most important form of primary inoculum and can be dispersed by wind for up to several kilometers, and the fruiting structures occur in crop residues (Gugel and Petrie, 1992; Petrie, 1994) for as long as the residue persists, which may be for three or more years (McGee, 1977). Baird et al. (1999) found that intact canola debris can serve as an inoculum source for at least three seasons or longer. Therefore, cultural practices such as residue management, rotation and weed control are effective in reducing the inoculum level and reducing the disease. These methods are the most economical and practical strategies of controlling blackleg.

Stubble management is a critical component of blackleg management programs. Deep tillage, rotation, and burning crop residues in heavily infested fields encourage the decomposition of infested stubble and the destruction of the inoculum (Gugel and Petrie, 1992; Kharbanda and Tewari, 1997).

Crop rotation is an effective method of reducing the inoculum level. Crop residues decompose due to the action of soil microbes under suitable environmental conditions. The fungus thus loses the nutrient base for survival. Rotation can keep fields out of canola and allows time for stubble decomposition. Because the blackleg pathogen can survive on intact canola debris for at least three seasons or longer (Baird et al., 1999) and the ascospores continue to be discharged from infected stubble for 3-5 years, a three- to four-year crop rotation is recommended (Kharbanda, 1993; Petrie, 1995). Lange and Kharbanda (1999) reported that crop rotation decreases the severity of blackleg, especially on the susceptible cultivar Westar. A rotation including barley, field peas and wheat for 3 years following canola can eliminate the potential disease sources (Turkington et al.,

2000b). There are possibilities of using the bird nest fungus *Cyathus olla*, as an inoculant, to accelerate stubble decomposition and shorten the crop rotation (Tewari and Briggs, 1995; Tewari et al., 1997)

Tillage in infested fields is effective in reducing the amount of inoculum on the soil surface by burying infested canola-plant debris (Gladders and Musa, 1980; Gugel and Petrie, 1992; McGee and Petrie, 1979). Buried canola straw underwent 1.6 to 2 times as much decomposition as straw on the soil surface (Blenis et al., 1999). Fast decomposition could reduce the inoculum in the soil. Conservation tillage has been increasingly used to conserve water and reduce soil erosion, and causes greater immobilization of mobile nutrients. However, reduced tillage can also cause yield losses due to the increased population of plant pathogens (Bailey, 1996) and weed (Derksen et al., 1996). For blackleg disease in canola, tillage had no significant effect on blackleg severity (Lange and Kharbanda, 1999; Turkington et al., 2000a). Direct seeding or reduced tillage will probably not increase the blackleg problem when a proper rotation is used and resistant cultivars are grown.

Controlling and destroying volunteer canola and susceptible weed hosts of *L. maculans* are other effective methods to reduce the inoculum of this pathogen. Kharbanda (1993) tested several wild cruciferous weeds and *Brassica* vegetables and found that wild mustard (*Sinapis arvensis*), dog mustard (*Erucastrum gallicum*), wild radish (*Raphanus raphanistrum*), and stinkweed (*Thlaspi arvense*) were susceptible to some isolates of *L. maculans*. All the cultivated vegetable crucifers such as broccoli (*Brassica oleracia* var. *botrytis*), cabbage (*Brassica oleracia* var. *capitata*), cauliflower (*Brassica oleracia* var. *botrytis*), and rutabaga (*Brassica napus* var. *napobrassica*) were extremely susceptible to the pathogen. These vegetable crops could play an important role in blackleg epidemiology.

Chemical Control

Fungicidal seed treatments provide the best defence against introduction of disease into new areas (Gugel and Petrie, 1992; Harris et al. 1989). Seed treatment with some systemic fungicides may also provide a protection from stem canker on adult canola plants (Sudarmadi and Wallace, 1984; Verma et al., 1983). Fungicides are one of the most effective means for controlling blackleg in the United Kingdom (Gladders, 1988; Gladders et al., 1998; Sansford et al., 1996). However, their use must be optimized to achieve the maximum economic response and to avoid unnecessary fungicide applications. Once the disease is established in an area, seed treatment is not effective since fungicidal seed treatments do not protect seedlings from

infection by wind-borne ascospores. Several workers have reported the failure of fungicidal seed treatments to control the blackleg of canola (McKenzie and Verma, 1988; Verma and McKenzie, 1982).

Applying flutriafol to canola as a coating on superphosphate fertilizer has effectively reduced yield losses due to blackleg in Australia (Ballinger et al., 1988a, 1988b), but this method was not effective in western Canada (Xi et al., 1991a). Incorporation into the soil of corn-cob granules impregnated with tebuconazole reduced the incidence and severity of blackleg (McKenzie and Verma, 1990, 1992a, b).

Foliar fungicide application is ineffective unless sprayed repeatedly during early stages of crop growth (Kharbanda, 1992; Kharbanda et al., 1996; Rawlinson et al., 1984). Spray timing is very important. Mckenzie and Verma (1992c) reported that single sprays of tebuconazole, 2, 3 and 4 weeks after emergence, reduced blackleg severity at crop maturity. Application of the fungicide Tilt® (propiconazole) can effectively control *L. maculans* but may be phytotoxic to canola when sprayed as a mixture with an adjuvant during early stages of crop growth (Kharbanda, 1992). Three triazole fungicides were tested for reducing blackleg and increasing the yield of spring canola (Rempel and Hall, 1995). It was found that foliar sprays of triazole fungicides at the late rosette stage reduced the disease incidence and severity, and increased the yield of canola. Effective fungicide application for disease control can be optimized by developing a disease-forecasting scheme (Fitt et al., 1997). This strategy, however, could increase production costs and become uneconomical, particularly if the disease pressure is high.

Post-harvest application of chemicals on crop residues in the fall could reduce pseudothecia formation in the following spring. The amount of viable fungus over-wintering on canola stubble was significantly reduced by directly applying several fungicides and herbicides, including glyphosate, dinoseb, benomyl, propiconazole and thiadimenol, to stubble (Humpherson-Jones and Burchill, 1982; Petrie, 1995). Negative results were obtained in the study by Rawlinson et al. (1984) and Turkinton et al. (2000b).

Breeding for Resistant Cultivars

The development of resistant cultivars is the most effective and economical method of blackleg control. Screening and evaluation of germplasms for resistance to blackleg has been carried out in many regions of the world, including Australia, Canada and Europe (Bansal et al., 1994, 1998; Rempel and Hall, 1996; Salisbury et al., 1995; Sjodin and Glimelius, 1988; Thurling and Venn, 1977). *Brassica* oilseed rape,

as well as the related oilseed species *B. juncea, B. carinata, B. nigra* and *B. oleracea,* are often included in blackleg screening studies. Rimmer and van den Berg (1992) have reviewed this topic extensively. Much research has been carried out since their publication.

There are two types of blackleg resistance in *Brassica,* seedling and adult plant, referring to the plant growth stage when the resistance to the pathogen is expressed. Adult-plant resistance is measured in the stem at maturity and attributed to the "A" genome of *B. juncea* in progenies of an interspecific cross between *B. juncea* and *B. napus* (Roy, 1978). Adult-plant resistance is under the control of two dominant genes, designated *Bl-1* and *Bl-2* (Sawatsky, 1989). Recently, ten quantitative trait loci contributing to field resistance to blackleg were detected by measuring the mean disease index and percentage of host plants on 152 doubled-haploid lines of winter rapeseed (Pilet et al., 1998). The rapeseed genetic map was constructed using 288 DNA markers on 19 linkage groups. Seedling resistance in *B. napus* is controlled by a single dominant gene (*Lm1*) in a French breeding line. A second linked gene was identified in the German winter rape cultivar Girita (Delwiche, 1980).

Resistance to blackleg has been found in *B. napus, B. carinata* and *B. insularis* (Purwantara et al., 1998; Rouxel et al., 1991; Sacristan and Gerdemann, 1986; Sjodin and Glimelius, 1988) and some wild crucifers such as *Arabidopsis thaliana* (Sillito et al., 2000). High degrees of resistance have been found in *B. juncea,* which has A- and B-genome chromosomes (Keri, 1991). Many efficient evaluation techniques have been developed (Bansal et al., 1994; Sippell and Hall, 1996). No high levels of resistance have been found in *B. rapa* and *B. oleracea* (Rimmer and van den Berg, 1992). Good levels of blackleg resistance under Canadian conditions have been found in summer and winter *B. napus* cultivars originating in Australia, Japan and Europe (Rimmer and van den Berg, 1992).

Inheritance of resistance has been investigated, and results showed that the resistance varied from monogenic- to two dominant genes, to polygenic systems (Rimmer and van den Berg, 1992). The gene-for-gene hypothesis has been demonstrated in the *Brassica-Leptosphaeria* system by genetic analysis of resistance in *B. napus* (cv. Major) to *L. maculans*. A single major locus (*LEM1*) controlling resistance to the *L. maculans* isolate PHW1245 (PG2) in *B. napus* cv. Major was mapped based on the interaction of phenotype on inoculated cotyledons, using a segregating population of doubled haploid lines (Ferreira et al., 1995). Studies by Keri et al. (1997) demonstrated that the resistance to *L. maculans* in *B. juncea* is

controlled by two nuclear genes with dominant recessive epistatic gene action. Amplified fragment length polymorphism markers linked to the *alm1* avirulent gene of *L. maculans* isolate PHW1245 (PG2) were later identified in the same system. A single gene model has been proven (Pongam et al., 1998). Another *avr* gene in *L. maculans* isolate a.2 (PG3), *AvrLm1*, controlling the cultivar specificity on "Quinta", has been described (Ansan-Melayah et al., 1995). Because of the high variability in *L. maculans*, they suggest that there are several *avr* genes in this fungus. Dion et al. (1995) have used the RFLP technique to map resistance in canola (*B. napus*) to *L. maculans*. Their results strongly supported the presence of a single major gene, named $LmFr_1$, controlling adult resistance in the spring rapeseed cv. Cresor. Resistance in *B. juncea* is monogenic, and the resistance gene is carried by the B genome determined by RAPD markers (Chevre et al., 1997). Molecular mapping of resistance to *L. maculans* in two Australian cultivars of *B. napus* has been conducted, and a single major locus is confirmed to be responsible for resistance (Mayerhofer et al., 1997). The inheritance of resistance to *L. maculans* was studied in near-isogenic lines derived from asymmetric somatic hybrids between *Brassica napus* and *B. nigra*, *B. napus* and *B. juncea*, and results showed that one single dominant allele controls the resistance in the *B. napojuncea* line, whereas two independent dominant loci were found in the *B. naponigra* line (Dixelius, 1999). Inheritance of virulence in *L. maculans* has been studied, and two genes for avirulence in the pathogen and two genes for resistance in the hosts were identified (Rimmer and van den Berg, 1992).

A large emphasis has been placed on the incorporation of blackleg resistance into *B. napus* (Pang and Halloran, 1996a). Stringam et al. (1995a) have developed a cultivar of canola resistant to blackleg with superior agronomic characteristics in *B. napus*, using doubled haploidy. This cultivar has been registered as Quantum (Stringam et al., 1995b) and has been widely used in western Canada in recent years. A new cultivar, Surpass 400 (*B. napus*), was produced by the Winnipeg-based seed company Advanta Seeds and has the trait "CR" or "Completely Resistant" (Anonymous, 2000), and this cultivar has been used in Australia in 2000 and has been scored as highly resistant to blackleg.

Interspecific transfer of resistance has been practiced in different countries (Rimmer and van den Berg, 1992; Roussel et al., 1999). In Canada, Gugel et al. (1994) have transferred blackleg resistance from *B. juncea* to *B. rapa* and produced canola-quality lines after only 4 cycles of recurrent selection. Zhu et al. (1993) have transferred blackleg resistance from *B. nigra* to *B. napus*. A new strategy to localize and

characterize interspecific introgressions in the genus *Brassica* was developed using RAPD and RFLP markers and an interspecific introgression of *B. juncea* that was localized on a linkage group of a previously determined *B. napus* genetic map (Barret et al., 1998). The cotyledons and leaves of *B. napus-B. juncea* recombinant lines are more resistant to A-group isolates of *L. maculans* tested under controlled conditions, whereas the recombinant lines are susceptible at the cotyledon stage and resistant at the adult stage to B-group isolates (Somda et al., 1998). Host-pathogen differential interactions on the durability of the monogenic resistance to *L. maculans* have been observed. Resistance of the recombinant line is unlikely to be durable in some isolates of *L. maculans* (Somda et al., 1999). The pathogen may adapt quickly to resistant lines and produce more virulent isolates. Multiple resistance genes should be used in plant breeding to prevent the breakdown of resistance. Somatic hybridization and protoplast fusion techniques are being used in the interspecific transfer of resistance (Siemens and Sacristan, 1996).

Biotechnological approaches for enhancing disease resistance involve either exploitation of the natural forms of resistance or the use of genetic-engineering approaches such as the introduction of chitinases, glucanases and other antifungal protein. Genetic engineering has been utilized to produce disease-resistant transgenic lines (Murphy and Mithen, 1995). Kazan et al. (1998) used hydrogen peroxide (H_2O_2) to induce defense genes to *L. maculans* in canola plants. The expression of a chimeric fungal glucose oxidase gene, driven by a pathogen and wound-inducible promoter, has been evaluated in transgenic canola. Inoculation with *L. maculans* on cotyledons of control and transgenic canola resulted in rapid cell death and cotyledon senescence. Defense genes from peas were transferred into canola in an attempt to incorporate resistance to blackleg into canola; transgenic lines showed decreased disease scores (Wang et al., 1999).

Biological control

"Biological control is the reduction of the amount of inoculum or disease-producing activity of a pathogen accomplished by or through one or more organisms other than man" (Cook and Baker, 1983). It is further defined as "pest suppression with biotic agents, excluding the process of breeding for resistance to pests, sterility techniques, and chemicals modifying pest behaviour." (Baker, 1984).

Biological control has been of increasing interest to researchers and is being successfully practiced on many crops (Baker and Cook, 1974; Tang et al., 1996), especially on controlling soil-borne diseases

(Martin et al., 1985). A number of fungal and bacterial microorganisms have been successfully developed as biocontrol products (Boyetchko, 1999). Also, several fungi and bacteria have exhibited biological control activities against plant pathogenic fungi of canola, including *Sclerotinia sclerotiorum* (Lib.) de Bary, *Rhizoctonia solani* Kuhn, *Pythium* spp., *Alternaria* spp. and *L. maculans*. Reducing initial inoculum, reducing disease spread, and preventing and controlling infections are the strategies used for biocontrol of these diseases.

The first step of biocontrol is to search for potential biological control agents. Under natural conditions, an ecosystem consists of numerous microorganisms competing for food and space. Some are parasitic on others. Therefore, non-pathogenic microorganisms that are parasitic or inhibitory to other pathogenic microbes may be beneficial and useful biocontrol agents. Agriculture & Agri-Food Canada and the University of Saskatchewan screened and evaluated 581 bacterial strains and found that 12 of them exhibited high inhibitory activity against five major fungal pathogens of canola (Boyetchko, 1999).

L. maculans is a stubble-borne fungus. The basidiomycetes *Cyathus olla* and *C. striatus* were investigated for their role in increased decomposition of canola residues, and for their potential for biocontrol of *L. maculans* (Tewari and Briggs, 1995).

A strain of *Penicillium verrucosum* (Dierckx) was found to produce a metabolite toxic to *L. maculans* (Kharbanda and Dahiya, 1990). The antifungal substance was obtained and identified as citrinin. It was heat-stable and inhibitory to *L. maculans* and some other pathogens of canola (Kharbanda, 1993). However, the reported nephrotoxic nature of citrinin makes it a non-suitable candidate for biological control of blackleg and other diseases of canola.

The antagonism of *Pantoea agglomerans* (syn. *Erwinia herbicola)*, a phyllosphere microorganism on canola, against *L. maculans* was tested *in vitro,* and it was found the bacterial suspensions significantly reduced the severity of blackleg disease. An antifungal substance that was partially thermolabile was found in the bacterial culture (Chakraborty et. al., 1994). No further work has been done to investigate the application of this bacterium under field conditions.

Strains of *Trichoderma* spp. (*T. viride* Pers.:Fr. and *T. harzianum* Rifai) have been tested for their protective ability against the pathogens *Phoma lingam* and *Sclerotinia sclerotiorum* (Starzycki et al., 1998). Strain differences in the inhibitory effect against the two pathogens were observed.

Partial suppression of virulent *L. maculans,* using the weakly virulent strain of the pathogen *in vitro* and *in vivo* has been reported (Petrie, 1982). Mahuku et al. (1996) observed the interaction between highly virulent (HV) and weakly virulent (WV) isolates of *L. maculans* on oilseed rape leaves and concluded that the WV type could coinfect and coexist with the HV type within a single lesion in leaves, and that inoculation of the WV type could induce systemic acquired resistance (SAR) in oilseed rape to the HV type. The weakly virulent isolates of *L. maculans* could potentially be used in limiting the development of virulent isolates on canola.

***Bacillus* spp. and *Paenibacillus polymyxa* in biological control of plant diseases**

Using bacteria to control fungal diseases of plants has been of increasing interest to researchers and growers of agricultural, forestry and horticultural plants. Many strains of *Bacillus* spp., *Paenibacillus* spp. and *Pseudomonas* spp. have been valuable and have received extensive investigation for biocontrol purposes in the past decades (Hill et al., 1997; Tang et al., 1996).

Approximately 40 *Bacillus* spp. are known to have antimicrobial activity under suitable conditions. More than 120 different *Bacillus* antibiotics have been identified, and more than two thirds of these were peptides (Kugler et al., 1990; Shoji, 1977; Vanittanakom et al., 1986). Antifungal substances from antagonistic *Bacillus* spp. were detected and identified by a chromatographic technique (Tschen et al., 1992).

Bacillus polymyxa [(Prazmowski 1880) Mace 1889] has been reclassified as *Paenibacillus polymyxa* [(Prazmowski 1880) Ash et al. 1994] based on 16S rRNA sequence similarity (Ash et al., 1993). The species within the genus *Paenibacillus* have 16S rRNA sequences with at least 89.6% similarity (Beatty, 2000). The bacterial cells are rod-shaped, generally 0.6 μ to 0.8 μ x 2.0 μ to 5.0 μ, and have abundant, peritrichous flagella. Endospores are ellipsoidal, subterminal or terminal and have a heavily ribbed surface (Anomynous, 1973). The ribs are generally longitudinal, parallel and distinctive. **Figure 1.4** shows the morphology of *P. polymyxa* cells and endospores.

Paenibacillus requires complex nutrients for growth; therefore, this species is more associated with plant roots in the soil or on rotting plant materials (Sonenshein et al., 1993). *P. polymyxa* can produce a large amount of extracellular polysaccharides and is known to produce several peptide antibiotics. Therefore, this species has many commercial uses in food, cosmetic and industrial areas, and has a potential use in agriculture. Many

antibiotics produced by *P. polymyxa* have been discovered by researchers; these peptides include polymyxins, polypeptins, jolipeptin, gavaserin, saltavalin, gatavalin, fusaricidins and polyxin (Beatty, 2000).

P. polymyxa strains as beneficial and biocontrol agents in agriculture and forestry were reported mainly in three aspects: (1) *P. polymyxa* as plant growth promoting rhizosphere (PGPR) bacteria (Bezzate et al., 2000; Shishido et al., 1996; Timmusk and Wagner, 1999); (2) *P. polymyxa* as nitrogen-fixation bacteria (Achouak et al., 1999; Petersen et al., 1996), and (3) strains of *P. polymyxa* as producing antifungal antibiotics and having potential as biocontrol agents (Alippi et al., 2000; Dijksterhuis et al., 1999; Godoy et al., 1991; Kado et al., 1987; Nielsen and Sorensen, 1997; Parmentier, 1988; Yuen et al., 1991). These effects of the bacteria were determined on various crops and trees, including legume plants, perennial grass, wheat, canola, potato, cotton, lodgepole pine, spruce and Douglas fir.

P. polymyxa strain 9A was used to protect potato plants against Verticillium wilt (Patent Number: 4663162, Kado et al., 1987). The young potato seedlings could be protected from *Verticillium* infection by applying the bacterium to potato-seed pieces. Antifungal and antibiotic effects of *P. polymyxa* on the growth of *Ceratocystis ulmi* (Parmentier, 1988) and on various potato pathogens (Lange et al., 1994) were demonstrated. *P. polymyxa* strains showed an ability to inhibit the fungus *Gaeumannomyces graminis* var. *tritici* (Ggt) on wheat (Weller et al., 1988). The strains isolated from the rhizoplane were a genetically homogenous population (Mavingui et al., 1992) and had homogenous and high chitinase and antifungal activities, whereas the population of *P. polymyxa* isolated from the soil and the rhizosphere was heterogenous (Mavingui and Heulin, 1994). Results suggested that the rhizoplane and rhizosphere provide selective pressures for specifical adaptation of *P. polymyxa* strains. Liang et al. (1996) screened 665 strains of rhizosphere bacteria and found that 23 strains of *Pseudomonas putida*, *P. fluorescens*, *P. polymyxa* and *B. subtilis* were antagonistic to *Pythium* sp. It was suggested that the bacteria had great potential as biocontrol agents for Pythium damping-off of safflower. Strains of *P. polymyxa* inhibited mycelial growth of *Sclerotinia sclerotiorum* and had a potential for biological control of the white mold disease of beans (Godoy et al., 1991; Yuen et al., 1991) and root rot of vegetables (Oedjijono et al., 1993). Sixteen isolates of *P. polymyxa* out of 100 from barley rhizosphere showed *in vitro* fungal antagonism against widely different plant-pathogenic fungi (*Aphanomyces cochleoides, Pythium ultimum and Rhizoctonia solani*) (Nielsen and Sorensen, 1997). Walker et al. (1998) demonstrated that *Bacillus* isolates from the spermosphere of peas and dwarf French beans had antifungal

activity against *Botrytis cinerea* and *Pythium* species. Shang et al. (1999) reported that *B. cereus* UW85 suppressed seedling damping-off diseases caused by Oomycetes and produced antibiotics. They concluded that *B. cereus* cultures affected zoospore behavior, and *B. cereus*-produced antibiotics, zwittermicin A and kanosamine, contributed to disease suppression and inhibition of germ-tube elongation in the presence of the plant root, and that the antibiotics also inhibited development of Oomycetes in culture. Dijksterhuis et al. (1999) studied the interaction of *Fusarium oxysporum* and *P. polymyxa* and found that *P. polymyxa* antagonizes the plant pathogenic *F. oxysporum* in liquid medium by means of an interaction process in which the presence of living bacteria is a prerequisite for continuous suppression of fungal growth.

Petersen et al. (1996) reported a synergism between *P. polymyxa* and *Rhizobium etli* (a symbiotic nitrogen-fixing bacterium). When *P. polymyxa* was applied as a co-inoculant, early rhizobial root populations and final root population densities were increased. It was concluded that *P. polymyxa* indirectly (via the host plant) stimulates and increases the *R. etli* population. Investigation by Moharram et al. (1997) showed that the addition of *P. polymyxa* as free-living nitrogen-fixing bacteria to the soil could increase the dry weight of wheat. Some strains of *P. polymyxa* have the effect of nitrogen fixation, and studies demonstrated that nitrogen fixation among aerobic endospore-forming bacteria seems to be restricted to a subset of species in the genus *Paenibacillus* (Achouak et al., 1999).

Strains of *P. polymyxa* promoted the growth of wheatgrass, ryegrass and white clover (Holl et al., 1988), western hemlock, pine and spruce seedlings (Chanway, 1995; Holl and Chanway, 1992; Shishido et al., 1995, 1996), and increased the yield of wheat (Rodriguez-Caceres et al., 1996). *P. polymyxa* strains could promote the growth of plants, and the genes controlling plant-growth-promotion rhizobacteria (PGPR) effects are co-regulated with genes of plant defenses against abiotic and biotic stress (Timmusk and Wagner, 1999).

Seed treatment of a biological control agent Kodiak, a *B. subtilis* strain GB03, (Gustafson, Inc., Plano, TX, USA) has been the first large-scale application of a biological control agent for suppression of seedling diseases and long-term chronic diseases of the rhizosphere of cotton (Brannen and Kenney, 1997) and sugar beets (Fukui et al., 1994) in the United States. Since *B. subtilis* is a spore-forming organism, it is extremely tolerant of environmental stresses and has many advantages over other biocontrol agents, such as *Pseudomonas* spp., for large-scale application. Colonization of the bacterium on cotton seeds was largely affected by seed factors (Mahaffee and Backman, 1993).

Foliar spray of *P. polymyxa* and other *Bacillus* spp. has shown different control effects over several foliar pathogens of wheat in greenhouse experiments (Alippi et al., 2000). *Paenibacillus polymyxa* and *B. cereus* can efficiently reduce disease severities infected by *Septoria tritici* and *Alternaria triticimaculans,* whereas *B. laterosporus* is effective against all the fungal pathogens including *Drechslera tritici-repentis* and *Bipolaris sorokiniana.*

Objectives of the Thesis

A bacterium strain *Paenibacillus polymyxa* PKB1 isolated from canola stubble was found to have an inhibitory effect against *Leptosphaeria maculans*, the causal agent of blackleg disease on canola. Research was conducted to determine if this bacterium was a suitable candidate for use as a biocontrol agent for blackleg of canola. To achieve this goal, the four objectives of this research were

1. To determine the effect of the bacterium *P. polymyxa* strain PKB1 on the blackleg disease *in vitro* and *in vivo*;
2. To determine the effect of *P. polymyxa* PKB1, in conjunction with the fungicide Tilt®, on the blackleg disease in canola in growth chambers and in field conditions and to investigate compost as a carrier of *Paenibacillus polymyxa* PKB1 for field application;
3. To develop a molecular probe for detection of *P. polymyxa* PKB1 applied to the environment;
4. To detect the effect of *P. polymyxa* PKB1 on different groups of *L. maculans,* based on the genetic variation among blackleg isolates collected in western Canada.

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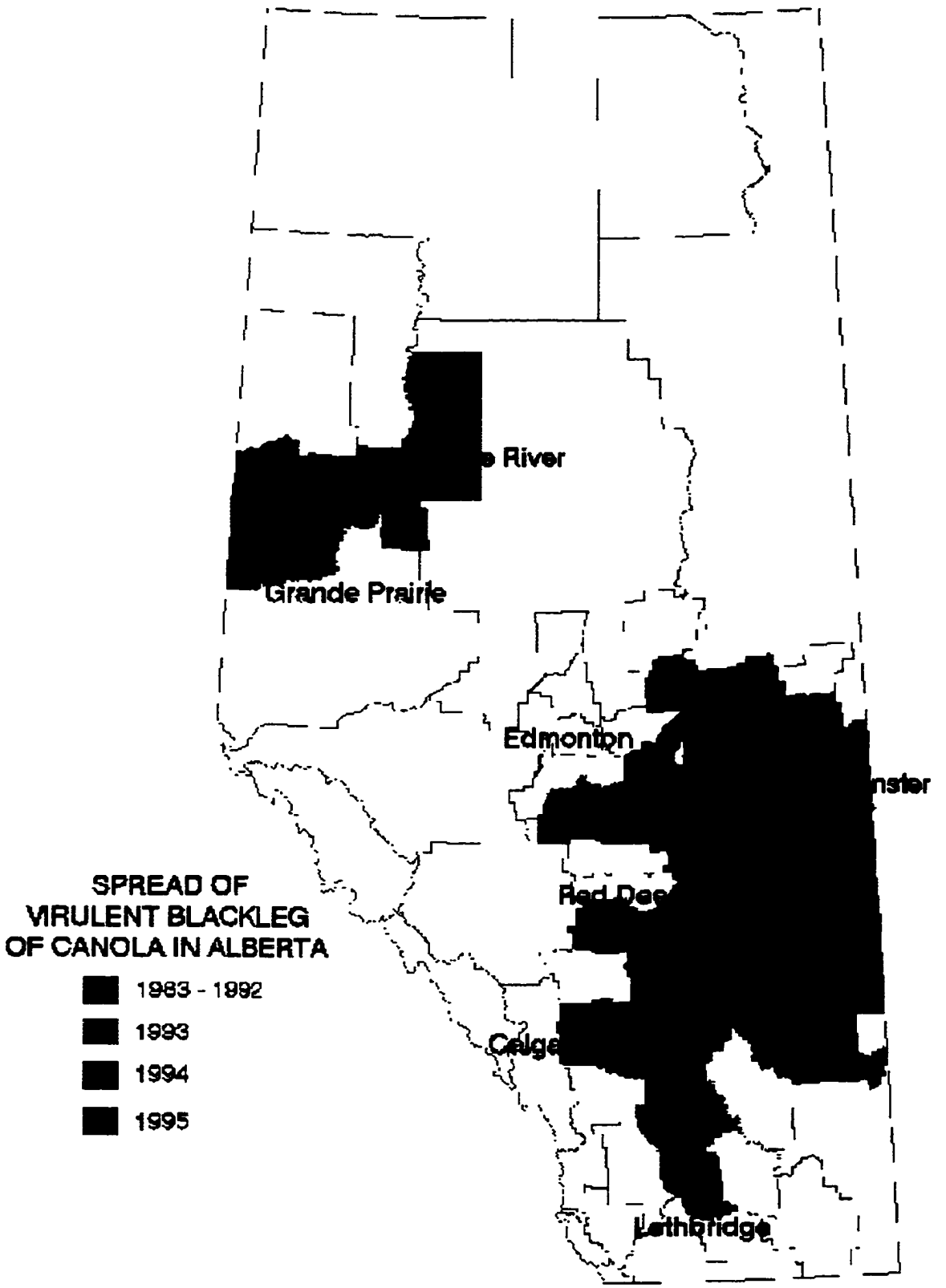

Figure 1.1. Distribution of virulent *Leptosphaeria maculans* in canola growing area in Alberta (with the permission of Dr. Kharbanda, Alberta Research Council, Vegreville, AB).

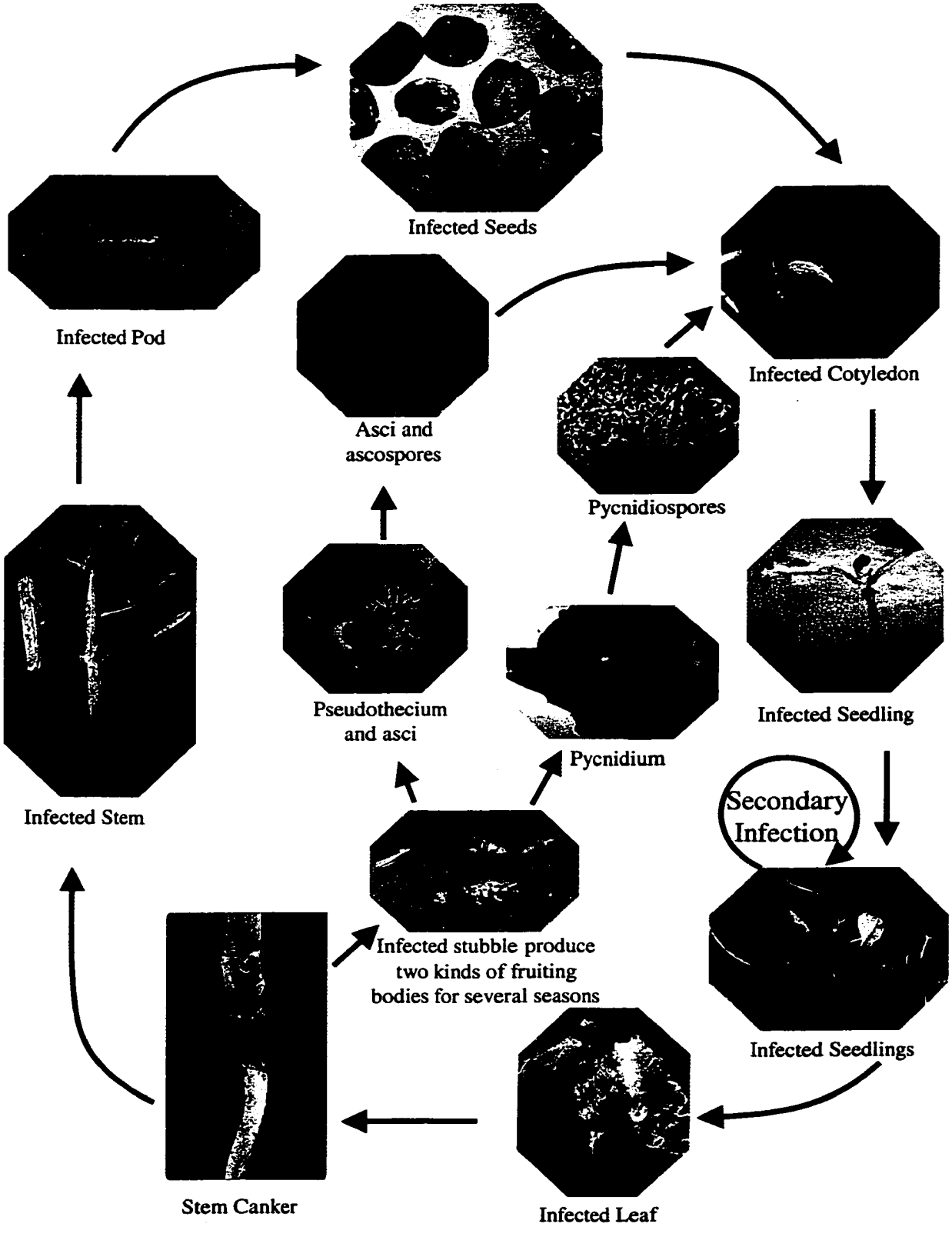

Figure 1.2. Disease cycle of blackleg caused by *Leptosphaeria maculans* on canola.

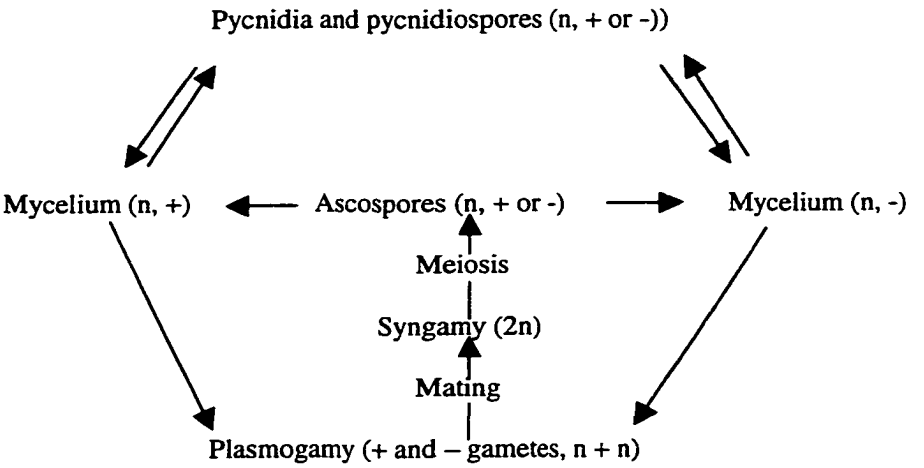

Figure 1.3. Life cycle of *Leptosphaeria maculans*

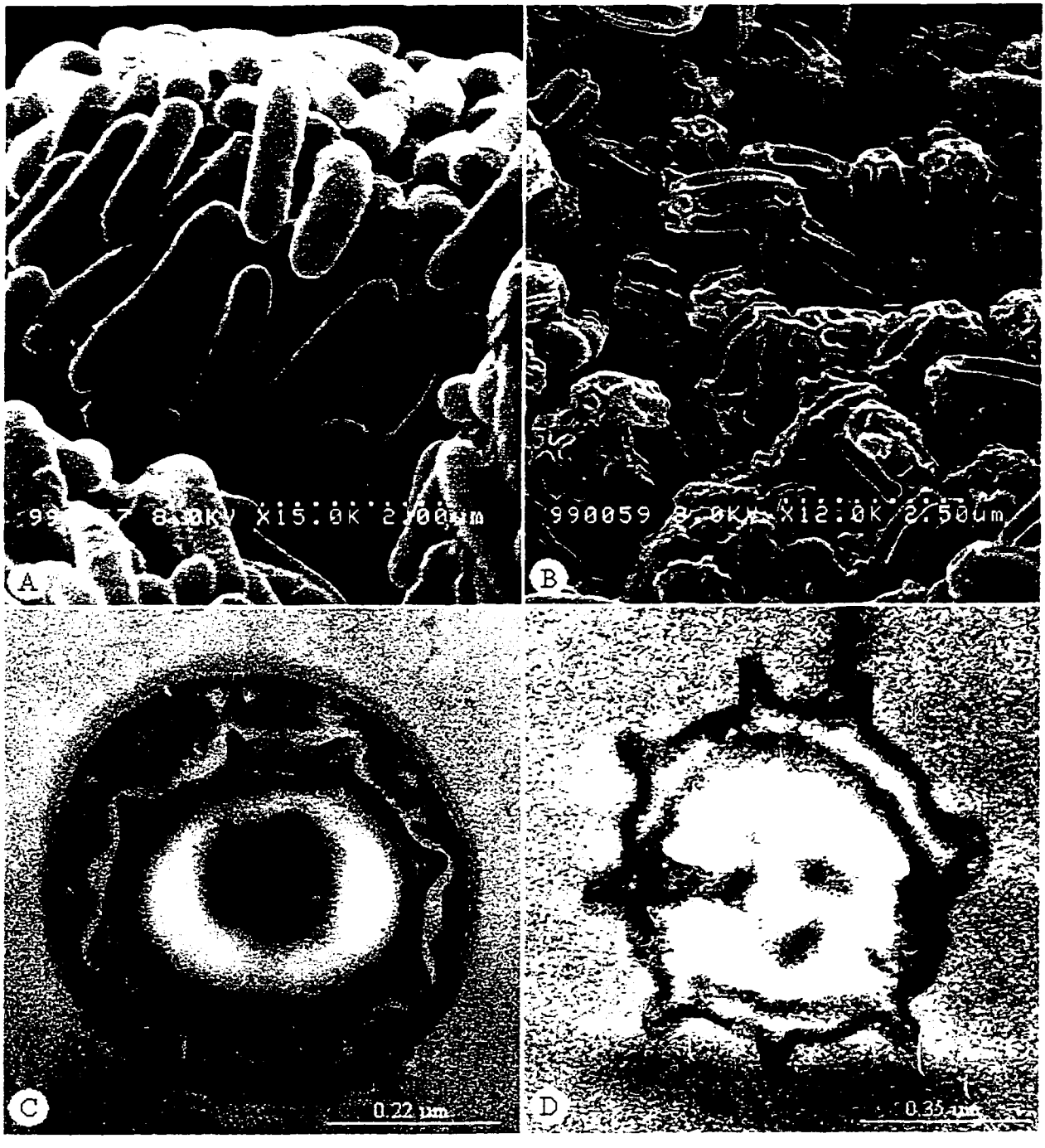

Figure 1.4. Scanning electron micrographs (A, B) and transmission electron micrographs (C, D) of a *Paenibacillus polymyxa* cell (A) and a cell containing an endospore (C) and endospores released from cells (B and D) (with the permission of Dr. P. D. Kharbanda and Dr. J. P. Tewari).

Chapter 2

Inhibitory Effect of *Paenibacillus polymyxa* PKB1 against *Leptosphaeria maculans*

Introduction

Blackleg is one of the most economically important diseases of canola in western Canada. Cultural and chemical control measures have been developed to manage the disease (Gugel and Petrie, 1992; Kharbanda, 1992, 1993). Disease-tolerant cultivars were developed to control the disease caused by the virulent strain of *L. maculans* (Stringam et al., 1995a, 1995b). This pathogen, however, shows high cultural variability, and attempts have been made to ascertain various pathogenicity groups. Blackleg-resistant *B. napus* cultivars currently registered in western Canada were screened mostly against isolates of single pathogenicity groups from the local geographic area during the selection process. Disease resistance could be lost through a new virulent race resulting from genetic change at a single virulence locus.

Fungicidal seed treatments provide the best defence against introduction of the disease into new areas (Gugel and Petrie, 1992). These treatments may also provide protection from stem canker on adult canola plants (Gabrielson et al., 1977; Maude et al., 1984). Fungicides are one of the most effective means for controlling blackleg in the United Kingdom (Gladders, 1988; Gladders et al., 1998; Sansford et al., 1996). However, seed treatments have failed to control the disease under some conditions in Canada (McKenzie and Verma, 1988). Furthermore, these treatments do not protect plants from wind-borne ascospores. Therefore, once the disease is established in an area, seed treatment is not important. Chemical control by applying flutriafol to canola as a coating on superphosphate fertilizer was not as effective in western Canada as in Australia (Xi et al., 1991). Foliar fungicide application was ineffective unless sprayed repeatedly during early stages of crop growth, and this practice could greatly increase the cost of disease control (Kharbanda, 1992; Rawlinson et al., 1984). Fungicide Tilt® (propiconazole) effectively controlled *L. maculans* but was phytotoxic to canola seedlings when sprayed as a mixture with an adjuvant during early stages of crop growth (Kharbanda, 1992). Post-harvest application of chemicals on crop residues in the fall could reduce pseudothecia formation in the following spring. The amount of viable fungus over-wintering on canola stubble was

significantly reduced (Humpherson-Jones and Burchill, 1982; Petrie, 1995). Some herbicides could also prevent ascospore formation. An effective and environmentally safe method of disease control is required.

In the literature, some work has been reported on the biological control of the blackleg disease of canola. Partial suppression of the virulent *L. maculans,* using the weakly virulent strain of the pathogen *in vitro* and *in vivo* was observed (Petrie, 1982). A strain of *Penicillium verrucosum* was found to produce a metabolite toxic to *L. maculans* (Kharbanda and Dahiya, 1990), but this bioactivity was not studied any further. Antagonism of *Erwinia herbicola*, a phyllosphere microorganism on canola, against *L. maculans* was tested *in vitro,* and an antifungal substance was found in bacterial culture (Chakraborty et al, 1994). Strains of *Trichoderma* spp. (*T. viride* and *T. harzianum*) were tested for their protective ability against the pathogens *P. lingam* and *Sclerotinia sclerotiorum* (Starzycki et al., 1998). A bacterial strain of *Paenibacillus polymyxa,* recovered from canola roots in Vegreville, Alberta, was found highly inhibitory to the growth of *L. maculans in vitro*. The bacterium was designated as PKB1. Studies were initiated to investigate the potential of *P. polymyxa* PKB1 as a biological control agent against *L. maculans*.

The objectives of this work were to evaluate the inhibitory effect of *P. polymyxa* PKB1 on spore germination and mycelial growth of *L. maculans in vitro* and on canola plants; to determine the influence of *P. polymyxa* PKB1 on the viability of the pycnidiospores of *L. maculans*; and to investigate the effect of *P. polymyxa* PKB1 on other pathogenic fungi on canola and the effect of some commonly used chemicals on *P. polymyxa* PKB1 growth.

Materials and Methods

In vitro assessment of the inhibitory effect of *Paenibacillus polymyxa* PKB1

Bioassay of the inhibitory effect of Paenibacillus polymyxa isolates A bacterial strain (96-1) isolated from canola stubble collected from Sedgewick, Alberta, was identified by Dr. R. Coleman in the Microbiology laboratory, Alberta Research Council, as *Paenibacillus polymyxa*. It was designated as PKB1. Strains 96-2 to 96-5 were isolated from the same source. Five other strains of *P. polymyxa* were obtained from other different sources (**Table 2.1**). Two *P. polymyxa* strains, 97-2 and 97-3, originally collected in Japan and U.S.A, respectively, were provided by Dr. S. Jensen, the University of Alberta.

Strain 96-17 was a type culture of *P. polymyxa* provided by Dr. R. Coleman. Strains 96-9 and 97-4 were collected in Alberta and provided by Dr. J. P. Tewari and Mr. R. Lange.

Bacterial cultures were grown on nutrient agar (NA, Difco) plates. A virulent isolate of *L. maculans* BLA from Dr. Kharbanda (Kharbanda, 1993) was cultured on V-8 juice (20%, v/v)-Rose Bengal (0.04%, v/v) agar plus antibiotic chloramphenicol (100 ppm) for 7 days. One 5 mm-diameter plug containing the *L. maculans* mycelium was transferred to the centre of a potato dextrose agar (PDA, Difco) plate, and a loop of bacterial cells was placed at four places around the agar plug. The plates were incubated under light at room temperature (22°C - 24°C) for one week. The inhibition zone around each bacterial strain was measured between edge of bacterial colony and fungal colony.

Inhibition of spore germination and germ-tube length The pycnidiospore suspension of BLA was prepared and adjusted to a concentration of $1x10^6$ cells mL^{-1}. A loopful of bacterium from a growing culture of *P. polymyxa* PKB1 on nutrient agar (NA, Difco) was inoculated into a 5 mL nutrient broth and incubated on a shaker (200 rpm) at room temperature for 3 days. The bacterial suspension was adjusted to about $1x10^7$ cells mL^{-1}, measured by direct counting under a microscope using a haemocytometer. A piece of autoclaved cellophane membrane was placed on the surface of 2% water agar in a 9 cm-diameter petri plate, and then a mixture containing 0.5 mL of the pycnidiospore suspension and 0.5 mL of the bacterial suspension was spread on to the surface of the membrane. Plates receiving 0.5 mL fungal spore suspension without the bacterium were used as controls. All treatments were replicated four times and arranged in a complete randomized design on a laboratory bench at room temperature under inflorescent lights. After 24, 48 and 72 h of incubation, a small piece of cellophane was cut from each plate, mounted on a microscope slide, and stained with leptophenol cotton blue to count spore germination and measure germ-tube lengths. Data were analyzed using the SAS ANOVA procedure (SAS Institute Inc., 1996).

In vivo assay of inhibitory effect of *Paenibacillus polymyxa* PKB1

Plants of the susceptible cultivar Westar and resistant cultivar Quantum (*Brassica napus* L.) were grown in 15-cm diameter pots in a greenhouse for 2 weeks. The bacterial spore suspension was made, as described previously, in a 1% (w/v) gelatine solution. Plants were sprayed with the pycnidiospore suspension of *L. maculans*, alone or in a mixture with bacterial suspension, 20 mL per pot, then covered

with a plastic bag to maintain high humidity, and incubated at 23°C in a greenhouse for two days. There were 10 pots per treatment, and each pot contained four plants. Six weeks after inoculation, disease severity was assessed based on symptoms on 6 cm crown-stem portion, using a scale of 0 to 4: 0 = no visible symptoms; 1 = lesion size is less than 1/3 around crown-stem portion, no pycnidia present; 2 = lesion size is 1/3 to 2/3 around crown-stem, some pycnidia present; 3 = Lesion size is more than 2/3 around crown-stem. Stem is cankered and extensive pycnidia present; 4 = Plant is completely girdled and dead. The experiment was repeated once. Data were analyzed using the SAS ANOVA procedure (SAS Institute Inc., 1996).

Fluorescence and confocal laser scanning microscopy

A mixture of *L. maculans* pycnidiospore (10 mL, $1x10^7$ spores mL^{-1}) and the bacterial cells of *P. polymyxa* PKB1 (1 mL, $1x10^8$ cells mL^{-1}) in 200 ml potato dextrose broth (PDB) was incubated at 28°C on a shaker (150 rpm). A fungal spore culture (10 mL inoculum into 200 mL PDB) without the bacterium was used as a check. To detect pycnidiospore viability, fungal spores were stained with a fluorescent dye, FungoLight (25 µl mL^{-1}, Molecular Probes, Inc., Eugene, OR, U.S.A.) for 30 min at 37°C, following the instructions provided by the manufacturer, and examined under a Zeiss fluorescent microscope at a 24 h interval for three days.

Interaction of the bacterium and blackleg fungus in culture and on the leaf surface of canola was studied with a fluorescent microscope and a confocal laser-scanning microscope (CLSM). In the culture study, the inoculation and incubation methods were the same as in the viability test. In the leaf test, a mixture of *L. maculans* pycnidiospore (10 mL, $1x10^6$ spores mL^{-1}) and *P. polymyxa* PKB1 cells and spores ($1x10^7$ cfu mL^{-1}) was sprayed onto detached canola (cv. Quest) leaves in a humidity chamber and incubated at room temperature. A fungal spore suspension ($1x10^6$ spores mL^{-1}) without the bacterium was used as a check. After 1, 2 and 3 days of incubation, a sample (1 cm^2) of leaf was cut, placed on a slide and stained with a 0.05% (w/v) acridine orange solution in a sodium acetate acid buffer, and then observed under a Zeiss epifluorescence microscope and a confocal laser-scanning microscope (Molecular Dynamics 2001). The laser used by the confocal microscope has an excitation wavelength of 488 nm. Acridine orange has

an emission of 525 nm (green) when bound to DNA and has an emission of 650 nm (red) when bound to RNA (Richard, 1996). The germinated fungal spores were counted under a fluorescent microscope.

Scanning electron microscopy

Pycnidiospore and bacterial suspensions, alone or in a mixture, were sprayed on a cellophane membrane placed on the water agar in a plate and on intact canola leaves. After 24, 48 and 72 h of incubation, samples were vapour fixed by osmium tetra oxide and "frizzed" for 15 min in liquid nitrogen at solid point (-270°C) in a cryo-stage chamber (Emitech K1250), 30 min at –40 °C to thaw ice crystals at the SEM stage, and then gold-coated at -178°C in a cryo-stage (Emitech K1250) chamber. The specimens were finally examined under a scanning electron microscope (JSM-6301F), and the images were photographed and saved on a computer.

Effect of *P. polymyxa* PKB1 on other pathogenic fungi on canola

The effectiveness of *P. polymyxa* PKB1 was tested against *Sclerotinia sclerotiorum*, *Rhizoctonia solani*, *Alternaria brassicae*, *Pythium* sp. and *Fusarium* sp. Individual plates were inoculated with the bacterium at four places close to the edge of the plate, and a PDA plug (5-mm-diameter) bearing mycelium of either one of the several fungi was placed at the centre of the plate. The inhibition zones between fungi and bacterial colonies were measured seven days after incubation at the room temperature under the light.

In another experiment, the bacterium was grown in nutrient broth (NB) for one week. A filtrate was then collected by centrifuging the bacterial suspension at 5000 g for 15 minutes and filtering it through a microfilter system (0.22 μm). The filtrate (1 mL) was mixed with 9 mL

potato dextrose broth (PDB, Difco) in a 5-cm diameter petri plate and inoculated with a PDA plug (5-mm diameter) bearing mycelium of one of the fungal isolates listed. Four replicated plates were used for each isolate. PDB plates without the bacterium filtrate served as a control. The mycelium dry weight was determined after incubation for one week at room temperature, and the data were analyzed statistically using the SAS ANOVA program (SAS Institute, Inc., 1996).

Effect of fungicides and herbicides on the bacterium

Sterile filter paper discs, 1-cm diameter, were immersed in the fungicides Tilt® (propiconazole, Novartis Crop Protection Canada Inc.), Sportak® (prochloraz), Rovral® (iprodione, Aventis Crop Science),

Helix®(Novartis Crop Protection), Vitavax RS®(carbathiin, Gustafson), Apron®(metalaxyl, Gustafson), Bravo ultrex® (chlorothalonil, Zeneca Agro), and Ronilan EG® (vinclozolin, BASF) suspensions, or the herbicides Lontrel® (clopyralid, Dow AgroSciences Canada Inc.), Poast® (sethoxydin, BASF) and Muster® (ethametsulfuron-methyl, Dupont Canada Inc.), Round Up® (glyphosate, Monsanto Canada Inc.), MCPA® (amine, ester, K and Na-salt, Novartis Crop Protection Canada Inc.) and Abound®, at the concentration of 100 ppm, and placed at the centre of 10-cm-diameter petri plates containing PDA. The plates were pre-seeded with the bacterium. Four replicate plates per treatment were arranged in a completely randomized design and incubated at room temperature. Inhibition zones around the fungicide discs were measured three and seven days after incubation. The test was repeated once.

Results

In vitro assessment

P. polymyxa PKB1, 97-1, 97-4 and all bacterial strains from Vegreville showed an inhibitory effect on the mycelial growth of *L. maculans,* whereas two cultures from other sources (97-3 and 96-17) had no effect to *L. maculans* (**Figure 2.1**). Because *P. polymyxa* PKB1 was the first one isolated from canola stubble in Alberta and had a high inhibitory effect against *L. maculans*, this strain was used throughout the study. The other strains might have been from the same bacterial population in the canola field; however, no further attempt was made to study these strains in detail.

The percentage of spore germination and the average germ-tube length of *L. maculans* were dramatically reduced in the presence of *P. polymyxa* PKB1 (**Figure 2.2**). At the beginning, some pycnidiospores germinated and produced germ tubes. As the incubation time increased, the percentage of spore germination was reduced.

In vivo assay

The two repeated experiments had a similar result. Data in the first experiment was analyzed. In greenhouse experiments, *P. polymyxa* PKB1 significantly reduced disease incidence and severity on susceptible canola cultivar Westar but not on resistant cultivar Quantum (**Figure 2.3**). Leaves sprayed with

blackleg fungal spores and bacterial suspension developed local lesions, but the stem canker was significantly reduced on Westar six weeks after inoculation.

Spore viability studied by fluorescence and confocal laser scanning microscopes

Pycnidiospores of *L. maculans* incubated with the bacterial suspension of *P. polymyxa* PKB1 had a dull yellow color under a fluorescent microscope, whereas spores without bacteria presence had a bright yellow-green color with red cylindrical objects inside the cells (**Figure 2.4**).

Similar results were observed under a fluorescent microscope and a confocal laser-scanning microscope. The viability and germination of *L. maculans* spores were dramatically reduced and inhibited by *P. polymyxa* PKB1. The germination of fungal spores was dramatically reduced in the presence of bacterium *in vitro* one day after incubation, and pycnidiospores had a defused, dull to transparent light green color (**Figure 2.5A and B**). On the second day of incubation, most of the pycnidiospores in the culture without bacterial cells germinated and grew actively while very limited growth of *L. maculans* was observed in the bacterium inoculated culture two days after incubation (**Figure 2.5C and D**). At day three, germinated spores of *L. maculans* grew into a mass of mycelium in the culture without bacterium whereas in cultures with *P. polymyxa* PKB1, the bacterium obtained a high concentration, and growth of *L. maculans* spores was completely inhibited (**Figure 2.5 E and F**).

Confocal laser scanning microscope images showed that in the presence of PKB1, pycnidiospores of blackleg had reduced and delayed germination, slower growth and lesser penetration through stomata on canola leaves than pycnidiospores without the presence of PKB1 on the leaf had at the same time (**Figure 2.6**). However, the bacterium caused slight damage to the leaf when the bacterial concentration was high.

SEM study

Light and scanning electron microscope studies showed no obvious morphological changes on the pycnidiospore surface treated with the bacterial cell suspension (**Figure 2. 7**). Both fungal spores and bacterial cells were covered with heavy mucilaginous materials. Pycnidiospores surrounded by the bacterial cells and spores had reduced germination and slower germ-tube growth both on the cellophane membrane (**Figure 2.7a to 2.7d**) and plant leaves (**Figure 2.7e and 2.7f**), compared with those without the presence of the bacterium. Bacterial endospores could be observed around germ tubes of *L. maculans* 3 days after treatment (**Figure 2.7d**).

Effect of bacterium on other pathogenic fungi on canola

P. polymyxa PKB1 was inhibitory to all the four fungi tested in petri-plates (**Figure 2.8**). A significant (P=0.05) inhibitory effect of the bacterial cell suspension on mycelial growth in liquid culture of all fungi tested was observed (**Table 2.2**). The result suggested that *P. polymyxa* PKB1 could be a potential biological control agent against not only blackleg but also other diseases of canola.

Effect of fungicides and herbicides on the bacterium

Two sets of data were combined and analysed. All the herbicides and the fungicides tested showed no inhibitory effect on the bacterium. Prochloraz® and Rovral® showed a small degree of inhibitory activity three days after the treatments started, but this was overcome by the bacterium by the seventh day (**Table 2.3**).

Discussion

The development of biocontrol organisms that are effective in controlling plant diseases is an important goal of biological control. Using bacteria to control fungal diseases on agricultural, forestry and horticultural plants has been of increasing interest to researchers. In the past several decades, many bacterial strains of *Paenibacillus* spp., *Bacillus* spp. and *Pseudomonas* spp. have been proven to be valuable agents and have been extensively investigated for biocontrol purposes (Godoy et al., 1991; Hill et al., 1997; Lange et al., 1994; Liang et al. 1996; Mavingui et al., 1992; Mavingui and Heulin, 1994; Tang et al., 1996; Weller et al., 1988; Yuen et al., 1991).

Antifungal and antibiotic effects of *P. polymyxa* on the growth of *Ceratocystis ulmi* (Parmentier, 1988) and on various potato pathogens (Lange et al., 1994) have been reported. Strains of *P. polymyxa* inhibited mycelial growth of *Sclerotinia sclerotiorum* showing potential for biological control of white mold disease of bean and root rot of vegetables (Oedjijono et al., 1993). Some strains of *P. polymyxa* have also shown the effect of promoting the growth of wheatgrass, ryegrass and white clover (Holl et al., 1988), western hemlock, pine and spruce seedling plants (Chanway, 1995; Holl and Chanway, 1992; Shishido et al., 1995, 1996), and increasing the yield of wheat (Rodriguez et al., 1996). Sixteen *P. polymyxa* out of 100 isolates from barley rhizosphere showed *in vitro* fungal antagonism against widely different plant-pathogenic microfungi (*Aphanomyces cochleoides, Pythium ultimum and Rhizoctonia solani*) (Nielsen and Sorensen, 1997). Walker et al. (1998)

demonstrated that *Bacillus* isolates from the spermosphere of peas and dwarf French beans had antifungal activity against *Botrytis cinerea* and *Pythium* spp. In the current study, the strain *P. polymyxa* PKB1 was highly inhibitory to the growth and development of *L. maculans*, demonstrating that strain PKB1 has a great potential for biological control of blackleg of canola. Other strains from the United States (*P. polymyxa* ATCC842 and *P. polymyxa* NCIB 8648), however, did not show an inhibitory effect on *L. maculans*. *P. polymyxa* subsp. *colistinus koyama* ATCC 21830 showed some antifungal effect on the blackleg fungus. The same results have been obtained by Beatty (2000), who found no antifungal activity of *P. polymyxa* NCIMB 8648 against *L. maculans*. Her study indicated that the effect of bacterial strains was specific to certain fungal pathogens.

It has been known that different biological mechanisms account for the biocontrol activities (Baker and Cook, 1974; Cook and Baker, 1983). These mechanisms include the production of secondary metabolite compounds such as antibiotics, direct competition between the microbial antagonist and the pathogen for nutrients and/or preoccupation of environmental niches, and direct parasitism. At least two groups of antibiotic metabolites were identified from *Pseudomonas* spp. (Keel et al., 1992; Thomashow and Weller, 1990) and seven or more other types from *P. polymyxa* (Beatty, 2000; Umezawa et al., 1978). Antibiotic metabolites played the most important role in biocontrol of plant pathogens using bacteria (Hill et al., 1997). As well, Weller et al. (1988) found that the production of antibiotics, siderophores, and chitinase was an important characteristic of some bacteria that suppress root and seedling diseases. Nielsen and Sorensen (1997) reported that 16 isolates of *P. polymyxa* were antagonistic to many pathogenic fungi and shared a characteristic profile of cell-wall-degrading enzymes. This enzyme profile was not observed in any of the non-antagonistic isolates. In a recent study, the mechanism involved in the inhibition effect of *P. polymyxa* PKB1 was most likely due to the production of an antifungal compound which is a mixture of four closely related oligopeptides, each containing nine amino acids (Beatty, 2000). The electron microscopy work in the current study also showed that the presence of bacterial cells did not cause obvious morphological changes of *L. maculans* spores. This result suggested that the inhibition effect was due to chemical but not physical or mechanical attack, including parasitism.

Research has demonstrated that production of antibiotic metabolite is typically associated with adverse growth conditions, nutrient limitation or entry into the stationary growth phase of the bacteria. Studies by

Beatty (2000) showed that *P. polymyxa* PKB1 produced anti-fungal metabolite after the onset of sporulation of the bacterium, and the antibiotic was associated mainly with the spores rather than with the free cells in the medium. In the germination test, the pycnidiospore of *L. maculans* treated with *P. polymyxa* PKB1 cell suspension could produce germ tubes at the early stage. However, as the number of bacterial cells increased and started to sporulate and produce an inhibitory substance, the germination and growth of fungal spores declined.

Systemic infection of *L. maculans* on canola has been proposed, in which the fungal spores cause infection on leaves and then the hyphae grow to the lower stem and cause stem canker (Hammond et al., 1985). In the current greenhouse test, *P. polymyxa* PKB1 sprayed on the leaves inoculated with *L. maculans* spores significantly reduced canker formation on the stems of susceptible cultivar Westar. This result suggested that the bacterium could inhibit the fungal mycelium growing from the leaf to the stem under greenhouse conditions. The bacterium had a significant inhibitory effect on both the growth and development of *L. maculans*. The mechanism involved in the infection needs to be further studied.

Fusarium spp., *R. solani*, and *Py*thium spp. are mainly soil-borne pathogens and cause damping-off, seedling blight and root rot diseases in canola. *P. polymyxa* PKB1 tested *in vitro* had a highly inhibitory effect against these pathogenic fungi. It is very encouraging that it would be extremely economical if several soil-borne diseases could be simultaneously controlled by a single application of the biocontrol agent. However, the biocontrol of a disease involves the interactions among the plant (host), causal agent (pathogen), biocontrol agent, and the biotic and abiotic environment. Further investigations need to be performed to understand the stability, suitable environmental conditions and delivery system for the biocontrol agent.

All fungicides and herbicides tested here did not affect the growth of *P. polymyxa* PKB1 *in vitro*. Results indicated that the application of commonly used herbicides and fungicides on canola would not have deleterious effects on the survival of the bacterium in field conditions. It would be safe to apply the bacterium along with the chemicals currently being sprayed on fields.

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Table 2.1. *Paenibacillus polymyxa* strains collected in Vegreville* and from other sources.

No	Code	Species	Source
1	96-1	*P. polymyxa* (PKB1)	Alberta Research Council, Vegreville,
2	96-2	*P. polymyxa*	Alberta Research Council, Vegreville
3	96-3	*P. polymyxa*	Alberta Research Council, Vegreville
4	96-4	*P. polymyxa*	Alberta Research Council, Vegreville
5	96-5	*P. polymyxa*	Alberta Research Council, Vegreville
6	96-9	*P. polymyxa*	University of Alberta, Dr. Tewari
7	96-17	*P. polymyxa* (ATCC 842)	Alberta Research Council, Dr. Coleman
8	97-2	*P. polymyxa* subsp. *colistinus koyama* (ATCC 21830)	University of Alberta, Dr. Jensen
9	97-3	*P. polymyxa* (NCIB 8648)	University of Alberta, Dr. Jensen
10	97-4	*P. polymyxa*	Alberta Research Council, Mr. R. Lange

* Strains 1 – 6 were identified by Dr. R. Coleman in Microbiology Lab at Alberta Research Council, Vegreville, Canada.
ATCC = American Type Culture Collection, U. S. A.
NCIB = National Collection of Industrial Bacteria, Scotland

Table 2.2. Effect of *P. polymyxa* PKB1 on mycelial growth of some pathogenic fungi on canola

		Mean mycelial dry weight (mg)	
Isolate	Pathogen	With bacterium	Control
S94-1	*Sclerotinia sclerotiorum*	5 a*	152 b
S94-2	*S. sclerotiorum*	3 a	89 b
88-1-8	*Pythium pythioides*	16 a	94 b
C51-25	*Rhizoctonia solani* AG2-1	13 a	147 b
R21	*R. solani* AG2-1	9 a	153 b
N15-6	*Fusarium avenaceum*	4 a	53 b
P66-30	*F. avenaceum*	3 a	43 b
Alt-1	*Alternaria brassicae*	3 a	80 b
CA2	*A. brassicae*	4 a	21 b

* Means of four replications; values in rows for each fungus followed by the same letter are not significantly different determined by Duncan's Multiple Range Test (P=0.05).

Table 2.3. Effect of fungicides and herbicides (100 ppm) on the growth of *P. polymyxa* PKB1 presented as the inhibition zone

Treatment	Mean inhibition zone (mm)	
	Day3	Day7
Tilt®	1	0
Rovral®	6	0
Prochloraz®	7	1.5
Abound®	0	0
Ronilan EG®	0	0
Bravo ultrex®	0	0
Helix®	0	0
Vitavax RS®	0	0
Apron®	0	0
Poast®	0	0
Muster®	0	0
Lontrel®	0	0
Round Up®	2.25	1.25
MCPA®	0	0
Control, water	0	0

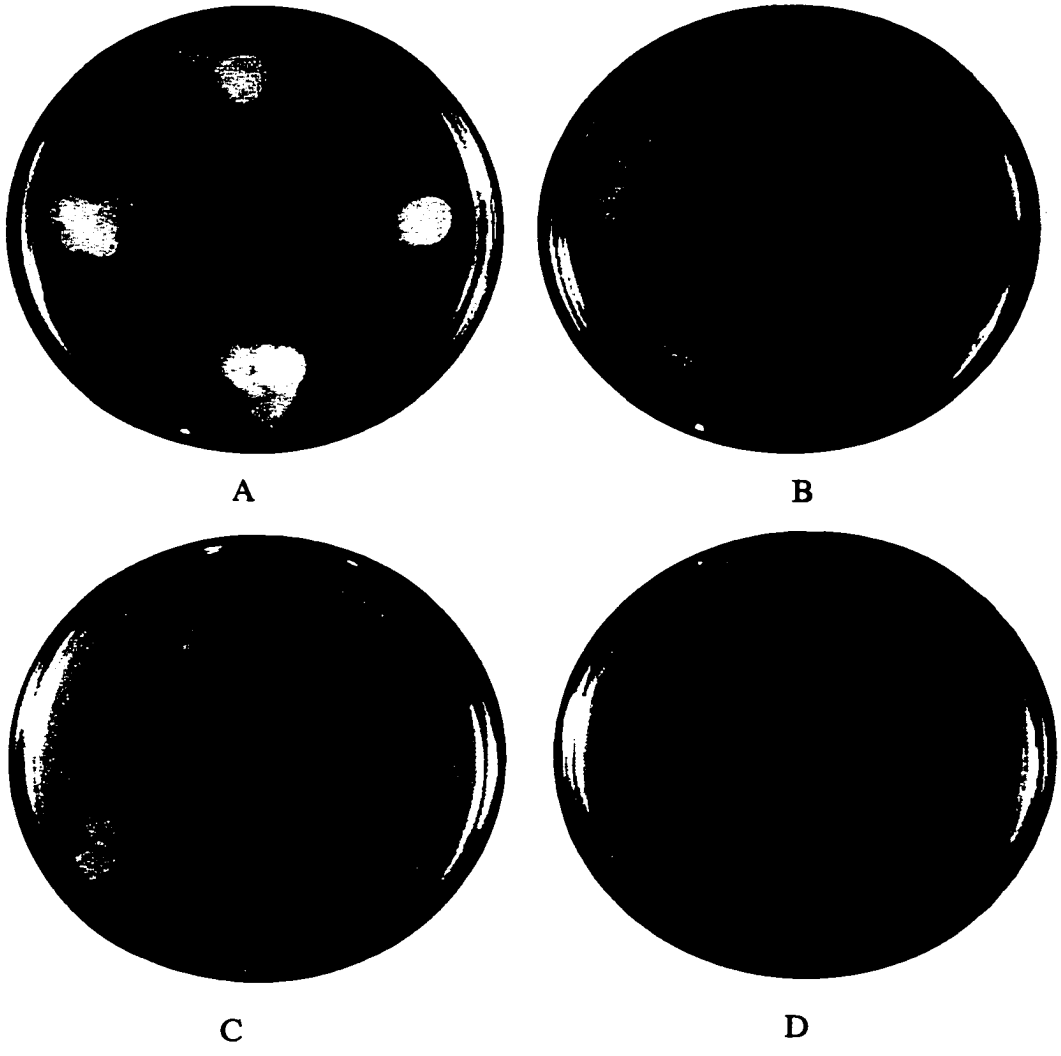

Figure 2.1. Inhibitory effect of *Paenibacillus polymyxa* PKB1 (A), *P. polymyxa* 97-2 (B), *P. polymyxa* 97-3 (C) *P. polymyxa* 96-17 (D) against *Leptosphaeria maculans*.

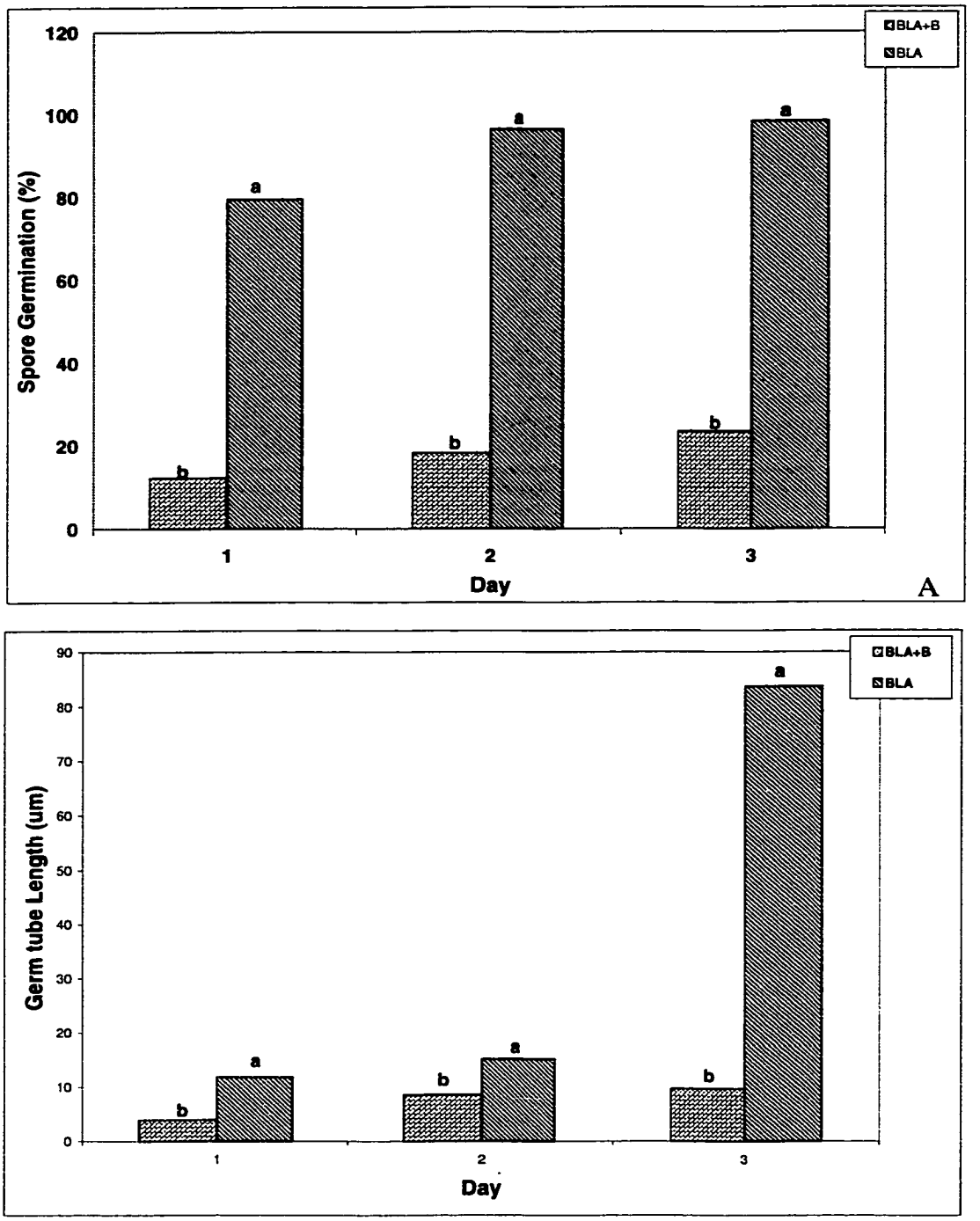

Figure 2.2. Inhibition effect of *P. polymyxa* PKB1 on spore germination (A, based on 400 spores), and germ tube length (B, average of 100 spores) of *Leptosphaeria maculans* (BLA). Means within days with different letters are significantly different according to Duncan's Multiple Range Test (P=0.05).

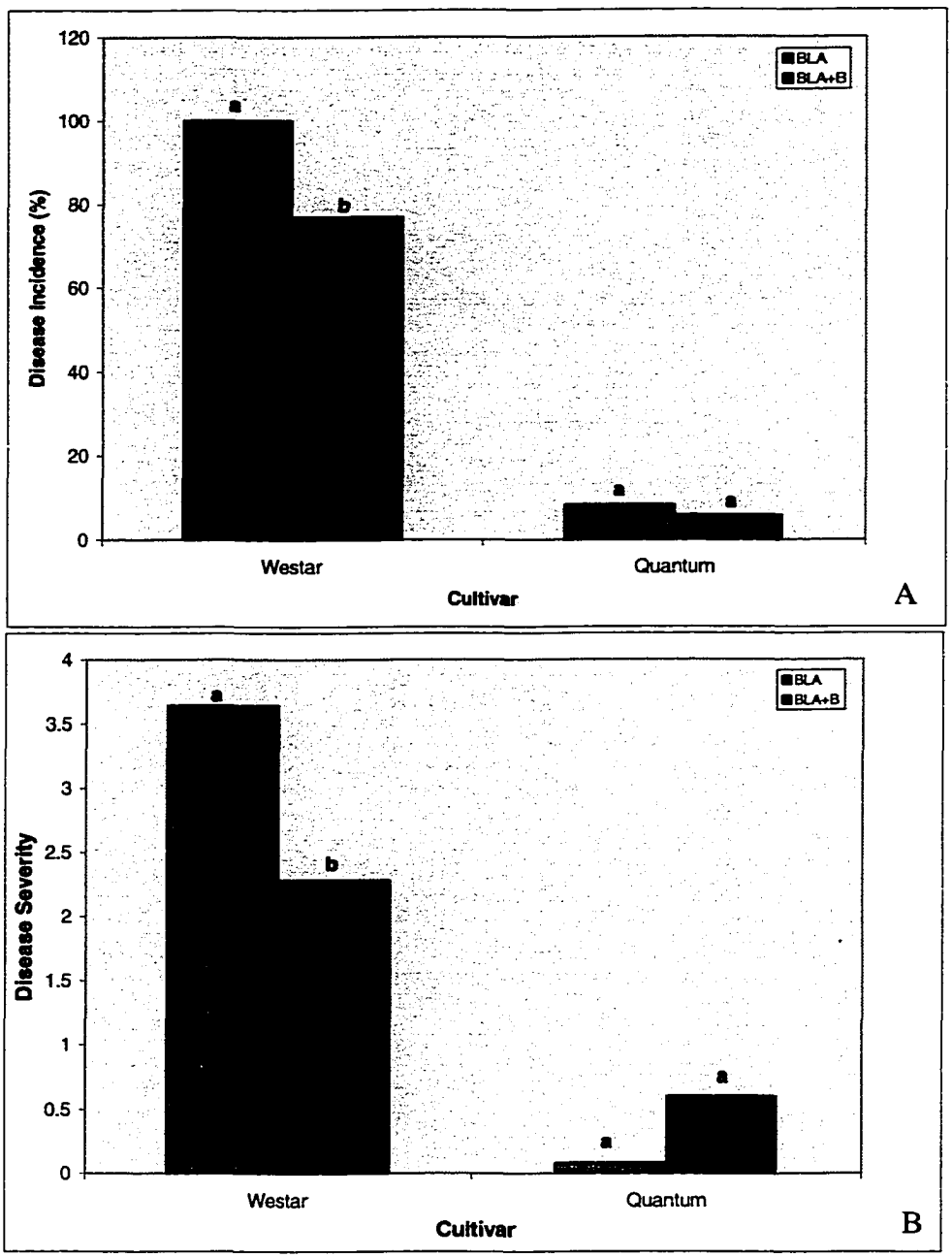

Figure 2.3. Effect of *P. polymyxa* PKB1 on (A) disease incidence (based on number of infected plants) and (B) disease severity (based on 0 – 4 scales, 0 = no visible symptoms; 1 = less than 1/3 stem lesion, no pycnidia; 2 = 1/3 to 2/3 stem lesion, some pycnidia; 3= more than 2/3 lesion on stem, cankered and extensive pycnidia; 4 = dead plant) caused by *Leptosphaeria maculans* (BLA) on canola plants in a greenhouse test. Means within cultivars followed by the same letter are not significantly different according to Duncan's Multiple Range Test (P=0.05).

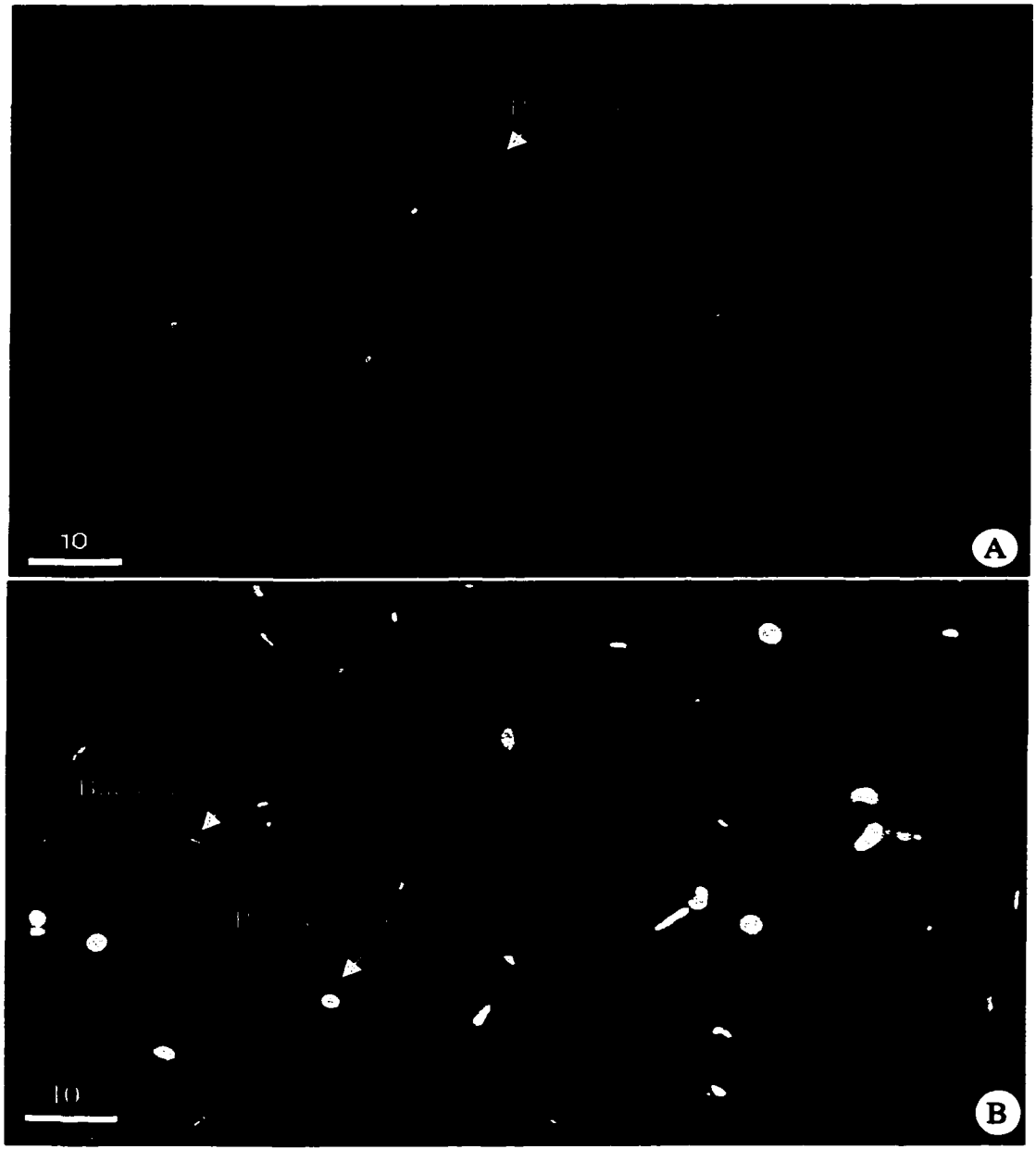

Figure 2.4. Fluorescent micrographs of (A) viable pycnidiospores of *L. maculans showing* bright green color with red cylindrical objects inside the cell, and (B) non-viable pycnidiospores stained dull yellow in the presence of *P. polymyxa* PKB1one day after incubation. (scale: μl).

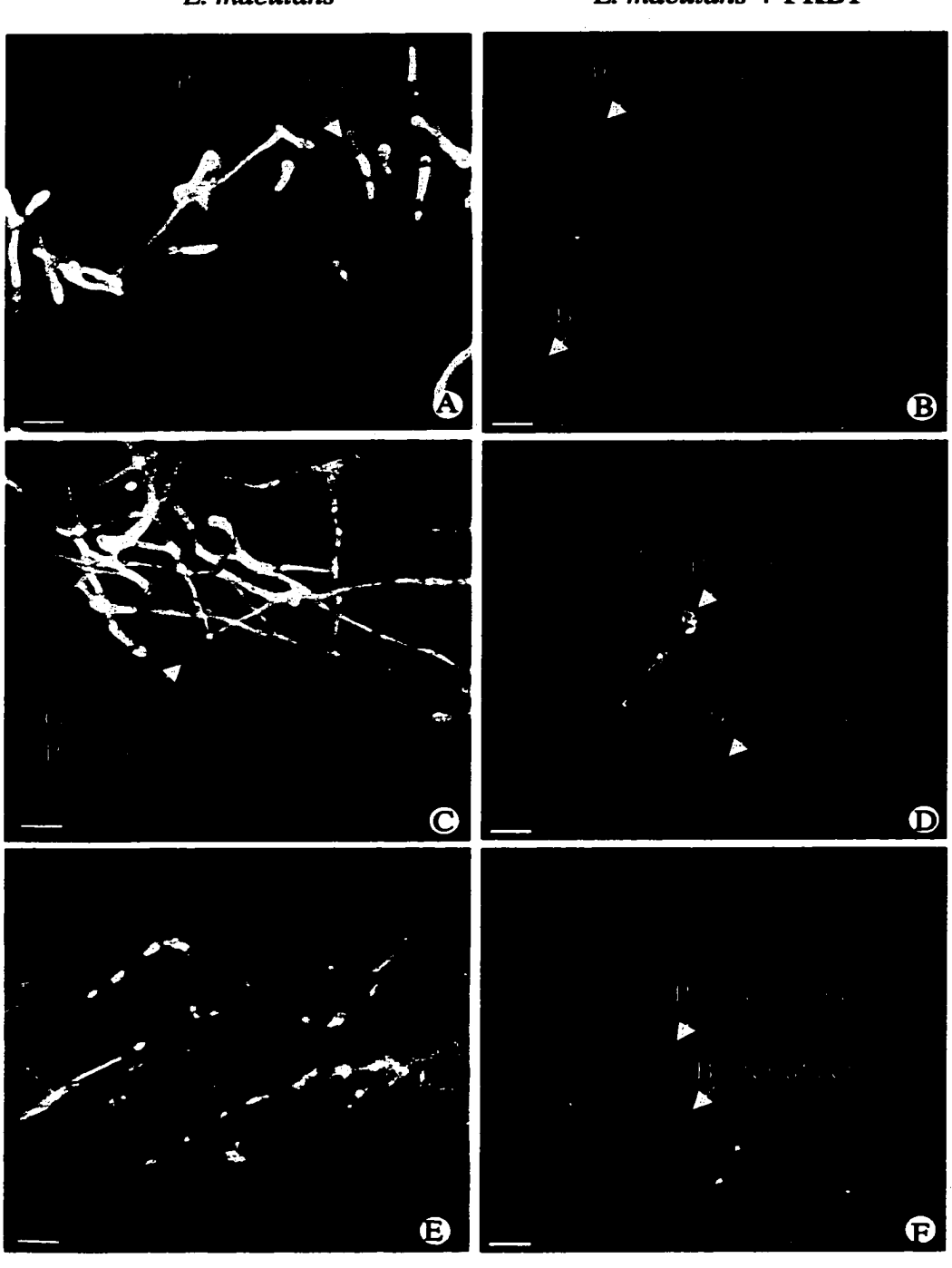

Figure 2.5. Fluorescent (A, C and E) and confocal laser scanning micrographs (B, D and F) showing the effect of *P. polymyxa* PKB1 to germination of pycnidiospores of *L. maculans* in liquid cultures 1, 2, and 3 days after incubation (B, D and F) . A, C and E are the control culture without bacterial cells presence 1, 2, and 3 days after incubation. (scale: μm).

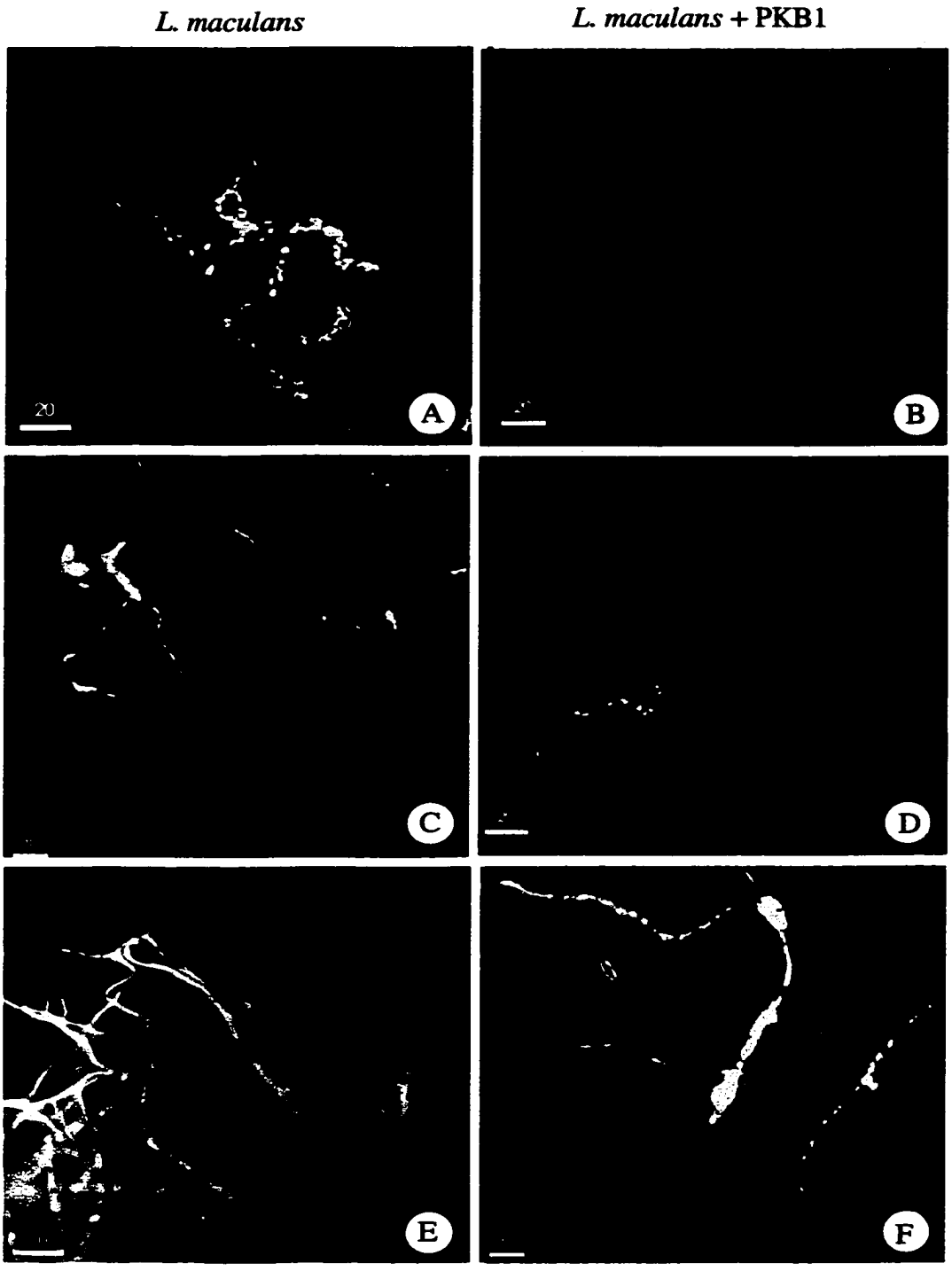

Figure 2.6. Fluorescent and confocal laser scanning micrographs showing the effect of *P. polymyxa* PKB1 on germination of pycnidiospores of *L. maculans* on detached canola leaves1, 2, and 3 days after incubation (B, D and E) . A, C and E are the control without bacterial cells presence 1, 2, and 3 days after incubation. (scale: μm).

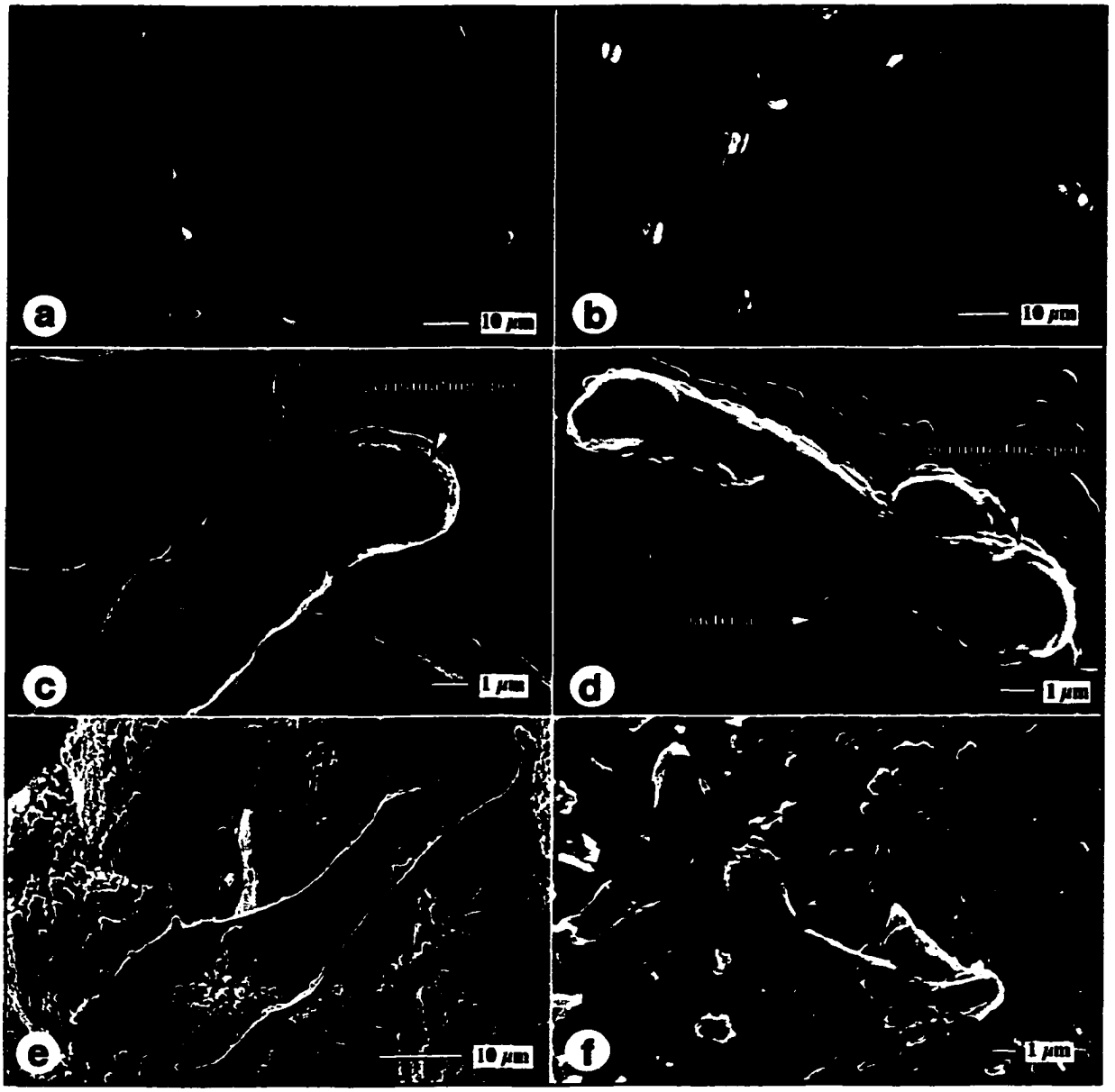

Figure 2.7. Scanning electron micrographs of germination of pycnidiospores of *Leptosphaeria maculans* (a) without bacterial treatment and (b) treated with bacterium on a cellophane membrane two days after incubation at room temperature; (c) without bacterial treatment and (d) treated with bacterium on a cellophane membrane three days after incubation at room temperature; (e) without bacterial treatment and (f) treated with the bacterium on a canola leaf surface three days after incubation in a greenhouse.

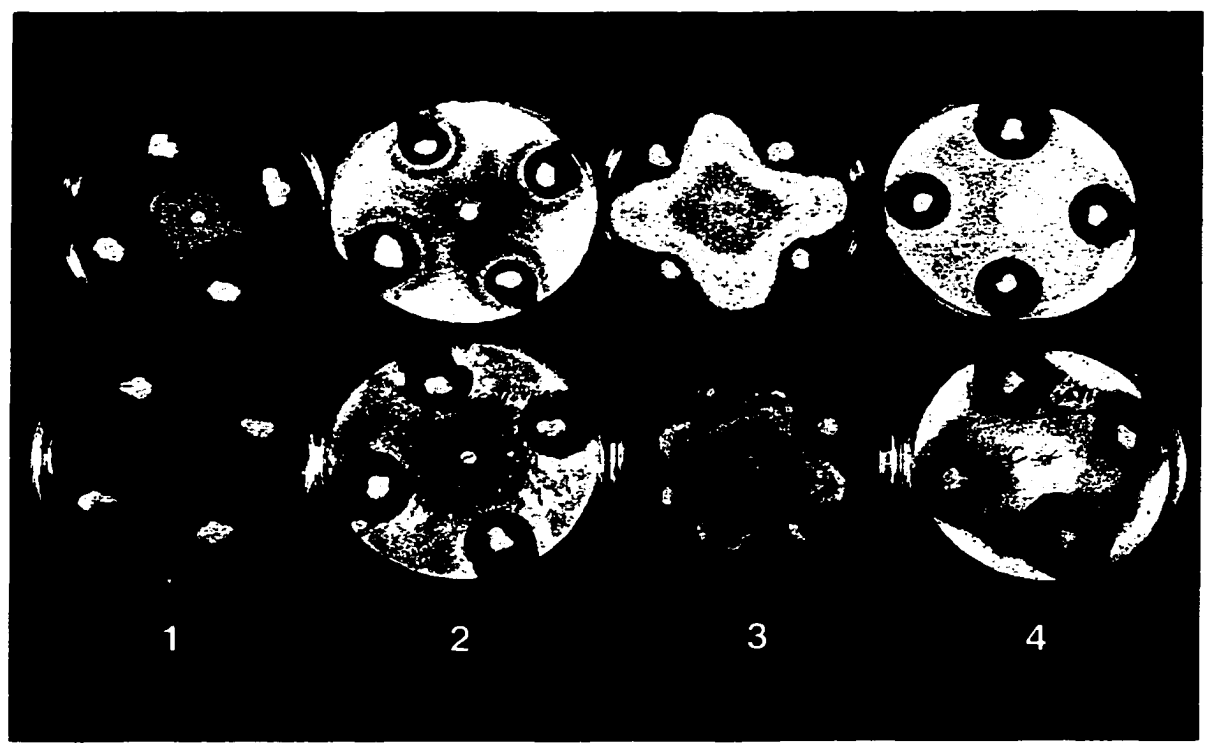

Figure 2.8. Inhibitory effect of the bacterium *Paenibacillus polymyxa* PKB1 on the growth of (1) *Fusarium avenaceum*, (2) *Sclerotinia sclerotiorum*, (3) *Rhizoctonia solani*, (4) *Pythium pythioides*.

Chapter 3

Integrated Control of Blackleg of Canola Using Tilt®, Compost and *Paenibacillus polymyxa* PKB1

Introduction

Leptosphaeria maculans affects all parts of the canola plant. The fungus is seed- and stubble-borne, and overwinters on infected canola stubble. It produces pycnidiospores and ascospores, which are important sources of primary inoculum (Bokor et al., 1975; Kharbanda, 1993; Ndimande, 1976). Air-borne ascospores can infect the crop throughout the season. Young seedlings are very susceptible to the disease, and plants develop tolerance to the disease as they mature beyond the six-leaf stage (Gugel and Petrie, 1992).

Fungicidal seed-slurry and seed-soak treatments have provided a good defence against introduction of the disease into new areas (Gabrielson et al., 1977; Maude et al., 1984). However, seed treatments have failed to control the disease under some field conditions (McKenzie and Verma, 1988). Furthermore, these treatments do not protect seedlings from air-borne ascospores. Applying flutriafol to canola as a coating on superphosphate fertilizer was not effective (Xi et al., 1991). Foliar fungicide application is ineffective unless sprayed repeatedly during early stages of crop growth (Kharbanda, 1992; Kharbanda et al., 1996; Rawlinson et al., 1984). Rempel and Hall (1995) found that foliar sprays of triazole fungicides at the late rosette stage reduced the disease incidence and severity, and increased yield of canola. Humpherson-Jones and Burchill (1982) demonstrated that fungicides applied on oilseed rape straw could suppress the development of the sexual stage of *L. maculans*. The amount of viable fungus over-wintering on canola stubble was reduced, and the formation of ascospores was prevented, reducing the initial inoculum. Application of the fungicide Tilt® (propiconazole) can effectively control *L. maculans* but is phytotoxic to canola when sprayed as a mixture with an adjuvant during early stages of crop growth (Kharbanda, 1992). Post-harvest application of Tilt® to infected stubble in the fall can eliminate the phytotoxicity problem and may reduce pseudothecia formation of *L. maculans* in the following spring.

Cultural and biological controls are two alternatives to synthetic chemical pesticides in disease management. Cultural control includes all aspects of crop husbandry, which influence disease development

such as crop rotation, tillage and nutrient management. Organic matter or compost amendment to agricultural soil has been successfully used to control soil-borne diseases (Hoitink and Fahy, 1986; Schuler et al., 1989; Tuitert et al., 1998; Voland and Epstein, 1994).

Biological control has increasingly interested researchers and is practised in many cropping systems (Adams, 1990; Tang et al., 1996). Some biocontrol products have been registered for control of plant diseases. For example, Quantum 4000 HB, a product based on *Bacillus subtilis* strain A-13/GB03, and a seed inoculant for peanuts, beans and cotton, provided control of Rhizoctonia root rot (Turner and Backman, 1991). In our previous study, a strain of *Paenibacillus polymyxa*, designated as PKB1, isolated from canola roots was found to be highly inhibitory to the growth of *L. maculans in vitro* (Chapter 2).

Integrated pest management (IPM) provides balanced, long-term and economical strategies for modern agricultural systems. The integration of biological, cultural and chemical controls into crop protection may combine the advantages of these strategies and provide both short and long-term disease controls.

Research was undertaken to determine the effectiveness of *Paenibacillus polymyxa* PKB1, alone or in combination with Tilt® or compost, in controlling blackleg of canola in the growth chamber and the field. The previous *in vitro* study had demonstrated that Tilt® had no deleterious effect on *P. polymyxa* PKB1 (Chapter 2). It should, therefore, be possible to apply both *P. polymyxa* PKB1 and Tilt® together to canola stubble. The objectives of this study were (1) to determine the inhibitory effect of *P. polymyxa* PKB1 and Tilt® on pseudothecia production by *L. maculans* under controlled conditions; (2) to determine the effectiveness of *P. polymyxa* PKB1 and Tilt® in inhibiting pseudothecia formation and reducing the viability of *L. maculans* on canola stubble under field conditions; and (3) to investigate the suitability of compost as a carrier of the bacterium for field applications.

Materials and Methods

Effects of Tilt ® and *P. polymyxa* PKB1 on pycnidia and pseudothecia formation and survival of *L. maculans* on canola stubble in the growth chamber

Effect of Tilt ® and *P. polymyxa* PKB1 on pycnidia and pseudothecia formation and fungal survival on canola stubble was determined in a growth chamber experiment. The experimental design was a three-factor factorial experiment with a split-plot design with four replications. Factor A was temperature (20°C, 10°C, -5°C and 20°C, 10°C, -5°C, 10°C, 20°C in succession), factor B was burial (buried and un-buried), and factor C was spray treatment (Tilt®, *P. polymyxa* PKB1 and water as control).

P. polymyxa PKB1 was cultured in potato-dextrose broth on a shaker (200 rpm) for 5 days. The canola cultivar Westar was planted in 15-cm-diameter pots, four seeds per pot, using an autoclaved greenhouse soil mix (soil: sand: vermiculite: peat, 1:1:1:1, v:v:v:v). When the plants reached the 5-leaf stage, stems of all the plants were inoculated by inserting pycnidia and pycnidiospores of *L. maculans* into an incision on the canola stem at approximately 2 cm above the soil level by using a mixture of two mating types (+ and -) of *L. maculans* capable of producing pseudothecia (obtained from Dr. P. H. Williams, Wisconsin University, U.S.A.). Six weeks after inoculation, all plants were chopped, leaving about 15cm stem above the soil level. All pots were then divided into three groups of 32 pots each; stems in the first groups were sprayed with Tilt® (125 g a.i./ha) + 1%gelatine, the second group was sprayed with the bacterial suspension (7.4×10^7cells and spores mL^{-1}), and the third group was sprayed with water as a control. Eight pots from each of the three groups were placed for 10 weeks either at -5°C, 10°C, or 20°C, or for 2 weeks at each of the following temperatures in succession, somewhat simulating outdoor conditions under the snowcover close to the ground level from fall to spring: 20°C, 10°C, -5°C, 10°C, and 20°C. Out of the eight pots in each group at each temperature regime, four were covered with sterilized soil mix so as to cut off light, simulating soil-covered stubble. At each temperature regime, the pots were completely randomized and arranged in four growth chambers.

Development of pycnidia was recorded for each stem (10 cm portion from root upwards) 10 weeks after application of Tilt® and bacterial suspension, by using a 0-3 rating system (0 = no pycnidia, 1 = less than 10% stem of 10 cm portion covered with pycnidia, 2 = 11 to 50% covered with pycnidia, and 3 = more than 50%

covered with pycnidia). Stems were also examined for the presence of pseudothecia. The viability of *L. maculans* in stem lesions and in the canola root was determined by culturing the stem and root tissues on a 20% (v/v) V8-juice agar medium amended with Rose Bengal (0.04%, v/v) and streptomycin (300 ppm). Viability of the bacterium was determined by culturing treated and untreated pieces of the stubble on nutrient agar amended with Tilt® (100 ppm) to suppress any fungal contaminants. The bacterial colonies were counted, and selected colonies were tested for the biocontrol effect. The experiment was repeated once.

Effects of Tilt ® and *P. polymyxa* PKB1 on pycnidia development, pseudothecia formation and survival of *L. maculans* on stubble under field conditions

(1) Effectiveness of Tilt® as post-harvest application: To determine the effect of fall-application of Tilt® on ascospore formation of *L. maculans* and disease control, a field experiment was conducted in Wainwright, Alberta from 1993 to 1995. Four 50 x 6 m blocks were established in a blackleg-infested canola field in Wainwright in 1993. Two randomly chosen blocks were sprayed with Tilt® (125 g a.i. ha^{-1}) and another two blocks were unsprayed as controls. Infected stubble pieces of canola were randomly selected at the time of fungicide application (Nov. 15, 1993) and microscopically examined in the laboratory for the presence of pseudothecia and ascospores on the infected stubble. Six month later, 25 stem pieces were collected at random on April 20, 1994 from each block and examined again in the laboratory for the presence of pseudothecia and ascospores. Incidence of ascospore-formed stem was calculated based on the presence (1) and absence (0) of ascospores on the stem checked. The density of ascospores on the stem in a 6 cm length was rated based on a scale of 0 – 3, in which 0 = no ascospores; 1 = less than 50% stem portion found to have ascospores, most pseudothecia immature (no ascospores inside pseudothecia except pseudoparaphysis); 2 = >50% pseudothecia found to contain ascospores; 3 = 100% pseudothecia found to contain ascospores). All blocks were seeded with barley (*Hordeum vulgare* cv. Harrington) on May 16, 1994. Stem pieces of canola crop residues were again collected from the field plot and examined on April 25, 1995 for the presence of pseudothecia and ascospores, and tested for viability of the pathogen on V8-juice agar plates. The method used for the viability test of *L. maculans* was the same as mentioned previously in the growth chamber test. In 1995, an experiment with a split-plot design and four replications was conducted in the same experimental area of 1993 and 1994 (50 m x 24 m).

Tilt®sprayed/unsprayed plots were the main plots, and cultivars were the subplots. Three canola (*B. napus*) cultivars Bullet, Legacy, and Westar, which are resistant, intermediate and susceptible to the virulent strain of *L. maculans*, respectively, were sown in the subplots. Each subplot was 6 m x 6 m and contained 30 rows, spaced at 20 cm apart. The field experimental designs for each year are illustrated in **Appendix 1**. Two hundred seeds were sown per row at a depth of 2 cm. The percent emergence and the percent infected seedlings were recorded one month after seeding. Plant stand, blackleg severity, and yield were determined at crop maturity. Blackleg severity was assessed using the scale described in growth chamber test.

Values were converted to mean disease severity (MDS) by using the formula:

$$MDS = \frac{\Sigma i P_i}{\Sigma P_i}$$

i : Numerical value of the disease category, $i = 0, 1, 2, 3, 4$
P_i: Number of plants in a disease category

(2) Effectiveness of *P. polymyxa* PKB1 and Tilt® Application: The efficacy of *P. polymyxa* PKB1 on reducing pseudothecia formation under field conditions was tested by applying the bacterial suspension (7.4 x 10^7 cells and spores mL^{-1}), Tilt® (2.5 mL L^{-1} water + Assist (adjuvant)), Tilt® plus bacterial suspension or sterile water to naturally infected canola stem pieces (15 cm long). The stubble were collected in the fall from Wainwright in 1994 and from Warburg in 1995. Two field experiments were conducted at the Vegreville experimental plots of the Alberta Research Council (ARC) from 1994 to 1996, using the treatments mentioned above. In the first experiment, pieces of canola stubble were trimmed to 13 cm long and treated by dipping the stems into (1) Tilt® suspension, (2) *P. polymyxa* PKB1 suspension, (3) Tilt® and *P. polymyxa* PKB1 suspension, and (4) water as a control. In the first experiment, ten treated canola stubble pieces from each treatment were placed on the surface of soil in a canola field in the fall of 1994 and marked for retrieval. Four replicates were used for each treatment. In the spring of 1995 and 1996, five stubble pieces of each treatment were collected and examined for ascospore production (rated using a scale of 0 to 3: 0 = no ascospores; 1 = less than 50% stem portion found to have ascospores, most pseudothecia immature that no ascospores inside pseudothecia except pseudoparaphysis; 2 = >50% pseudothecia found to contain ascospores; 3 = 100% pseudothecia found to contain ascospores). At least 10 pseudothecia were examined for the presence of ascospores.

The second field experiment was designed as a 3-factor factorial experiment and all treatments were arranged in a split-split plot design with four replications. Factor A was spray treatment [(1) Tilt® suspension, (2) *P. polymyxa* PKB1 suspension, (3) Tilt® and *P. polymyxa* PKB1 suspension, and (4) water as a control], factor B was cultivars [Westar *(B. napus)* and Tobin (*B. rapa*)], and factor C was burial (buried and surface). A total of 320 stubble pieces (13 cm long) of each cultivar were collected from infected canola fields and treated with four different spray treatments. Five treated stubble pieces of each cultivar were sealed into a nylon onion bag. There were 16 bags per treatment for each cultivar. Half of the bags were buried to a depth of 15 cm. There were two bags per replicated plot, in an uncultivated field at the Vegreville experimental site, ARC. The remaining eight bags of each cultivar were placed on the soil surface in the same plot of buried bags. The experimental design is illustrated in **Appendix 2.** In the spring of 1996, one bag was retrieved from each treatment of each plot and stems were checked for pseudothecia presence, fungal, and bacterial survival by using the same methods as described above. In the fall of 1996, stubble of one bag from each treatment of each replicated plot was checked again by using the same method. The percentage of ascospore-containing stubble pieces was calculated. The data was analyzed using SAS ANOVA procedures (SAS Institute, Inc., 1996).

Effects of compost, *P. polymyxa* PKB1 and Tilt® on *Leptosphaeria maculans*

To ensure the efficiency of the biological control agent, a large-scale and reliable inoculation method is essential for the establishment of the bacterium in soil. A field experiment, therefore, was carried out to investigate the utilization of compost as a carrier of *P. polymyxa* PKB1 to control blackleg of canola under the field conditions.

Preparation of the *P. polymyxa* PKB1 amended-compost:

The composts were prepared at the Compost Technology Centre (CTC), Olds, Alberta.

In 1996, composts were prepared to determine the most suitable raw material as well as the incubating conditions to enhance the colonization of the introduced *P. polymyxa* PKB1 spores to develop disease-suppressive compost. The type of compost chosen was a cattle manure mixed with wood chips. Of this, mature and near mature materials were utilized. In both, the thermophylic stage of composting had been completed, and the risk of annihilating the introduced spores with high temperatures was minimal.

Mature and near mature composts were pasteurized at 80°C for 5 h to destroy the resident microorganisms, thus eliminating competition and enhancing the viability of the intended *P. polymyxa* PKB1 spore inoculum. The spore inocula (prepared from freeze-dried spores) were introduced after the compost was cooled below 45°C at a rate of 3.7 - 4.2 x 10^6 spores g^{-1} dry mass compost. The inoculated composts were then incubated in 0.25 m^3 composting chambers with or without a soy-meal addendum (5 and 10%, v/v) and with or without forced aeration. The incubation period lasted 52 days. Temperature, oxygen, pH and moisture were measured each day (Data were provided by CTC, Olds, Alberta). Samples were taken on days 0, 15, 40 and 52 and checked for bacterial concentration and inhibitory effect against *L. maculans*. In all, three batches of the composts with different treatments were prepared in 1996 (**Table 3.1**) in order to determine the best conditions for bacterial growth and enhanced biocontrol effect.

In order to detect the bacterium applied to the compost, a mutant (*P. polymyxa* PKB1-rif^r) resistant to antibiotic rifampicin (200 mg L^{-1}) was selected as a marker (Kharbanda, 1998). In 1998, compost amended with the antibiotic- resistant *P. polymyxa* PKB1-rif^r mutant was prepared at CTC, Olds College by using a procedure similar to that in 1996 and described below.

Ten cubic meters of cattle manure and wood chips, which had been composting for three months, were isolated and intensively turned to enhance the oxygen concentration until the material was nearing the maturing phase. One cubic meter near mature compost (41.7% moisture, 539.8 kg m^{-3} bulk density and pH 7.5) was steamed for 5 hours at 80°C and cooled to below 45°C (**Figure 3.1**). To half of this material, 13.5 kg of soybean meal (5%, w/v) was added and well mixed in. The spore suspension was prepared in distilled water (pH 7.4) from the freeze-dried spores. A total of 18.91 g of freeze-dried *P. polymyxa* PKB1-rif^r spores ($4.7x10^{10}$ cfu mg^{-1}) were dispersed in 1 L of distilled water, and the suspension made up to 3.785 L and sprayed evenly over this half of the pasteurized compost and mixed thoroughly. The resultant inoculum concentration was $3.3x10^9$ cfu g^{-1} dry mass of compost. The inoculated compost was then incubated in two 0.25 m^3 composting chambers (labelled as S1 and S2) with forced aeration for 45 days. Another half of the compost was sprayed with water, incubated in two other 0.25 m^3 composting chambers (labelled as C1 and C2) under the same conditions and served as a control. Temperature and moisture in each composting chamber were measured daily.

Assessment of *P. polymyxa* PKB1 in composts:

At weekly intervals during the composting period, samples from each compost chamber (inoculated with *P. polymyxa* PKB1 or without inoculation) were taken and assessed for bacterial concentration and inhibitory effect against *L. maculans*.

In vitro assessment: One gram (wet weight) of compost sample was suspended in 10 mL sterile distilled water and then heated to 80°C in a hot-water bath for 30 min to kill any vegetative cells of non-spore-forming bacteria and fungi. Dilution series (10 fold) were made, and then 0.1 mL of each dilution was plated onto PDA plates. Bacterial colonies were counted after 48h of incubation at 22°C. The replicas of bacterial colonies on PDA plates were made on PDA + rifampicin (100 mg L^{-1}) plates and incubated at 25°C for 48 h, and the final counts of colony forming units (cfu) were determined per gram wet weight of the compost.

The inhibitory effect of *P. polymyxa* PKB1 re-isolated from compost was confirmed by using two methods. In the first method, a loopful of the bacterium was placed at four places on a PDA plate, around an agar plug of *L. maculans* culture. Inhibition zones in the culture around the bacterial colonies were checked after 10 days incubation at 22°C. In the second method, 0.1 mL of the diluted compost suspension and 0.9 mL of *L. maculans* spore suspension were mixed and then spread onto four PDA plates. Inhibition of fungal growth was observed after one week of incubation at room temperature.

Effect of compost plus *P. polymyxa* PKB1 on blackleg:

Growth chamber tests:

To determine the effect of compost, and compost + *P. polymyxa* PKB1 on blackleg disease of canola, one liter of compost:vermiculite mixture (1:1, v:v) was mixed well with 20 mL *L. maculans* isolate BLA spore suspension (5 x 10^6 spores mL^{-1}). Pots (10 cm-diameter, plastic) were first filled with 500 mL autoclaved greenhouse growth medium (Promix, a peat-based growing medium, WestGro). Ten canola seeds (*Brassica napus*, cv. Westar) were then placed on the surface of the soil mixture and covered with 100 mL of compost-bacterium-vermiculite-blackleg spore mixture. Pots receiving vermiculite plus blackleg spores alone were used as one of the control treatments. Another set of pots covered with compost-vermiculite mixture without blackleg spores was used as another control. Each treatment was

replicated four times. Pots were covered with plastic bags to maintain high humidity, arranged in completely randomized design and incubated in a growth chamber at 20°C and 16 h light and 10°C, 8 h night per day. One week later, seedling emergence and infection of cotyledon by *L. maculans* were recorded. Plant height, shoot dry weight and root dry weight were recorded 21 days after seeding. Three batches of composts (**Table 3.1**) were made in CTC, Olds and composts from batch A and C were tested to determine the best compost-soybean meal-bacterium combination. Composts from batch B were not tested because no *P. polymyxa* PKB1 was inoculated.

In the second growth chamber experiment, compost inoculated with *P. polymyxa* PKB1 was mixed with Promix at a ratio of 1:3 (v:v). Pots (13-cm-diameter, plastic) were first filled with 800 mL compost-Promix mixture. Ten canola seeds (*Brassica napus*, cv. Westar) were then placed on the surface of the mixture and then covered with 200 mL of perlite-blackleg spore mixture, prepared at the inoculum level of 50 mL pycnidiospore suspension (1.3×10^7 spores mL^{-1}) in one liter perlite. There were five treatments in this test, (1) bare seeds grown in compost without bacterial inoculation, covered with 200 mL *L. maculans* infested perlite; (2) bare seeds grown in compost with bacterial inoculation, covered with *L. maculans* infested perlite; (3) bare seeds grown in Promix, covered with *L. maculans* infested perlite; (4) bare seeds grown in Promix, covered with autoclaved perlite; (5) Vitavax RS (fungicide) treated seeds, covered with *L. maculans* infested perlite. Pots were arranged in a complete randomized design with four replications in a growth chamber programmed with 20°C, 16 h light and 18°C, 8 h night per day. The seeding, growth condition and disease rating were the same as in the first experiment. This experiment was repeated once. Data were analyzed by using the SAS ANOVA procedure (SAS Institute, Inc., 1996).

Field experiment:

L. maculans infected canola stubble pieces were collected from fields in Lloydminster, Alberta and treated with (1) a dip in *P. polymyxa* PKB1 spore suspension (1×10^8 spores mL^{-1} in 1% (w/v) gelatine water solution); (2) a dip in the fungicide Tilt® solution (2.5 mL L^{-1} 1% gelatine water solution); (3) a dip in both *P. polymyxa* PKB1 and Tilt® solutions; (4) burial in 500 mL compost inoculated with *P. polymyxa* PKB1 spores (compost had a total bacteria count of 3×10^8 cfu mL^{-1}); (5) burial in compost without bacterium inoculation; and (6) a dip in 1% (w/v) gelatine water solution as a control. Five treated stubble

pieces were sealed into a bag made from nylon screen. A total of 48 bags per treatment were divided into three groups and placed in three placement positions: (1) buried to a depth of 15 cm; (2) hung 40 cm above the soil, and (3) placed on the soil surface. The treatments were replicated four times, four bags for each treatment in each replication, and placed in a canola field plot arranged in a randomized complete block design on Oct. 14, 1996 in Vegreville (**Appendix 3**). This field was destined to grow canola for the following two years. One bag from each treatment of each plot was removed from the field on each of the following dates: April 19, Oct. 21, 1997 and May 8, Oct. 24, 1998 and kept in a freezer (-20°C) until examined.

To examine pseudothecia formation on canola stubble, all the five pieces of a bag from each treatment were examined microscopically. The percentage of stems with ascospore-containing pseudothecia was calculated. The viability of *L. maculans* on/in the stubble was tested by culturing three small segments (5 mm diameter) of a stem on the antibiotic PDA medium described earlier. The number of pieces from which *L. maculans* had grown out was counted two weeks after incubation at room temperature. The data were analyzed by using the SAS general linear model procedure (SAS Institute, Inc., 1996).

Evaluation of disease suppressiveness of composts

In current experiments, compost without inoculation of *P. polymyxa* PKB1 also showed biocontrol effect of inhibiting *L. maculans*. Therefore, compost samples from different sources were collected from the Composting Centre, Olds College, Alberta and the High River Composting Centre (**Table 3.2**) and evaluated for their efficacy in disease control. The purpose was to understand the mechanisms involved in the disease suppression by compost, including presence of other disease suppressive microorganisms.

Observation of microflora in compost:

The microflora in compost were observed by two types of microscopic systems: a Zeiss epifluorescent microscope and a Molecular Dynamics 2001 confocal laser scanning microscope (CSLM). One gram of sample was placed in a test tube containing 10 mL of 0.05% acridine orange solution. The test tube was swirled and inverted. The sample was examined under the fluorescent microscope after 10min staining. The DNA of microorganisms were stained bright green by acridine orange (Chalmers et al. 1997).

Microbial activity in different composts:

The total microbial activity was determined by the fluorescein diacetate (3', 6'-diacetylfluorescein (FDA)) method (Schnurer and Rosswall, 1982). FDA is hydrolyzed by a number of different enzymes such as protease, lipases, and esterases. The product of this enzymatic conversion is fluorescein, which can be seen within cells by fluorescent microscopy and can also be quantified by fluorometry or spectrophotometry. The production of fluorescein is proportional to the microbial activity.

FDA (Sigma Chemical Co.) was dissolved in acetone (analytical grade, Sigma Chemical Co.) and stored as a stock solution (2 mg mL^{-1}) at -20°C. For all determination of FDA hydrolysis activity, the FDA final concentration was 10 μg mL^{-1}. A compost sample (1 g) was dispersed in 50ml of sterile potassium phosphate buffer (60 mM, pH 7.6) and then amended with FDA. The suspension was incubated at 25°C on a rotary shaker (120 rpm) for 3 hours, and then 50ml acetone was added to stop the reaction. A 30 mL suspension was removed from the flask and centrifuged at 6,000 rpm for 5 min, and the amount of fluorescein was measured as absorbance at 490 nm with a spectrophotometer (SmartSpec™ 3000, Bio-Rad Laboratories Inc.). FDA added to the buffer was used as blank control. A standard curve of fluorescein was prepared from a stock solution containing 200 mg of fluorescein (Sigma Chemical Co.) in 20 mL of hot ethanol. A serial dilution was made to yield a final concentration of 0.625, 1.25, 2.5, 5, and 10 μg fluorescein ml^{-1}.

Total bacterial population:

The total bacterial population was determined by serial dilution and plating method. Compost samples were air-dried, and a 1 g sample was suspended in 10 mL distilled water. The well-mixed suspension was divided into two sets, 5 mL in each. One set of samples was treated in hot water bath at 80°C for 30 min to kill non-spore-forming bacteria and fungi. A 10-fold serial dilution was made up to 10^8 dilution, and 100 μl suspension of each dilution was spread on PDA plates. These plates were incubated in an incubator at 28°C for 4 days, and the bacterial and fungal colonies were counted.

Bioassay of bacteria and fungi against plant pathogens:

Representative bacterial and fungal isolates were transferred and purified. *Trichoderma* spp. and bacterial isolates were selected and tested for their inhibitory effect on *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Bacterial isolates showing inhibitory effect against pathogenic fungi were purified and stored

in 20% (w/v) glycerol at -80°C for further study. Three bacterial strains were sent to the Microbiology Lab in ARC, Vegreville, Alberta for identification.

Comparison of beneficial bacteria* population between inoculated and non-inoculated composts:

In order to determine whether or not the introduction of *P. polymyxa* PKB1 into compost could enhance the beneficial bacteria in the compost, the percentage of beneficial bacteria from total bacteria growing on the plates containing compost with or without inoculation of *P. polymyxa* PKB1 were examined. A total of 100 bacterial single colonies from plates containing compost with inoculation of *P. polymyxa* PKB1 were randomly selected and tested for their inhibitory effect against *R. solani* AG 2-1 on PDA plates. The same number of bacterial colonies was selected from plates containing compost without inoculation of *P. polymyxa* PKB1 as a comparison. Bacterial isolates showing an inhibitory effect against *R. solani* AG 2-1 were counted, subcultured, and stored in a 20% (w/v) glycerol solution for further identification.

Suppression of plant diseases by composts:

Effect of compost on disease suppressiveness was tested in a growth chamber experiment on a soil-borne disease of canola, Rhizoctonia root rot. Composts (**Table 3.2**) were mixed with greenhouse growth medium Promix (1:3, v:v) and incubated for two days. The mixture was used to fill 13-cm fiber pots, and 25 canola seeds (cv. Quest) were seeded on the surface of the Promix-compost mixture. Five barley grains infested with *Rhizoctonia solani* AG 2-1 were placed in each pot, and the seeds were covered with the Promix-compost mixture. There were four pots for each treatment, and all the pots were incubated and completely randomized in a greenhouse.

Results

Effect of Tilt® or *P. polymyxa* PKB1 on the development of pycnidia and pseudothecia, and viability of the fungus in the stubble in growth chamber tests

Significant differences in the pycnidial density of *L. maculans* on canola stubble were observed among spray treatments, temperature and burial in the growth chamber tests. There were significant interactions

* Beneficial bacteria means bacteria showing an inhibitory effect against pathogenic fungi in the petri plate test.

among temperature, burial and spray on the pycnidial density of *L. maculans* on canola stubble based on two sets of the experimental data (**Table 3.3**).

Multiple mean comparison for each single effect was conducted using Duncan's Multiple Range test at the level of $P \leq 0.05$. Canola stems treated with Tilt® had significantly fewer pycnidia than the corresponding untreated checks under most temperature regimes, excepting samples kept at 20°C and buried samples under various temperatures (**Table 3.4**). Stubble burial significantly increased the number of pycnidia over the pycnidia number found on unburied stubble pieces within each temperature and Tilt® or bacterium treatment. Stubble incubated at 10°C developed more pycnidia than those at any other temperature did. Lower temperature and darkness were found to be favourable for fungal development. In most treatments, the bacterium alone had an insignificant effect on pycnidium development on the surface of stubble. No pseudothecia were found on stubble in any treatments under growth chamber conditions.

Significant differences in the viability of *L. maculans* on canola stubble were observed among treatments, temperature and burial in the growth chamber tests (**Table 3.5**). Significant interactions among temperature, burial and spray on the viability of *L. maculans* on canola stubble were also found.

The bacterium had a profound effect on reducing viability of the fungus in canola stubble in the growth chamber (**Table 3.6**). Survival of *L. maculans* was significantly less on the buried than on the unburied stubble at 10°C and 20°C but not at -5°C or at the +varied temperature.

Effect of Tilt® on the formation of pseudothecia and disease severity of canola under field conditions

Two-year-old stubble from field plots sprayed with Tilt® produced a small number of pseudothecia (**Figure 3.2A**), and these pseudothecia contained none or very few immature ascospores (**Figure 3.2B**). This result suggests that application of Tilt® on canola stubble could inhibit the development of pseudothecia and thereby reduce the production of ascospores under field conditions.

In the field experiment in Wainwright, 1993-1995, the Tilt® x cultivar interaction was significant with respect to mean disease severity (**Table 3.7**) but not to percent emergence, infection, plant stand and yield ($P<0.05$). Tilt® treatment on canola stubble significantly reduced mean disease severity in the susceptible cultivar Westar and reduced the percentage of infected seedlings in cultivar Legacy. Tilt® treatment on canola stubble did not significantly increase the percentage of emergence, plant stand and yield of Westar, the trend

was that Westar growing in plots treated with Tilt® had a higher percent emergence, plant stand and yield than that in non-Tilt® treated plots (**Table 3.8**).

Effect of Tilt® and *P. polymyxa* PKB1 on pseudothecia formation and viability of *L. maculans* under field conditions

In the first field experiment in Vegreville, both Tilt® and bacterium, applied individually or in a combination, significantly reduced pycnidia formation of *L. maculans*. However, their inhibitory effect on ascospore formation was not apparent. The combination of Tilt® and bacterium application significantly reduced the ascospore formation on the canola stubble (**Table 3.9**).

In the second field experiment in Vegreville, no ascospores of *L. maculans* were found in the first sampling (six months after treatments applied) from both buried and un-buried samples. Therefore, the fungal viability on each treated stubble sample was tested and recorded. No significantl effects of burial and cultivar were found on viability of *L. maculans* (**Table 3.10**). Tilt® or bacterial treatment, alone or in combination, significantly reduced pathogen viability in stubble tissue (**Table 3.11**). In the second sampling (12 months after treatments applied), no ascospores were found in buried samples. Data collected from surface samples were analysed only. Tilt® or bacterial treatment did show a significant effect on the formation of ascospores on cultivar Westar, but bacterium alone did not show a significant effect on ascospore formation on the cultivar Tobin. The two cultivars responded differently to the bacterial treatment. On Westar, canola stem treated with bacterium showed significantly reduced ascospore formation, whereas on Tobin, the reduction was not significant (**Table 3.11**). Overall, the viability of *L. maculans*, measured by counting stubble pieces yielding viable fungus on a special medium (20% V-8 juice + Rose Bengal (0.04%)), was low in all the treatments on Westar and a significant difference was detected.

Assessment of the bacterium in the compost

Compost- *P. polymyxa* PKB1 Incubation:

All compost bins showed an immediate increase in oxygen concentration and a precipitous drop in temperature as a result of the utilization of the soybean meal after *P. polymyxa* PKB1 spores and soy meal were inoculated into the compost bins.

The spore-containing bins (S1 and S2 in **Figure 3.3**) showed a more reaction than the control bins (C1 and C2) with higher temperatures (TS1 and TS2 in **Figure 3.3**) and more microbial activity, as evidenced by the quicker return to a higher oxygen concentration (OS1 and OS2 vs. OC1 and OC2 in **Figure 3.3**). The oxygen concentration remained stable at 10% level six days after incubation, and temperature stabilized at 48°C eight days after. This result was due to the activity of a larger viable bacterial population in the inoculated bins after the steam-pasteurization treatment. This result also indicated that the bacterial population had reached the maximum or saturated stage after one week of incubation.

The concentration of total bacteria in composts with or without inoculation of the bacterium ranged from 2.0 x 10^7 to 3.4 x 10^8 cfu mL^{-1}on PDA plates. To confirm that the uninoculated compost did not contain *P. polymyxa* PKB1-rif^r, the bacterial colonies isolated from uninoculated compost on PDA amended with rifampicin were tested. Very few bacterial colonies from compost without inoculum survived on rifampicin-amended plates. Subsequent incubation of rifampicin-amended PDA indicated that *P. polymyxa* PKB1-rif^r recovered from inoculated compost was strongly inhibitory to *L. maculans* mycelial growth.

Effects of compost-bacterium on blackleg in the growth chamber

In experiments conducted to determine the general effect of the prepared composts (batch A) on plant growth, their effect on plant growth was significantly beneficial, for no phytotoxic effects were observed (**Table 3.12**). The plants grown in pots with compost treatments had significantly higher plant height, and in some composts, they had significantly higher shoot dry weight and root dry weight compared with those of the control (plants grown in greenhouse growth medium without compost amendment). The results demonstrated the beneficial effect of composts on plant growth. Compost batch B had no PKB1 inoculation, therefore, no data was presented here. In the experiment of compost batch C, the effect of compost on cotyledons of canola infection by *L. maculans* was determined in the growth chamber (**Table 3.13**). Although there was no significant difference among the treatments, there was a similar trend in both tests using different batches of compost (batch A and C), in that the plants grown in the control pots (CK, compost without bacterium) consistently had the lowest percentage of emergence and the highest percent infection, and lowest shoot and root growth. The results showed that the bacterium present in the infested

compost could protect the young seedling from post-emergence seedling blight due to the blackleg fungus. The method of making compost batch C4 was used for subsequent compost making because C4 gave a significantly lower percent blackleg infection compared to that of the control.

In the second growth chamber test, results of the two experimental repetitions were fundamentally similar. Data of the first repetition are analyzed and presented here. Compost inoculated with *P. polymyxa* PKB1 showed no significant effect on the percent blackleg disease infection on canola seedlings but had a significant effect on the percent emergence (**Table 3.14**). Compost-, compost + PKB1- and Vitavax RS-treated plants had reduced disease severity, although 40 percent of the plants became infected in compost- and compost + PKB1-treated plots. **Figure 3.4** shows differences among the treatments. Compost-, compost + PKB1- and Vitavax RS-treated plants had higher survival compared with those in the untreated control pot.

Effects of Tilt®, *P. polymyxa* PKB1, and compost on viability of *L. maculans* and ascospore formation on canola stubble under the field conditions

Effect on Viability of *L. maculans*:

The effect of *P. polymyxa* PKB1 and Tilt®, *P. polymyxa* PKB1 + compost on the viability of *L. maculans* on canola stubble was significant (**Table 3.15**, F>P=0.005). The viability of the fungus was significantly reduced when the stubble were treated with Tilt®, Tilt® + *P. polymyxa* PKB1, and compost with or without *P. polymyxa* PKB1 six months after treatment, while bacterium alone was not very effective (**Table 3.16**).

Effect on Ascospore Formation:

No ascospores were found on any canola stubble by the first sampling date, six months after treatments applied. Therefore, no data were included in the analysis. There were significant differences in ascospore production in samples from subsequent samplings due to different treatments and different burial methods (**Table 3.17**). It was clear that the treatments of compost + *P. polymyxa* PKB1 and Tilt® + *P. polymyxa* PKB1 had a significant inhibitory effect on the formation of ascospores on the canola stubble (**Figure 3.5**). On suspended samples, only immature pseudothecia could be seen. On samples placed on the soil surface,

it was observed that many mature ascospores were produced on the check stem and a few on the stem treated with bacterium alone, but not on the stems in the other four treatments. The results suggested that the Tilt®, Tilt® plus bacterium, bacterium plus compost and compost treatments effectively delayed the formation and development of ascospores. Bacterium alone had less effect, perhaps due to the poor establishment of the bacterium in the field. The bacterium-inoculated compost had a significantly greater inhibitory effect on ascospore formation compared with that of bacterium alone. Burial effects on pseudothecia formation were analyzed within each spray treatment for each sampling date (**Figure 3.6**).

Detection of disease-suppressive compost

Image of compost particle:

A confocal microscope image of compost particles showed that microbes were present on the compost based on the green fluorescent area on the compost particle (**Figure 3.7**).

Determination of chemical composition and microbial activity in composts:

The chemical compositions of various composts are listed in **Table 3.18**. Wood chips-based compost had a very high C/N ratio, and the municipal solid waste-based compost had a very high concentration of lead. Most of the composts met the standard requirements for compost in Canada (Compost Council of Canada, www.compost.org).

Comparisons of the capacity of microbial communities to hydrolyze FDA indicated differences in activity among various composts (**Table 3.19**). Pulp sludge-based compost had the lowest fluorecein production while municipal yard waste-based compost had the highest.

Figure 3.8 shows the microbial activity of compost inoculated with *P. polymyxa* PKB1 and compost without inoculation. There was no difference between the total microbial activities in the two treatments.

Bacterial population in composts:

The total bacterial population in different composts was very similar except in screened compost from High River (**Table 3.19**). Microbes in the composts mostly consisted of various bacteria, actinomycetes and fungi, including *Penicillium* spp., *Trichoderma* spp., *Gliocladium* sp., *Rhizopus* sp., *Fusarium* spp., *Pythium* sp., *Aspergillus* sp. and some unidentified fungi. After heat treatment, only thermophilic microorganisms survived. Screened compost from High River had a lower microbial population.

Bioassay and identification:

Representative isolates of bacteria were selected and tested for their inhibitory effect on pathogenic fungi *Sclerotinia sclerotiorum* and *Rhizoctonia solani* AG2-1. Many bacteria showed various degrees of inhibition against these fungi (**Figure 3.9**). Bacteria with white and pinkish colonies were the most often observed within inhibitory bacteria. Three representative isolates were identified as *Bacillus subtilis* and *B. licheniformis* at the Microbiology lab, ARC, Vegreville. More bacterial strains have been isolated, and their identification is in progress. Municipal waste and cattle manure had more beneficial bacteria whereas wood chip-based compost had more fungi such as *Tricoderma* spp. Beneficial bacteria were not found in heat-treated wood chip-based compost. Screened compost from High River had a lower population of bacteria.

Comparison of beneficial bacteria population between inoculated and non-inoculated composts:

Out of 100 bacterial isolates from *P. polymyxa* PKB1-inoculated compost, 76% showed an inhibitory effect against *R. solani,* whereas only 38% of isolates from control compost (without inoculation) had various degrees of inhibition.

Disease suppression of different composts:

Composts started with different materials showed different disease suppression effect on Rhizoctonia damping-off of canola (**Table 3.20**). Cattle manure compost inoculated with bacterium *P. polymyxa* PKB1 and 1999 screened compost significantly increased the percent of emergence and reduced the mean disease severity. Wood chip-based compost performed the worst compared with other composts and the control, greenhouse growth medium without any compost amendment.

Discussion

Effect of *P. polymyxa* PKB1

Studies reported here demonstrated good prospects and encouraging biocontrol properties of *P. polymyxa* PKB1 against *L. maculans* on canola *in vitro,* under a controlled environment and, combined with Tilt® or compost, under field conditions. *P. polymyxa* PKB1 inhibited the growth and development of *L. maculans* in growth chamber studies. The survival of the fungus on canola stems was significantly

reduced when they were treated with *P. polymyxa* PKB1 spore suspension. However, the effect was not significant when the bacterium-alone-treated stubble were placed in the field. The reason could be that the environmental conditions and competition from local microbes in the field were not suitable for the establishment and survival of the bacterium when applied in a small amount. Probably, the bacterium could not multiply fast enough to compete with the local microbes. In the current study, growth chamber test has shown that temperature, moisture and light can influence both fungus growth and bacterium performance. Direct manipulation of a specific biocontrol agent is rather difficult in order to achieve good disease control efficacy. This difficulty is not surprising since biological control in nature is the result of the multiple, additive, and diverse activities of populations of organisms (Schroth and Hancock, 1985). It is, therefore, important to study the ecology, physiology and genetics of *P. polymyxa* PKB1 and identify the environmental and nutritional factors that favour its antagonistic behaviour.

The bacterium had a profound effect on reducing viability of *L. maculans* in canola stubble in the growth chamber. In the presence of the bacterium, survival of the fungus was significantly poorer on buried than on unburied stubble at 10°C and 20°C temperature regimes but not at -5°C. The reason is possibly due to higher soil moisture level under buried conditions, which is favourable for the growth of bacterium. Under most conditions, survival of the fungus was significantly poorer on the bacterium-treated stubble than on the untreated check or stubble treated with Tilt® alone. This result suggests that the bacterium may inhibit the fungus viability inside the plant tissues and inhibit fungal development, whereas action of Tilt® is limited to the superficial layers of each stubble piece. These results also suggest that the bacterium could reduce the survival of *L. maculans* on canola stubble and thereby reduce the overall inoculum potential of the fungus. Studies by Beatty (2000) have shown that *P. polymyxa* PKB1 produces an antifungal metabolite during sporulation and this antifungal metabolite could inhibit the growth of several gram-positive and negative bacteria and other fungi.

Effect of *P. polymyxa* PKB1 combined with fungicide Tilt®

The present study showed that ascospore production could be markedly suppressed by chemical treatment on infected stubble. When *P. polymyxa* PKB1 and Tilt® were applied together to canola stubble, the inhibitory effect on *L. maculans* was more pronounced than when they were applied individually under

field conditions. Tilt® significantly reduced the number of pycnidia on canola stubble, and *P. polymyxa* PKB1 significantly reduced the survival of *L. maculans* under controlled conditions, possibly because Tilt® applied to the surface of stubble could rapidly kill the fungus on the stem surface so that the pycnidia could not develop, whereas *P. polymyxa* PKB1 produced an antifungal metabolite and inhibited the fungus growth inside the stubble. Under field conditions, *P. polymyxa* PKB1- and Tilt®-treated canola stubble had significantly fewer mature ascospores and yielded fewer *L. maculans* colonies, indicating that the treatment had affected fungal viability. The Tilt® and bacterial treatment either completely inhibited formation of pseudothecia of *L. maculans* or permitted formation of only a small number of pseudothecia containing immature ascospores. The results showed that with the combination of bacterium and Tilt® treatment, the fungal inoculum could be tremendously reduced. The combined effect of rapid killing with the chemical and longer-term control with the biocontrol agent could be obtained. It was obvious that other factors in the soil or environment affected the survival of the fungus. Because field conditions are complicated, further study is needed to investigate the effect of environmental factors on the bacterium and Tilt® efficacy.

Tilt® application to canola stubble in the fall reduced inoculum in a field but did not significantly reduce disease incidence on canola plants grown in this field. The reason is that air-borne ascospores from nearby infected fields could cause foliar infection. The effect of Tilt® applied in the fall was time-limited and did not prevent plants from infection during the next summer. Disease pressure was low in Wainwright in 1995 due to dry weather conditions. The mean disease severity in untreated Westar (no Tilt® treatment) was only 1.89 (**Table 3.7**). This result may be an additional reason why the effect of Tilt® treatment was not evident in the field experiment, especially on tolerant cultivars.

Effect of *P. polymyxa* PKB1 combined with compost

P. polymyxa PKB1-inoculated compost significantly reduced the viability and pseudothecia formation of *L. maculans* under field conditions. Compost alone also had a significant effect. The reason of this is due to the presence of some beneficial bacteria in the compost (Hoitink et al., 1993). Two years of data in current study showed consistent results and indicated that compost was a good carrier for the bacterium for field application. Because the bacterium can produce endospores, bacterial spores applied to the compost could survive for long time under unfavorable conditions. This study showed that the bacterial population

remained very high in compost after 45 days incubation and that *P. polymyxa* PKB1 rif^r strain still showed an inhibitory effect against *L. maculans*. The total bacterial population in the compost inoculated with *P. polymyxa* PKB1 remained high ($1x10^9$ cfu mL^{-1}) after storage in a freezer (-20°C) for 18 months. Use of *P. polymyxa* PKB1 in combination with compost could offer efficacy nearly equal to that of Tilt® treatment (**Table 3.13** and **Figure 3.6**). Moreover, compost applied to the agricultural soil could provide crops nutrient as high as those obtained from artificial fertilizer. This compost system would have additional advantages of environmental safety and a long-term biocontrol effect that would help the ecosystem balance, recycle and reuse organic wastes (Dick and McCoy, 1993). The nutrient composition of each tonne of compost has been reported to be close to 18 kg N, 25 kg P, 20 kg K, 3 kg S, and is quite consistent (Tom Clark, CTC, Olds, personal communication).

Compost alone had a highly inhibitory effect on ascospore formation of *L. maculans*. This effect was due to the presence of some other microbes inhibitory to pathogenic fungi in the compost. In this study, the compost was found to contain *Bacillus* spp. (*B. subtilis* and *B. licheniformis* were identified), *Trichoderma* spp., *Gliocladium* sp. and some *Penicillium* spp. The microbial population and activity were high in all the composts, except wood chips-based compost, tested by plating and FDA methods. General suppression was achieved due to the presence of beneficial microbes in the composts in this case (Hoitink et al., 1993) because some bacteria isolated from compost had an inhibitory effect against pathogenic fungi. The disease-suppression effect might have also been caused by other inhibitors in compost. It has been reported that some composts, particularly those prepared from tree barks, release inhibitors of plant pathogens (Dissanayake and Hoy, 1999; Granatstein, 1997; Hoitink and Fahy, 1986) and induce systemic acquired resistance in plants to some bacterial pathogens (Zhang et al., 1998). Disease-suppressive soil and compost are a well-known phenomenon and are related to shifts in the microbial population. However, the suppression is sometimes variable due to random recolonization of compost by effective biocontrol agents (Kuter et al., 1983). Introduction of beneficial bacteria into the compost could ensure increased bacterial population in compost, enhance its disease-suppressive effect (Kwok et al., 1987) and obtain consistent effects. Out of 100 bacterial isolates from *P. polymyxa* PKB1-inoculated compost, 76% showed an inhibitory effect against *R. solani*, whereas only 38% of isolates from control compost (without inoculation of *P. polymyxa* PKB1) had various degrees of inhibition although the total bacterial population were quite

similar. This result indicated that *P. polymyxa* PKB1 re-colonized the inoculated compost very quickly and became dominant in the compost while other microbes became dominant in uninoculated compost with less beneficial bacteria in it. This experiment also demonstrated that the compost could be a good delivery medium for the biocontrol agent. The re-colonization and survival of the biocontrol agent depends on many factors, including moisture, pH value, carbon source in the compost and the method of composting (Hoitink et al., 1997, Mandelbaum and Hadar 1990) and will be studied to standardize development of disease-suppressive compost using *P. polymyxa* PKB1.

Application of an effective biocontrol agent on canola stubble could result in eliminating the need for fungicide sprays, and thus reduce the production cost and environmental contamination. Use of compost would also make use of animal manure, 14.5 million tonnes of which is produced in Alberta every year.

Integrated control: Biological, cultural and chemical control

Combined use of biological, chemical and cultural controls can provide a high efficacy of disease control. The results for the most effective treatments were consistent over the two-year experiment and indicated that the production of ascospores on canola stubble was highly sensitive to Tilt®, bacterium and compost. Applying any two of these together in the field could effectively reduce the pathogen population in the soil. *P. polymyxa* PKB1 had a highly inhibitory effect against other pathogenic fungi on canola, such as *Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium* spp. and *Pythium* spp. (Yang et al., 1996). The integration of biological (*P. polymyxa* PKB1), cultural (compost) and chemical (Tilt®) controls would be extremely economical if the important soil-borne diseases could be simultaneously controlled. Since these fungi also affect other crops such as peas, beans, and cereals, the long-term effect of the biocontrol agent would be realized in subsequent crops in rotation. Studies by other researchers also showed the effectiveness of integrated pest management in controlling soil- and stubble-borne diseases (Martin et al., 1985).

In buried samples, pseudothecia and ascospores were not observed, possibly because pseudothecia formed earlier on buried stubble, and ascospores were released from the fruiting bodies. It was reported that pseudothecia of *L. maculans* could be found on buried stubble from the same-year crop in November while they could not be found on stubble above the soil until the following summer (Kharbanda and

Ostashewski, 1997). Kharbanda and Ostashewski also observed that buried stubble tend to produce the perfect stage of *L. maculans* more rapidly than stubble left standing on the soil surface. A consistent seasonal pattern of ascospore discharge, which started in July, by *L. maculans* from canola residues was detected in Saskatchewan (McGee and Petrie, 1979). The current study could not find ascospores in any of the samples retrieved six months after application but found ascospores in the subsequent samples. This result is in agreement with McGee (1974) and Petrie's finding (1994).

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Table 3.1. Different treatments and composition supplements to the composts prepared from cattle manure and wood chips at Composting Technology Centre, Olds, Alberta in 1996.

Sample	Treatment
First batch	
A1	Mature compost, no heat treatment
A2	Mature compost, heat treated for 5 h
A3	Mature compost + PKB1 spores (added after heat and cooling treatments)
A4	Mature compost + PKB1 spore + 10% soybean meal
A5	Young compost, no heat treatment
A6	Young compost, heat treated for 5 h
A7	Young compost, + PKB1 spores (added after heat and cooling treatments)
A8	Young compost + PKB1 spore + 5% soybean meal
Second batch	
B1	Mature compost, passive aeration
B2	Near mature compost, passive aeration
B3	Mature compost + 10% soybean meal, active aeration
B4	Near mature compost + 5% soybean meal, active aeration
B5	Mature compost, passive aeration in pile outdoors
Third batch	
C1	Mature compost + PKB1 spores, passive aeration
C2	Near mature compost + PKB1 spores, passive aeration
C3	Mature compost + 10% soybean meal + PKB1 spores, aerated incubation
C4	Near mature compost + 5% soybean meal + PKB1 spores, aerated incubation
C5	Mature composts + PKB1 spores, incubated in outdoor piles
C6	Mature compost, non-treated source material
C7	Near mature compost, non-treated material

Table 3.2. Compost samples tested for detection of disease suppressiveness of compost and population of microbes in compost.

Number	Compost	Source*
1	Municipal yard waste	1
2	Cattle manure	1
3	Pulp sludge	1
4	Wood chips	1
5	Screened municipal solid waste	1
6	Screened compost	2
7	1998 cattle manure compost	2
8	1999 unscreened compost	2
9	Cattle manure + PKB1, windrow, row 98	2
10	Cattle manure, windrow, row 99	2
11	Cattle manure + soy meal + PKB1, compost bin	1
12	Cattle manure + soy meal, compost bin	1

* 1 = Composting trechnology Centre, Olds College, Olds, Alberta
2 = EcoAg Initiatives, Inc., High River, Alberta

Table 3.3. ANOVA table for pycnidial density on the canola stubble tested in a growth chamber.

Source	DF	Sum Square	Mean Square	F	P
Block	1	0.26	0.26	2.07	0.1528
Temperature	3	3.48	1.16	2.89	0.2035
Error (a)	3	1.20	0.40		
Burial	1	38.84	38.84	307.38	0.0001
Temp x Burial	3	4.28	1.43	11.30	0.0001
Spray	2	16.82	8.41	66.55	0.0001
Temp x Spray	6	7.58	1.26	10.00	0.0001
Burial x Spray	2	4.92	2.46	19.47	0.0001
Temp x Burial x Spray	6	4.97	0.83	6.55	0.0001
Error (b)	20	5.85	0.29		
Error	144	18.19	0.13		
Total	191	143.33			

Table 3.4. Occurrence of pycnidia on canola stubble treated with Tilt® or *P. polymyxa* PKB1 in a growth chamber in 1995.

		Density of pycnidia on canola stubble		
Temperature	Burial	Tilt® Spray	Bacterium Spray	Check
-5°C	No	0.25 b[1] A[2]	1.00 a A	1.31 a A
-5°C	Yes	0.81 b B	1.29 b B	2.88 a B
10°C	No	0.50 b A	1.66 a A	1.63 a A
10°C	Yes	2.44 b B	2.92 ab B	3.00 a B
20°C	No	0.25 b A	0.71 a A	0.44 ab A
20°C	Yes	0.63 a A	0.84 a A	1.75 a B
Various T[3]	No	0.44 b A	0.49 b A	1.06 a A
Various T[3]	Yes	1.81 ab B	1.31 b B	2.31 a B

[1] Pycnidial density was evaluated on a scale of 0 - 3, where 0=no pycnidia, 1=less than 10% of the stem with one inch length covered with pycnidia, 2=11 to 50% covered with pycnidia, and 3=more than 50% covered with pycnidia. Means within rows followed by the same small letters are not significantly different according to Duncan's Multiple Range Test (P= 0.05).

[2] Means within columns followed by the same capital letters within each temperature treatment are not significantly different according to least significant difference test (P= 0.05).

[3] Each sample was kept for 2 weeks at each of the 5 different temperatures, 20°C, 10°C, -5°C, 10°C, 20°C in a cycle.

Table 3.5. ANOVA table for the viability of *Leptosphaeria maculans* on the canola stubble tested in a growth chamber.

Source	DF	Sum Square	Mean Square	F	P
Block	1	0.28	0.28	0.44	0.5083
Temperature	3	3.22	1.08	74.74	0.0026
Error (a)	3	0.04	0.01		
Burial	1	97.78	97.78	154.08	0.0001
Temp x Burial	3	41.94	13.98	22.03	0.0001
Spray	2	90.50	45.25	71.30	0.0001
Temp x Spray	6	62.83	10.47	16.50	0.0001
Burial x Spray	2	16.87	8.43	13.29	0.0001
Temp x Burial x Spray	6	20.91	3.49	5.49	0.0001
Error (b)	20	1.68	0.08		
Error	144	91.39	0.64		
Total	191	452.39			

Table 3.6. Viability of *L. maculans* on canola stubble treated with Tilt® or *P. polymyxa* PKB1 at various temperature and burial status in growth chamber tests, 1995.

		Mean number of infectious stubble pieces (%)		
Temperature	Burial	Tilt® Spray	Bacterium Spray	Check
-5°C	No	4.19 a[1] A[2]	1.89 b A	4.19 a A
-5°C	Yes	3.38 a A	2.13 a A	3.44 a B
10°C	No	4.13 ab A	2.88 b A	4.94 a A
10°C	Yes	3.31 a B	0.40 b B	4.00 a B
20°C	No	4.75 a A	2.94 b A	4.56 a A
20°C	Yes	2.44 a B	0.18 b B	2.63 a B
Various T[3]	No	4.00 a A	4.25 a A	4.75 a A
Various T[3]	Yes	3.56 a A	1.13 b B	4.38 a A

[1] Means followed by the same small letters within rows are not significantly different according to Duncan's Multiple Range Test (P=0.05). Values indicate the mean number of stubble pieces out of five yielding *L. maculans* colonies.

[2] Means followed by the same capital letters within columns within each temperature treatment are not significantly different according to least significant difference (lsd) test (P=0.05). Values indicate the mean number of stubble pieces out of five yielding *L. maculans* colonies.

[3] Each sample was kept for 2 weeks at each of the 5 different temperatures, 20°C, 10°C, -5°C, 10°C, 20°C in a cycle.

Table 3.7. ANOVA table for the mean disease severity tested on three *Brassica napus* cultivars in field at Wainwright, 1995.

Source	DF	Sum of Squares	Mean Square	F	P
Rep	3	0.16	0.05	5.43	0.0137
Tilt	1	0.58	0.58	5.64	0.0981
Rep x Tilt (E_a)	3	0.31	0.10	10.49	0.0001
Cultivar	2	2.22	1.11	112.52	0.0001
Tilt x Cultivar	2	0.36	0.18	18.15	0.0002
Error	12	0.12	0.01		
Total	23	3.75			

Table 3.8. Performance of three *Brassica napus* cultivars in a field containing blackleg infested stubble treated with Tilt® at Wainwright, 1995.

Cultivar	Spray	Emergence*	% Infection*	Plant Stand*	MDS*	Yield (g/plot)
Bullet	Tilt®	267.8 a	20.2 a	138.5 a	0.86 a	226.4 a
Bullet	No Tilt®	273.3 a	22.8 a	139.0 a	0.97 a	231.2 a
Legacy	Tilt®	306.3 a	23.2 a	159.3 a	0.83 a	273.0 a
Legacy	No Tilt®	288.5 a	30.7 b	150.8 a	1.01 a	235.3 a
Westar	Tilt®	300.8 a	30.7 a	168.0 a	1.24 a	252.5 a
Westar	No Tilt®	215.8 a	40.0 a	108.0 a	1.89 b	238.2 a

* Mean of four replications; means within columns within each cultivar followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.05).

Table 3.9. Occurrence of pycnidia and ascospores of *L. maculans* on canola stubble unsprayed or sprayed with Tilt® and *P. polymyxa* PKB1 in the field in Vegreville, Alberta, 1995 -1996.

Treatment	Pycnidia Rating[1]	Pseudothecia Rating[2]
Check	2.31 a[3]	0.69 a
PKB1	0.94 b	0.56 ab
Tilt	0.94 b	0.50 ab
Tilt + PKB1	0.63 b	0.25 b

[1] Rated as 0 to 3 scales, 0 = no pycnidia; 1 = less than 1/3 surface of stem has pycnidia; 2 = 1/3 to 2/3 stem surface has pycnidia; 3 = more than 2/3 stem surface has pycnidia.

[2] 0 = no mature ascospores; 1 = a few, less than 50% matured pseudothecia containing ascospores; 2 = >50% matured pseudothecia; 3 = 100% matured pseudothecia.

[3] Mean of 4 replications; means within columns within each cultivar followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.05).

Table 3.10. ANOVA table for the viability tested on two canola cultivars in field at Vegreville, 1995 - 1996.

Source	DF	Sum of Squares	Mean Square	F	P
Rep	3	224	74	0.27	0.8477
Spray	3	2654	884	8.27	0.0059
Rep x Spray (E_a)	9	962	106		
Cultivar	1	715	715	2.54	0.1368
Spray x Cultivar	3	2076	692	2.46	0.1129
Rep x Spray x Cultivar (E_b)	12	3376	281		
Burial	1	540	540	1.95	0.1176
Spray x Burial	3	1081	360	1.30	0.2965
Burial x Cultivar	1	68	68	0.25	0.6245
Spray x Burial x Cultivar	3	564	188	0.68	0.5732
Error	24	6643	276		
Total	63	18907			

Table 3.11. Occurrence of pycnidia and ascospores of *L. maculans* on canola stubble unsprayed or sprayed with Tilt® and *P. polymyxa* PKB1 in the field in Vegreville, 1995 -1996 [1].

Treatment	Burial	Viability (%) 6 months after treatment	Ascospore (%) 12 months after treatment
Tobin			
Check	Buried	40.0 a[3]	0.0
Bacterium	Buried	0.0 b	0.0
Tilt®	Buried	8.5 b	0.0
Tilt® + Bac	Buried	5.0 b	0.0
Check	Surface	35.0 a	83.3 a
Bacterium	Surface	25.0 a	67.8 ab
Tilt®	Surface	10.0 b	41.3 b
Tilt® + Bac	Surface	15.0 b	0.0 c
Westar			
Check	Buried	15.0 a	0.0
Bacterium	Buried	0.0 b	0.0
Tilt®	Buried	5.0 b	0.0
Tilt® + Bac	Buried	5.0 b	0.0
Check	Surface	35.0 a	70.9 a
Bacterium	Surface	10.0 b	10.4 c
Tilt®	Surface	20.0 b	52.1 b
Tilt® + Bac	Surface	10.0 b	20.8 c

[1] The first sampling was conducted six months after the treatments were applied.

[2] The second sampling was conducted 12 months after the treatments were applied.

[3] Mean of four replications; means within columns within each cultivar and each burial treatment followed by the same letter do not differ significantly according to Duncan's Multiple Range Test (P=0.05).

Table 3.12. Effect of composts (Batch A) with or without *P. polymyxa* PKB1 on emergence, plant height, shoot dry weight and root dry weight of canola cultivar Westar in a growth chamber test (without disease inoculation).

Compost	Emergence (%)	Plant height (mm)	Shoot dry weight (g)	Root dry weight (g)
A1	97.5 ab*	87.7 d	0.543 c	0.135 de
A2	91.2 abc	99.7 bc	0.699 b	0.139 de
A3	95.0 abc	101.5 bc	0.731 b	0.160 cde
A4	91.3 abc	112.9 a	0.964 a	0.171 bc
A5	88.8 bc	99.2 bc	0.661 bc	0.185 bc
A6	90.0 abc	99.1 bc	0.690 bc	0.197 ab
A7	86.3 c	97.9 c	0.672 bc	0.163 cd
A8	98.8 a	108.8 ab	1.001 a	0.214 a
CK	92.5 abc	76.7 e	0.541 c	0.131 e

* Means in a column followed by the same letter are not significantly different determined by Duncan's Multiple Range Test (P=0.05).

A1: Mature compost, no heat treatment

A2: Mature compost, heat treated for 5 h

A3: Mature compost + PKB1 spores (added after heat and cooling treatments)

A4: Mature compost + PKB1 spore + soybean meal (10%)

A5: Young compost, no heat treatment

A6: Young compost, heat treated for 5 h

A7: Young compost, + PKB1 spores (added after heat and cooling treatments)

A8: Young compost + PKB1 spore + soybean meal (5%)

Table 3.13. Effect of compost (Batch C) + *P. polymyxa* PKB1 on the growth of canola plants (cv. Westar), and infection of cotyledons of canola by *Leptosphaeria maculans* in a growth chamber test.

Compost	Emergence (%)	Infection (%)	Shoot weight (g)	Root weight (g)
C1	95.0 a*	7.5 ab	1.254 a	0.400 a
C2	97.5 a	10.0 ab	1.406 a	0.379 a
C3	95.0 a	12.5 a	1.520 a	0.379 a
C4	90.0 a	5.0 b	1.512 a	0.347 a
C5	97.5 a	10.0 ab	1.130 a	0.357 a
C6	90.0 a	15.0 a	1.307 a	0.362 a
C7	97.5 a	15.0 a	1.402 a	0.352 a
CK	85.0 a	17.5 a	1.084 a	0.267 a

* Means in a column followed by the same letter are not significantly different determined by Duncan's multiple Range Test (P=0.05).

C1: Mature compost + PKB1 spores, passive incubation

C2: Near mature compost + PKB1 spores, passive incubation

C3: Mature compost + 10% soybean meal + PKB1 spores, aerated incubation

C4: Near mature compost + 5% soybean meal + PKB1 spores, aerated incubation

C5: Mature composts + PKB1 spores, incubated in outdoor piles

C6: Mature compost, non-treated source material

C7: Near mature compost, non-treated material

Table 3.14. Effect of compost, compost + PKB1 and Vitavax RS on blackleg disease of canola seedlings in a growth chamber test*

Treatment	Emergence (%)	Infection (%)	Mean Disease Severity
Bare seed, Compost, BLA**	82.5 a	43.1 a	2.76 ab
Bare seed, Compost+PKB1, BLA	95.0 a	45.0 a	2.44 ab
Bare seed, no compost, no BLA	98.0 a	0.0 b	0.00 c
Bare seed, BLA inoculated	60.0 b	41.0 a	3.25 a
Vitavax RS, BLA	97.5 a	2.5 b	2.20 b

* Means in a column followed by the same letter are not significantly different determined by Duncan's multiple Range Test (P=0.05).

** BLA = a virulent isolate of *Leptosphaeria maculans*

Table 3.15. ANOVA table for effect of Tilt®, *P. polymyxa* PKB1 and compost on the viability of *Leptosphaeria maculans* on canola stubble in the field based on first sampling six months after treatment applied.

Source	DF	Sum of Squares	Mean Square	F	P
Rep	3	0.08	0.027	2.07	0.1211
Spray	5	0.73	0.146	5.38	0.0050
Rep x Spray (E_a)	15	0.41	0.027		
Burial	2	0.004	0.002	0.17	0.8443
Spray x Burial	10	0.14	0.014	1.08	0.4003
Error	36	0.47	0.013		
Total	71	1.84			

Table 3.16. Effect of Tilt®, *P. polymyxa* PKB1 and compost on the viability of *Leptosphaeria maculans* on canola stubble in the field based on first sampling six months after treatment applied.

Treatment	Mean Tissue Pieces with viable *L. maculans* (%)
Check	40.6 a*
PKB1 Alone	33.3 ab
Compost without PKB1	24.4 bc
Compost + PKB1	16.7 c
Tilt® + PKB1	15.0 c
Tilt® Alone	13.3 c

* Means in a column followed by the same letter are not significantly different determined by Duncan's Multiple Range Test (P=0.05).

Table 3.17. ANOVA table for effect of Tilt®, *P. polymyxa* PKB1 and compost on the percent pseudothecia formation of *Leptosphaeria maculans* on canola stubble in the field based on second sampling 12 months after treatment applied.

Source	DF	Sum of Squares	Mean Square	F	P
Rep	3	727.8	242.6	1.31	0.2861
Spray	5	19027.8	3805.5	33.47	0.0001
Rep x Spray (Ea)	15	1705.6	113.7	0.61	0.8439
Burial	2	13611.1	6805.5	36.75	0.0001
Spray x Burial	10	11188.9	1118.9	6.04	0.0001
Error	36	6666.7	185.2		
Total	71	52927.8			

Table 3.18. Some chemical properties in various composts

Compost	C/N Ratio	Concentration of Trace Elements ($\mu g\ g^{-1}$)										
		As	Cd	Cr	Co	Cu	Pb	Hg	Mo	Ni	Se	Zn
Municipal yard waste	7.23	5.0	0.58	26.0	5.7	24.2	18.3	0.19	1.54	15.3	1.0	116.0
Cattle manure	7.18	5.2	0.34	22.0	4.85	36.9	9.9	0.30	2.59	13.6	1.3	160.0
Pulp sludge	18.62	0.47	1.80	9.1	1.25	53.3	4.82	0.88	2.63	4.38	0.8	187.0
Wood chips	65.37	0.94	0.82	5.9	1.07	5.69	1.69	0.70	0.39	3.03	0.2	115.0
Screened municipal solid waste	10.20	5.62	0.57	30.9	6.0	42.7	2689.0	0.27	2.09	17.4	1.0	133.0
1999 screened compost	7.79	3.72	0.345	20.2	5.65	48.2	6.24	0.32	2.53	10.7	1.5	220.0
1998 compost	11.27	3.91	0.39	30.5	6.46	50.3	6.89	0.20	2.41	12.6	1.5	223.0
1999 unscreened compost	11.48	1.87	0.286	10.7	3.94	52.3	3.68	0.35	3.35	5.5	2.0	250.0
Cattle manure + PKB1 + soybean meal	7.69	2.81	0.29	15.2	3.08	27.7	5.52	0.90	3.74	11.2	1.4	136.0
Cattle manure + soybean meal	7.58	3.7	0.387	17.2	3.33	27.3	6.20	0.86	2.90	12.0	1.3	127.0
Cattle manure+PKB1	12.99	5.52	0.41	31.1	6.93	44.9	8.23	0.22	2.39	15.9	1.9	200.0
Cattle manure	12.47	5.36	0.44	27.1	6.8	46.0	7.93	0.12	2.45	14.7	1.9	206.0

Table 3.19. Total microbial activity in various composts determined by fluorescein diacetate hydrolysis assay.

Compost	Source	Microbial Activity Fluorecein ($\mu g\ g^{-1}$)	Total Bacterial Population ($CFU\ g^{-1}$)
Municipal yard waste	Olds	12.735	3.28E+08
Cattle manure	Olds	10.493	2.60E+08
Pulp sludge	Olds	4.852	2.51E+08
Wood chips	Olds	10.051	5.46E+08
Screened municipal solid waste	Olds	9.602	3.00E+08
1999 screened compost	High River	10.657	1.41E+04
1998 compost	High River	8.079	2.82E+08
1999 unscreened compost	High River	8.111	1.45E+08
Cattle manure + PKB1 + soybean meal	Compost Bins, Olds	8.509	5.52E+12
Cattle manure + soybean meal	Compost Bins, Olds	11.813	2.60E+11
Cattle manure+PKB1, 2 weeks after inoculation	Windrow, High River	10.120	1.70E+10
Cattle manure (CK), 2 weeks after inoculation	Windrow, High River	10.574	1.12E+10
CK (#99), High River, 1st sample	Windrow, High River	7.056	7.70E+10
Compost before PKB1 (#98), High River, 1st sample	Windrow, High River	10.259	5.00E+10
Compost after PKB1 (#98), High River, 1st sample	Windrow, High River	6.532	5.14E+10

Table 3.20. Effect of various composts on Rhizoctonia damping-off of canola in a greenhouse test

Treatment	Emergence (%)	Survival (%)	Mean Disease Severity
Municipal yard waste	57.0 de*	35.0 cd	2.75 bc
Cattle manure	72.0 bcd	35.0 cd	3.33 abc
Pulp sludge	70.0 bcd	49.0 c	3.17 abc
Wood chips	40.0 e	10.0 d	4.52 a
Screened municipal solid waste	82.0 ab	40.0 c	3.64 abc
1999 screened compost	58.0 cde	45.0 c	2.34 c
1998 compost	72.0 bcd	46.0 c	2.97 abc
1999 unscreened compost	85.0 ab	46.0 c	3.58 abc
Cattle manure + PKB1 + soybean meal	73.0 abcd	38.0 c	3.11 abc
Cattle manure + soybean meal	83.0 ab	54.0 bc	2.95 abc
Cattle manure+PKB1	95.0 a	80.0 ab	2.60 c
Cattle manure, no PKB1 inoculum	82.0 ab	57.0 bc	3.21 abc
Cattle manure, windrow #99	79.0 abc	39.0 c	3.98 ab
Cattle manure, windrow #98	79.0 abc	44.0 c	3.42 abc
Cattle manure, windrow #98, inoculated with *P. polymyxa* PKB1	90.0 ab	51.0 c	3.46 abc
CK1, Promix, inoculated with *R. solani*	69.0 bcd	43.0 c	3.97 ab
CK2, Promix, no *R. solani* inoculum	95.0 a	96.0 a	0.00 d

* Means followed by the same letters are not significantly different from each other determined by Duncan's Multiple Range test (P=0.05).

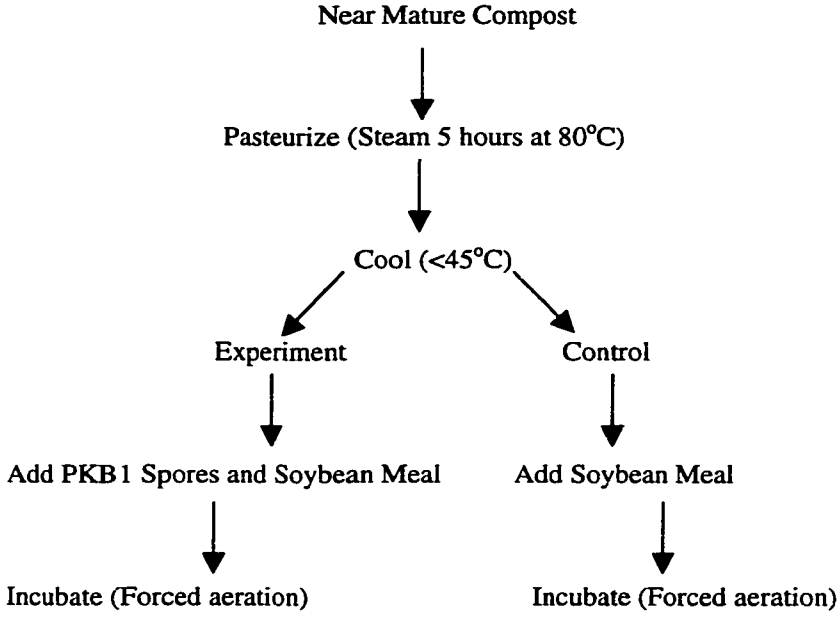

Figure 3.1. Procedure of preparation of bacterium amended compost mafe from cattle manure plus wood chips at Composting Technology Centre, Olds, Alberta.

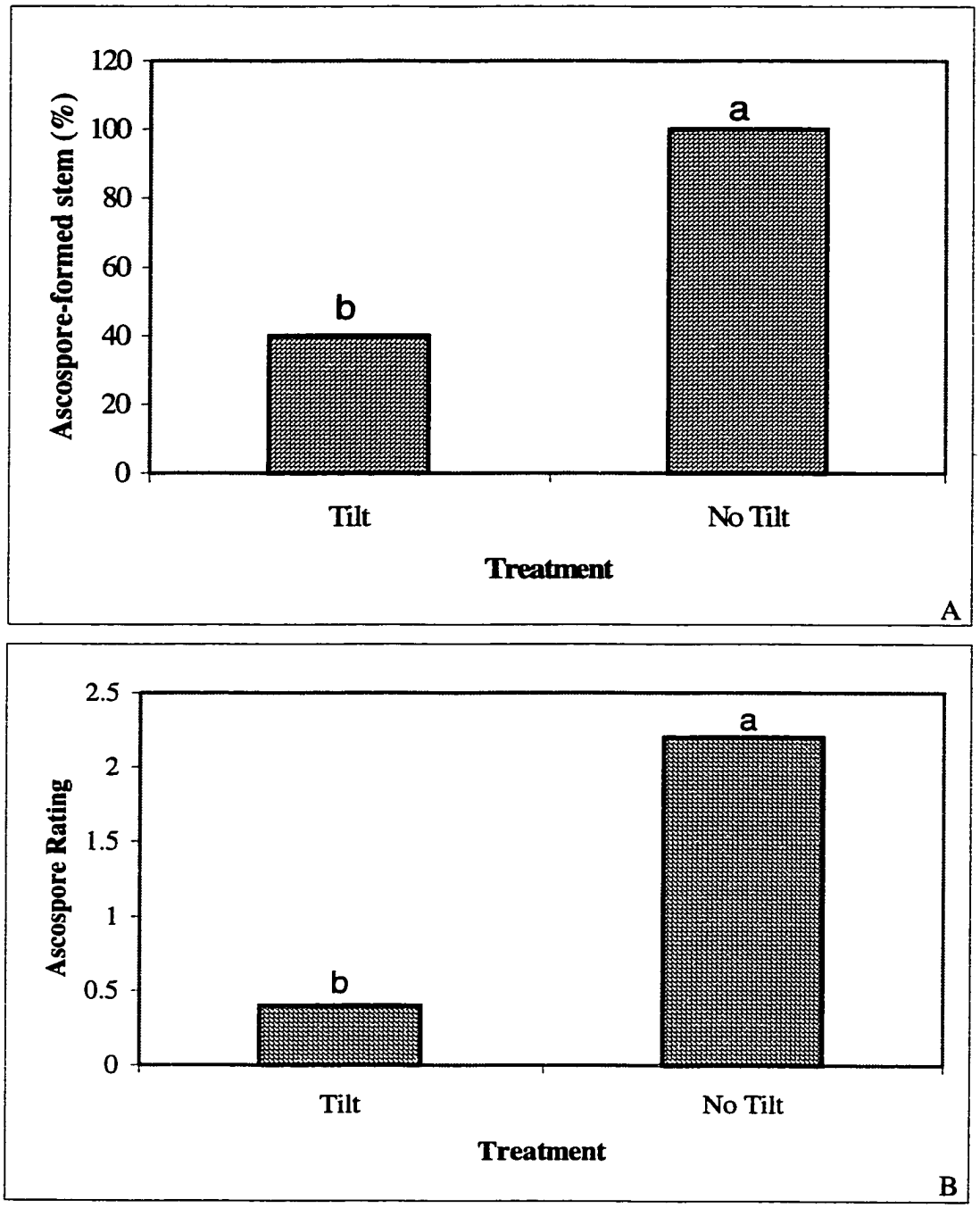

Figure 3.2. Occurrence of ascospores (A) and ascospore density rating (B) on canola stubble unsprayed or sprayed with Tilt® in the field in Wainwright, Alberta, 1994 -1995. Bars labelled with the same letter are not significantly different according to least significant difference test (p=0.05), means of 25 stem samples.

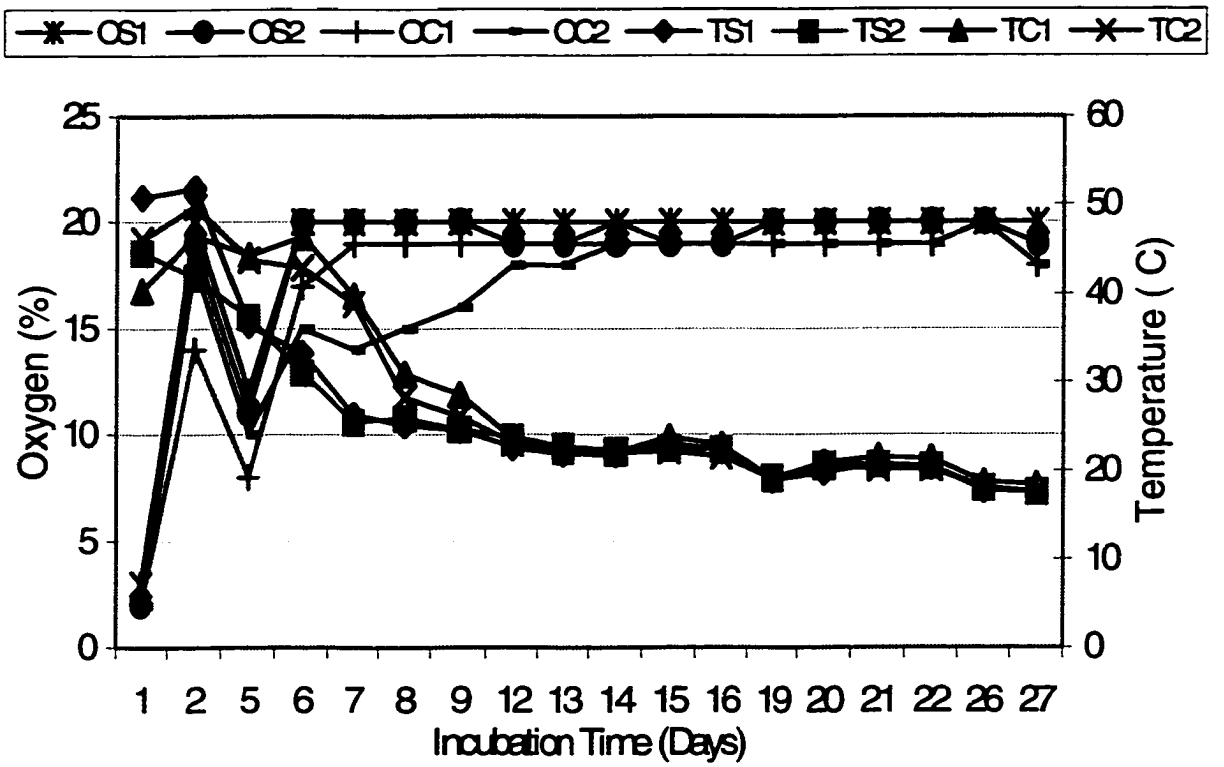

Figure 3.3. Oxygen (%) and temperature (°C) measurements during incubation of compost after *Paenibacillus polymyxa* spores were added into different compost bins, Olds, 1998. S1 and S2 were compost bins inoculated with *P. polymyxa* PKB1. C1 and C2 were compost bins without inoculation. O is oxygen measurement and T is teperature measurement.

Figure 3.4. Effect of compost amended with *Paenibacillus polymyxa* PKB1 spores in reducing blackleg disease severity of canola in a growth chamber test. CK1 = No compost, no blackleg inoculum; CK2 = Compost, no inoculum; CK3 = No compost, inoculated with *Leptosphaeria maculans*. BLA = a virulent isolate of *L. maculans*.

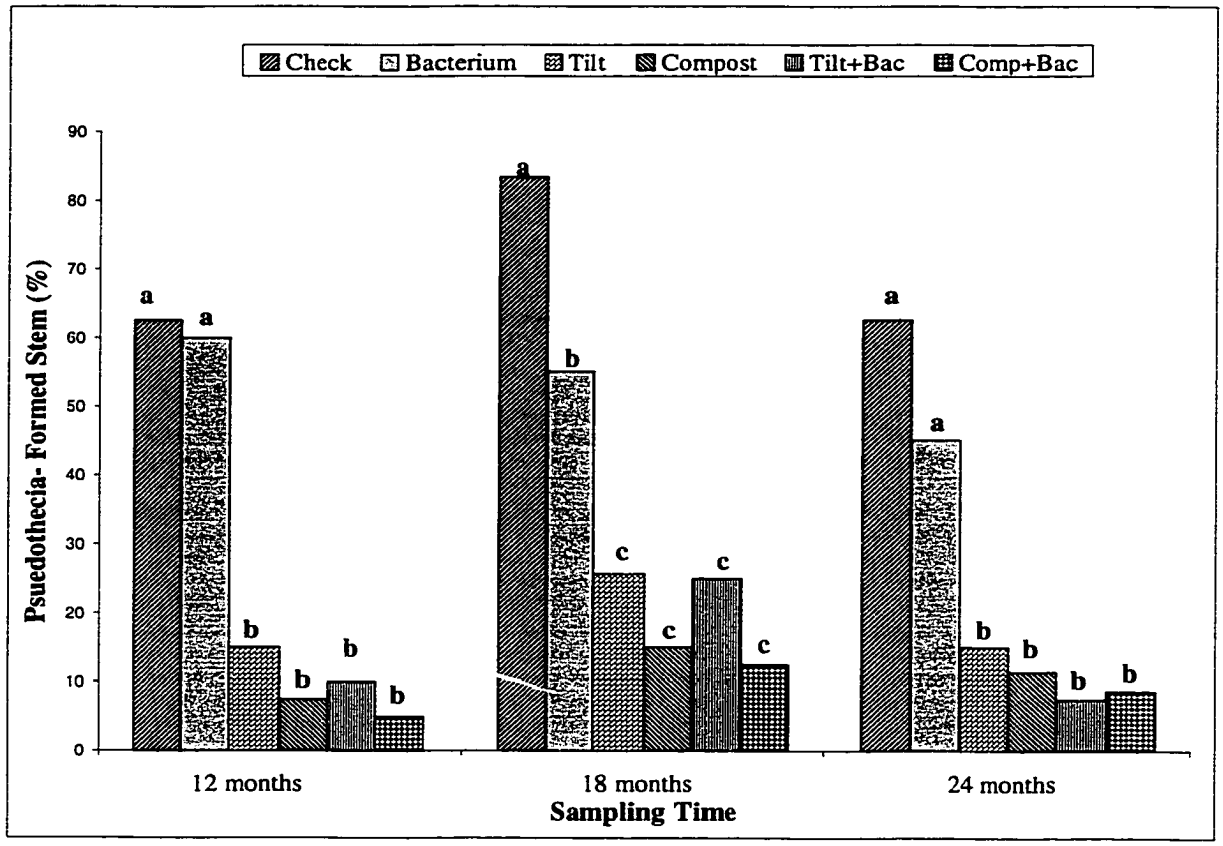

Figure 3.5. Effect of Tilt®, *P. polymyxa* PKB1 and compost on the pseudothecia formation of *Leptosphaeria maculans* on canola stubble in field conditions. Bars labelled with the same letter within each treatment are not significantly different according to Duncan's Multiple Range test ($P<0.05$).

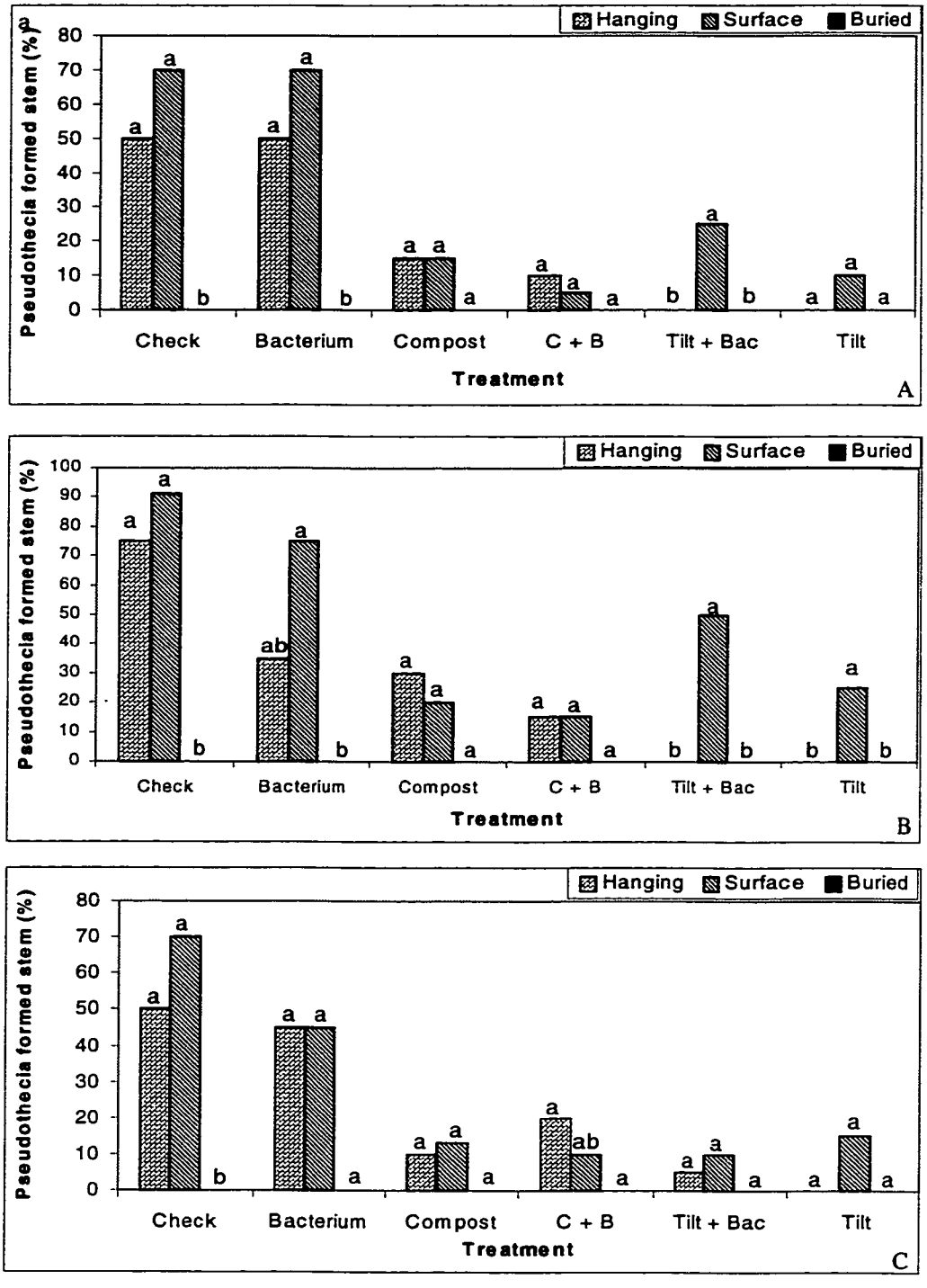

Figure 3.6. Effect of burial on the pseudothecia formation of *Leptosphaeria maculans* on canola stubble 12 month (A), 18 month (B) and 24 month (C) after treatments applied in field conditions. Bars labelled with the same letter within each treatment are not significantly different according to Duncan's Multiple Range test ($P<0.05$).

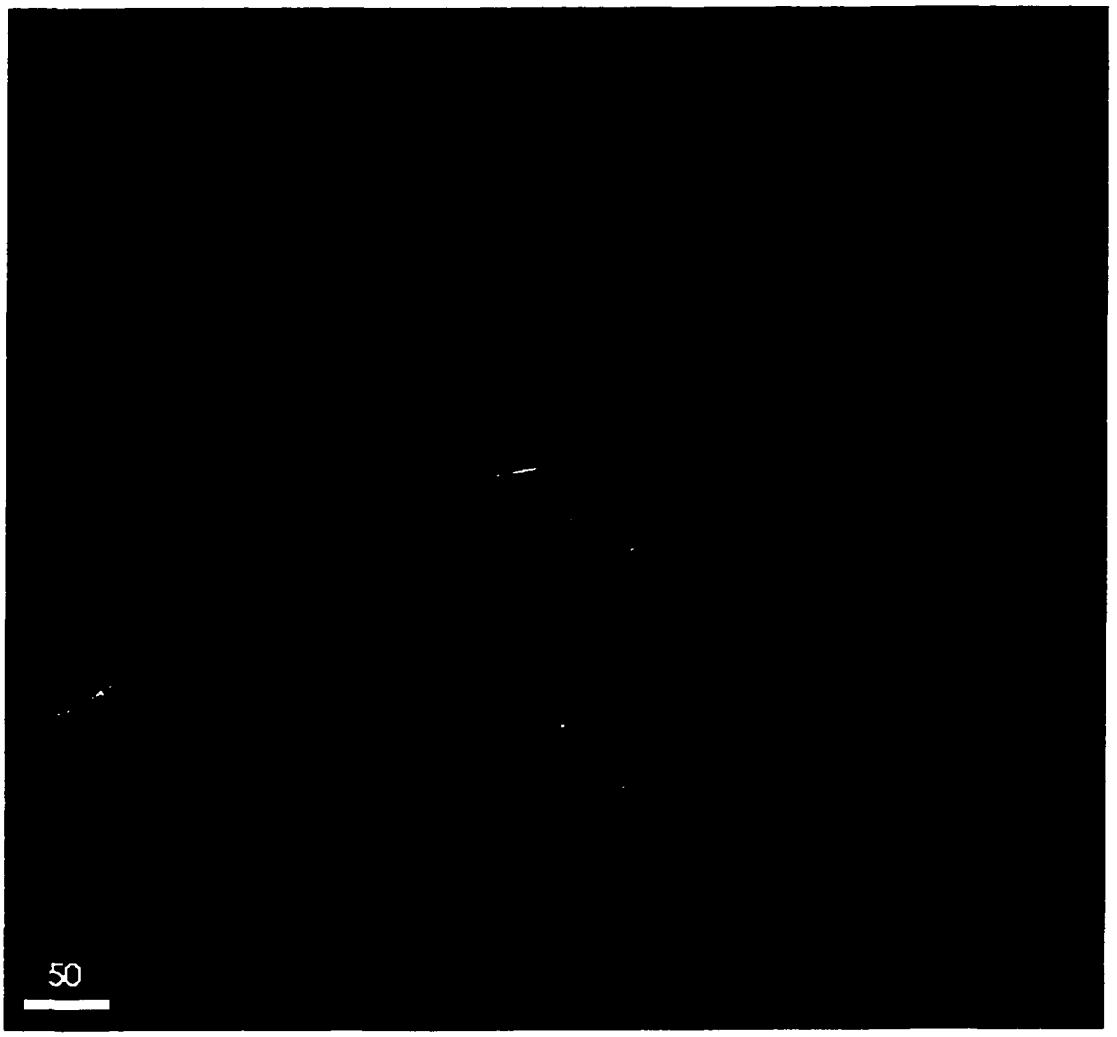

Figure 3.7. Confocal laser scanning microscope image showing microbes on a compost particle inoculated with *Paenibacillus polymyxa* PKB1 stained with acridine orange.

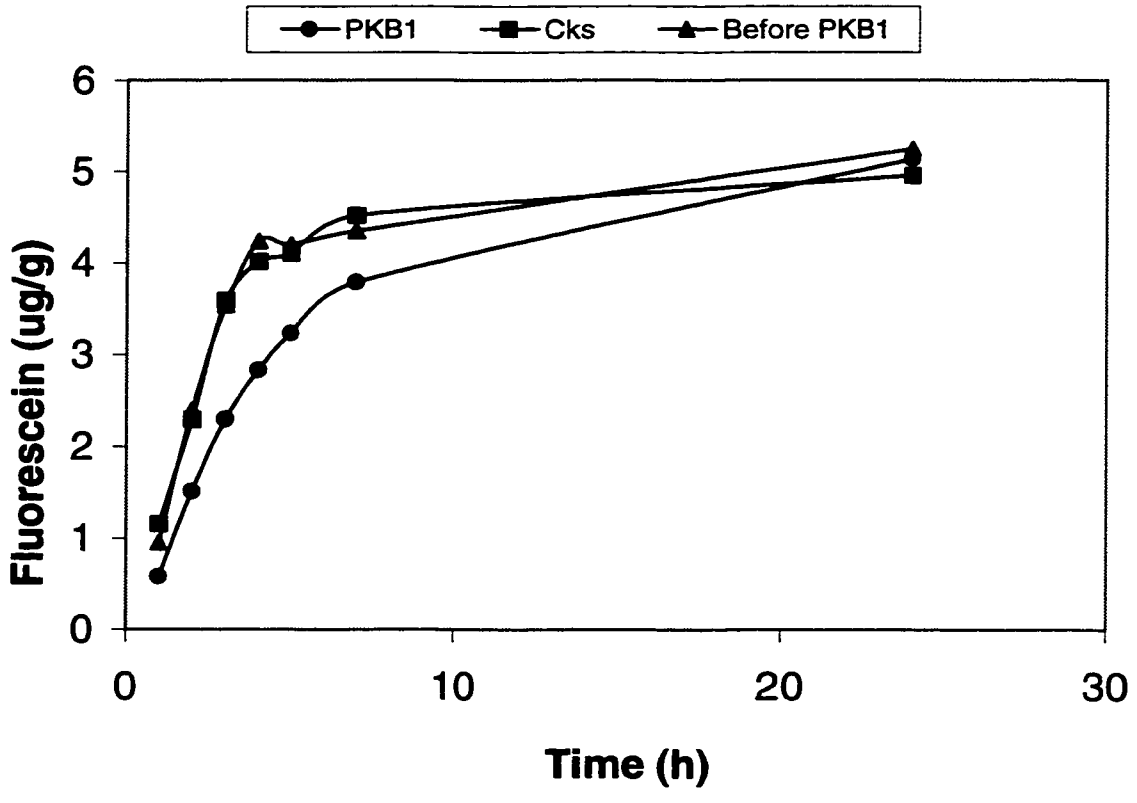

Figure 3.8. Microbial activity in compost presented as fluorescein diacetate (FDA) hydrolysis (mean of three samples).

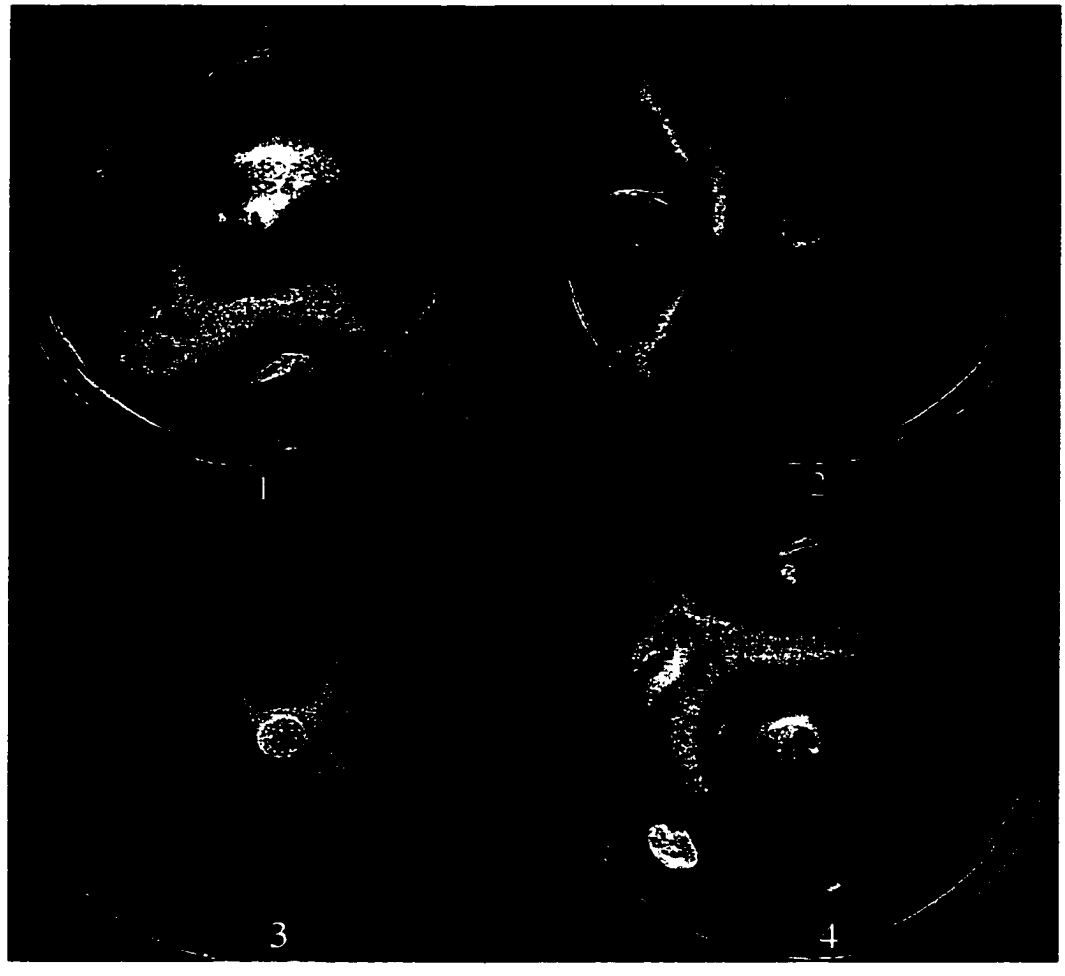

Figure 3.9. Effect of various bacteria isolated from composts to *Rhizoctonia solani* AG2-1 in a plate test. 3 = Control, no bacteria were placed on to the plate.

Chapter 4

Development of Specific DNA Probes for Molecular Detection of *Paenibacillus polymyxa* PKB1

Introduction

The effectiveness of *Paenibacillus polymyxa* PKB1, alone or in combination with Tilt®, in controlling the blackleg of canola caused by *Leptosphaeria maculans* in growth chamber and in field tests has been investigated in the current study. Infested canola stubble pieces were dipped in a *P. polymyxa* PKB1 spore suspension and placed in the field. The results showed that *P. polymyxa* PKB1 significantly reduced the formation of ascospores in *L. maculans* on canola stubble (Chapter 3) and had a good potential as a biocontrol agent.

The ability to track the microorganism is a prerequisite to any study relating to the behaviour of a microorganism artificially introduced into the environment. When efforts were made to use compost as a carrier of *P. polymyxa* PKB1 for field application, difficulties were encountered in retrieving the bacterium from the compost and the treated canola stubble in field (Chapter 3). Therefore, this project was started to develop a fast and reliable recognition technique for this specific bacterial strain in the biocontrol study.

The genus *Bacillus* consists of many species (Gordon et al., 1973). Traditional bacterial identification methods are complicated and time-consuming. In the past, naturally occurring markers were often found to be insufficient; therefore, mutagenesis was used to generate autotrophic, morphological, or drug-resistant mutants (Nautiyal, 1997). However, these markers, such as antibiotic resistance, were not always stable and were sometimes lost after several generations; as well, researchers underestimated the wild-type populations (Lochner et al. 1991; Mahaffee et al., 1997).

The isozymes, restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR), and amplified fragment length polymorphism (AFLP) techniques are currently being applied to develop molecular markers. These markers have been used to detect specific phytopathogenic fungi and bacteria (Arnau et al., 1995; Delye et al., 1997; Goodwin and Annis, 1991; Kageyama et al., 1997; Kaiser et al., 1997; O'Neill et al., 1997; Pongam et al., 1999; Schafer and Wostemeyer, 1994; Schilling et al., 1996; Yashitola et al., 1997). A PCR assay was developed to detect spore-forming bacteria by using specific primers designed based on the known nucleotide sequences of the sporulation sigma

factor (E) (sporIIGB) from *Bacillus subtilis* and *B. thuringiensis* (Arcuri et al., 1999). Spacers of ribosomal DNA (rDNA), mainly the noncoding internal transcribed spacers (ITS) that vary between species within a genus, are being increasingly used in studies of fungal molecular taxonomy (Balesdent et al., 1998). The advantages of molecular markers over other types of markers are that the molecular markers are highly sensitive, stable, and reproducible (Michelmore and Hulbert, 1987).

A non-radioactive labelling technique has been used to develop specific molecular probes for detecting fungal species or pathotypes. RAPD-PCR-derived, digoxigenin-labelled DNA probes have been developed to detect aggressive or non-aggressive strains of *Leptosphaeria maculans* (Schafer and Wostemeyer, 1994), to differentiate *Alternaria brassicae* from other *Alternaria* spp. (Sharma and Tewari, 1998), *Rhychosporium secalis* from other phytopathogenic fungi (Sharma et al. 1996), and *Fusarium culmorum* from *F. graminearum* (Koopmann et al., 1994). Digoxigenin (Dig)-labelled RNA probes were applied to certify virus-free tomatoes in Italy (Saldarelli et al., 1996). This technique is safe for human health and the environment compared to radioactive isotope labelling.

Initial observations on genetic variation within *P. polymyxa* have been reported (Yang et al., 1996). Genetic polymorphism was observed among strains of *P. polymyxa* and *Bacillus* spp. The objectives of this study were (1) to detect the molecular polymorphism among *P. polymyxa* and seven *Bacillus* spp. by using the RAPD-PCR technique; (2) to develop specific molecular probe(s) for reliable detection of *P. polymyxa* PKB1 by using non-radioactive Dig-DNA labelling techniques; and (3) to develop specific primers for *P. polymyxa* PKB1 strain identification.

Materials and Methods

Strains of *Paenibacillus* and *Bacillus* spp.

Thirteen strains of *P. polymyxa* and 10 strains of seven other *Bacillus* spp. were used in this investigation (**Table 4.1**). Strains 96-1 to 96-5 and 97-1 were isolated from canola stubble in Vegreville, Alberta and identified by Dr. R. Coleman at the Microbiology Laboratory, Alberta Research Council, Vegreville, Alberta. All these strains showed an inhibitory effect on *L. maculans* (Chapter 2). Two strains, however, were ineffective as inhibitors of *L. maculans*. *P. polymyxa* 97-2, originally isolated in Japan, could produce antibiotics but had only a moderate inhibitory effect on *L. maculans*, and *P. polymyxa* 97-3,

isolated in U.S.A., could not inhibit the growth of *L. maculans*. Two mutants of *P. polymyxa* PKB1, R1 and R2, resistant to antibiotic rifampicin at 100 and 200 ppm, were selected (Kharbanda, 1998). The resistance to antibiotic rifampicin was used as a marker, for detection of the bacterium (Yang et al., 1999). Strain 96-17 was collected and provided by Dr. R. Coleman, and had no inhibitory effect on *L. maculans*. Strains 96-9 and 97-4 were collected in Alberta and had a moderate inhibitory effect on *L. maculans*.

In the previous biocontrol experiments (Chapter 3), naturally infested canola stems were collected from fields and treated with *P. polymyxa* PKB1 spore suspension. These stems were placed in the field for 6, 12, and 18 months, then retrieved and brought back to the laboratory to check for the production of ascospores of *L. maculans*.

Extraction of genomic DNA

The bacterial strains were grown in a 5mL nutrient broth (Difco) at 200 rpm on a shaker at room temperature for 2 days. Genomic DNA was extracted according to the procedure described by Sambrook et al. (1989) with some modifications. The bacterial suspension was centrifuged to collect the bacterial cells. The crude DNA was first extracted with 567 µl TE buffer (10 mM Tris HCl, pH 8.0, and 0.1 mM ethylenediamine tetraacetoc acid (EDTA)), 3 µl proteinase K (Life Technologies Inc., 20 mg/ml), and 30 µl 10% sodium dodecyl sulfate (SDS) at 37°C for 1 h, and then digested with RNAse A (Sigma, 20 mg/mL) at 37°C for 1 h. Polysaccharides of the bacterium were removed by adding 100 µl of 5 M NaCl and 80 µl of cetyltrimethyl-ammonium bromide (CTAB) and incubating at 65°C for 10 min, followed by chloroform/isoamyl alcohol (24:1) and phenol/chloroform/ isoamyl alcohol (25:24:1) extractions. The DNA was precipitated with one volume of isopropanol, washed with 70% ethanol, dried, and dissolved in 100 µl TE buffer. The amount of DNA was measured with a Gene Quant., RNA/DNA Calculator (Phamacia LKB Biochrom Ltd., Cambridge, U.K.). The quality of DNA was checked by electrophoresis on 1% (w/v) agarose gel and stained with ethidium bromide.

DNA amplification

Twenty random primers (Operon Technologies Inc., Alameda, CA, U. S. A.) were used for PCR reactions. Four primers giving good DNA amplification patterns were selected for further studies. These

primers were OPA-07: 5'-GAAACGGGTG; OPA-08: 5'-GTGACGTAGG; OPA-13: 5'- CAGCACCCAC; and OPA-14: 5'- TCTGTGCTGG. PCR reactions were carried out in a solution of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT (dithiothreitol), 200 µM each of dNTPs, 0.5 µM primer, 1.25 U Taq DNA polymerase, and 20 ng genomic DNA in 25 µl aliquots by using a thermal cycler (Thermolyne, Temp. Tronic, Barnstead/ Thermolyne Corporation, Dubuque, IA, U.S.A.). The thermal cycling profile consisted of 40 cycles at 95°C for 1 min, 35°C for 1 min, and 72°C for 2 min. In addition, all profiles were preceded by an initial denaturation at 94°C for 5 min, 35°C for 1 min for low stringency annealing of primer, and 72°C for 1 min for primer extension, followed by a final extension at 72°C for 5 min. Electrophoresis of amplified products were carried out in 1% (w/v) agarose gels, stained with ethidium bromide, and photographed over a 302 nm UV Transillumunator with Polaroid 57 positive/negative film. The gel was also scanned into a computer using a computer program Molecular Analyst® (© Bio-Rad Laboratories Inc. 1995-96). A one kilobase (kb) DNA ladder (Gibco-BRL) was used as a DNA size marker. All PCR reactions were performed twice.

Probe synthesis

Details of four probes along with their sizes are given in Table 4.2. DNA of *P. polymyxa* PKB1 was amplified with the primers OPA-07, OPA-08, OPA-13 and OPA-14 in a volume of 50 µl in a thermocycler. PCR products amplified with each primer were separated on 1% (w/v) agarose gel (low melting point) by electrophoresis. Four DNA fragments of 0.61, 0.71, 0.27, and 0.62 kb were excised from the gel and purified with a DNA Purification Kit (Boehringer Mannheim) following the supplier's instructions. The purified DNA fragments were separately labelled with alkali-labile Dig-11-dUTP in a simultaneous amplification in a thermocycler using the same PCR profile as described previously. The labelling reaction was performed in a 50 µl volume reaction solution containing 5 µl 10X PCR buffer (200 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 µl of 50 mM $MgCl_2$, 0.25 µl (1-1.5 unit) Taq DNA polymerase, 1 µl of each of 10 mM dATP, dGTP, dCTP, 10 µl Dig-11-dUTP+ dTTP (1:4), 2.5 µl of PCR product-specific 10 mM primer, and 2.5 µl template DNA. Estimation of the yield of the Dig-labelled probe was done according to the method given in the Genius System User's Guide for Membrane Hybridization, Version 3.0 (Boehringer Mannheim). The labelled probes were stored at -20°C for later use.

Southern blotting and hybridization

The genomic DNA (2 μg) of each of the 23 bacterial strains was digested separately with restriction endonucleases, Eco RI, Pst I, and Hind III (Gibco-BRL) at 37°C for 1 h. The DNA digests were separated on 1% (w/v) agarose gel in 0.5X TBE buffer by electrophoresis, stained with ethidium bromide, and photographed. A 21 kb dig-labelled marker was used (Boehringer Mannheim).

The DNA fragments on agarose gel were depurinated in 250 mM HCl for 10 min, denatured in 0.5 M NaOH, 1.5 M NaCl solution (pH 7.0) for 1 h, and then neutralized in 1.0 M Tris-HCl plus 1.5 M NaCl solution (pH 8.0) for 1 h. The DNA fragments were transferred onto a positively charged nylon membrane (Boehringer Mannheim) in a 10X SSC (175.3 g NaCl, 88.2 g sodium citrate in 2 L water, pH 7.0) solution overnight. The membrane was briefly washed in 5X SSC after blotting, placed between two sheets of 3M Whatman filter paper, and baked at 80°C for 1 h to fix the DNA. Pre-hybridization was performed for 2 h in a pre-hybridization solution containing 5X SSC, 1% (w/v) blocking reagent (in 100 mM maleic acid, 150 mM NaCl, pH 7.5), 0.1% N-lauroylsarcosine, and 0.02% sodium dodecyl sulphate (SDS) in a hybridization bag at 65-68°C. Hybridization was performed with each probe separately for 16-18 h in a hybridization solution (pre-hybridization solution containing Dig-labelled probe in a ratio of 1:20). After hybridization, the probe solution was stored at -20°C for further use and the membrane was washed twice in 2X SSC + 0.1% SDS for 5 min per wash at room temperature, followed by two additional washings (15 min each) in 0.5X SSC + 0.1% SDS at 65°C.

Chemiluminescence detection

Probed DNA was detected with Lumigen PPD [4-methoxy-4-(3-phosphatephenyl)-spiro-(1,2-dioxetane-3,2'-adamantane) disodium salt] by using a DIG Luminescence Detection Kit (Genius 7, Boehringer Mannheim) and following the procedure given by the supplier (Genius System User's Guide, Boehringer Mannheim). The membrane was exposed to X-ray film (Fuji) for 2-3 h at room temperature and was developed in X-OMAT Processors (Kodak M35 & M35A). The image on the film was scanned into a computer and analyzed by using the molecular analysis program GeneJockey II (© P. L. Taylor, 1992-1995). The Southern hybridization test was repeated twice.

Dot blotting

The specificity of probes was also determined by the dot-blotting method. Thirteen strains of *P. polymyxa* and 10 strains from seven *Bacillus* spp. were used. Genomic DNA (1 µl) of each bacterial strain were placed onto a positively charged nylon membrane (Boehringer Mannheim) and fixed in an oven (80°C) for 1 h. The hybridization and chemiluminescence detection procedures were the same as those described above.

DNA cloning and sequencing

Four DNA probes were cloned in *Escherichia coli* supercompetent cells by using a pCR-Script™ Amp SK (+) Cloning Kit (Stratagene®) and following the supplier's instructions. Purified and unlabelled DNA fragment was polished, ligased and inserted into a vector (plasmid Amp^r). The plasmids with inserts were transformed into *E. coli* cells by heat pulse at 42°C for 45 second. *E. coli* cells were cultured on LB-ampicillin-methicillin agar (10 g of NaCl, 10 g of tryptone, 5 g of yeast extract, 20 g of agar, 20 mg ampicillin and 80 mg of methicillin, 1 L water) plates containing X-gal and IPTG (isopropylthio-β-D-galactoside) for 16 h. White bacterial colonies were selected, transferred to LB-ampicilin broth and incubated at 37°C on a shaker (250 rpm) for 16-18 h.

The plasmid DNA was extracted by using a PlasmidPURE™ DNA Miniprep Kit (Sigma Bio Sciences™) and following the supplier's instructions. The yield and quality of the purified plasmid DNA were examined by electrophoresis on 1% (w/v) agarose gel. The plasmid DNA was digested with restriction endonuclease Kpn I and Sac I at 37°C for 1 h to check for the insert. The electrophoresis of digests were run on 1% (w/v) agarose gel and stained with ethidium bromide.

Confirmed plasmid DNA with insert was sequenced by using an automated DNA sequencer (Applied Biosystems/Perkin Elmer 373). Two universal primers T3 and T7 were used. The data were edited and analyzed by using the computer software GeneJockey II (© P. L. Taylor, 1992-1995).

Developing specific primers

DNA sequences were entered into the computer program GeneJockey II (© P. L. Taylor, 1992-1995) and searched for possible primers. Eight compatible primers, six from P1-8 and two from P1-14 (Table 4.3), were designed by using the computer software Amplify 1.2 (© Bill Engels, 1992). The primers were

synthesized according to the sequence and examined in PCR reactions with 23 strains of *P. polymyxa* and *Bacillus* spp. for specificity to *P. polymyxa* PKB1.

RFLP Analysis

Ten strains of *P. polymyxa* were amplified by the designed primers listed in **Table 4.3**. PCR products amplified with the designed primers J1 and JY1 (**Table 4.3**) from these 10 bacterial strains were digested with three restriction endonucleases, Rsa I, MseI, and Sau3A I separately to check for restriction site polymorphisms in the DNA fragments from these 10 bacteria. The reaction mix contained 2 µl DNA from the PCR product, 1 µl (5 units) restriction endonuclease, 2 µl reaction buffer, and 15 µl water. The reaction was carried out in a 37°C water bath for 1 h. The digests were separated on 1.5% agarose gel at 100 V. The gel was stained with ethidium bromide and photographed under UV light.

Because none of the three restriction enzymes could differentiate the nucleotides amplified by primers J1 and JY1, the PCR products amplified with primers J1 and JY1 from strains *P. polymyxa* PKB1 and 97-4 were sequenced and compared. The sequencing was done by using an automated DNA sequencer (Applied Biosystems/Perkin Elmer 373).

Detection of bacteria in compost inoculated with *Paenibacillus polymyxa* PKB1

Bacterial strains isolated from compost that had been inoculated with *P. polymyxa* PKB1 (Chapter 3) were detected by using PCR and dot-blotting techniques. Bacterial strains from compost without inoculation were also included in the test. Primer OPA-07 was used in the tests.

These tests were also used to confirm the specificity of probes and specific primers for the detection of *P. polymyxa* PKB1 within bacterial strains isolated from compost that had been inoculated with *P. polymyxa* PKB1.

Bacterial isolates from composts with and without inoculation of *P. polymyxa* PKB1 were tested for their inhibitory effect against fungal pathogens, *L. maculans*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*, of canola (Chapter 3). Isolates showing an inhibitory effect were selected and purified. DNA was extracted and amplified with OPA-07 in a PCR reaction by using the same protocol as that mentioned above.

Results

DNA amplification patterns

The bacterial strains 96-1 and 96-2 were amplified with twenty primers in a preliminary study. Four of these 20 primers were selected for further use because they produced good and countable bands. Amplification patterns of 13 strains of *P. polymyxa* and 10 strains of seven *Bacillus* spp. with four primers, OPA-07, OPA-08, OPA-13 and OPA-14, are given in **Figure 4.1.** These four primers gave multiple products in the 0.3-6 kb size range. OPA-07 and OPA-13 gave clearer amplification patterns (**Figures 4.1A** and **4.1C**). When these four primers were used, all the strains of *P. polymyxa* collected from Vegreville showing antifungal activity to *L. maculans* (lane 2 to 9 in **Figure 4.1**) had one or more fragments in common for each primer. Strains of *P. polymyxa* in lanes 10 to 14 were from different locations and showed different DNA amplification patterns. Within the group of Vegreville strains in lanes 2 to 9, the variation was less, and they were easily distinguishable from other *P. polymyxa* strains or strains of other *Bacillus* species. At least three groups could be classified within the 13 strains of *P. polymyxa* according to PCR profiles: (1) Vegreville strains; (2) strains 97-2, 97-3 and 96-17; and (3) 96-9 and 97-4. Strains in lanes 15 to 24 were from seven different *Bacillus* species (**Table 4.1**). Their DNA amplification patterns were quite different from each other. Four DNA bands of PKB1 (96-1 in lane 2) amplified by each primer were selected to prepare probes, as indicated by arrows in **Figure 4.1.**

Detection of probes

All four probes (**Table 4.2**) were used to hybridize with the Southern blots of Hind III, Eco R1, and Pst I restriction digests of different bacterial strains (**Figure 4.2**). Probes P1-7, P1-8, P1-13 and P1-14 were species-specific and hybridized with only the DNA of PKB1 and strains of *P. polymyxa* (96-1 to 97-4 in upper lanes 2-12 in **Figures 4.2A-4.2B** and upper lanes 2-12, lower lanes 2-3 in **Figures 4.2C-F**) but not with that of other *Bacillus* spp. (96-8 to 96-19 in lower lanes 2-12 in **Figures 4.2A-4.2B**, and 96-10 to 96-19 in lower lanes 4 -12 in **Figures 4.2C-F**). DNA of the antibiotic-resistant mutants could also be probed (R1 and R2 in **Figures 4.2C-F**). P1-7 and P1-8 hybridized with the Hind III and Eco RI digests, and polymorphic bands were obtained among the isolates. It seemed that strains 96-17, 97-2, and 97-3 had similar patterns (**Figures 4.2 A** and **4.2E**). Strains 96-9 and 97-4 had similar patterns and belonged to the same group

(**Figures 4.2 D** and **4.2E**), and all Vegreville strains were the same except that 96-2 did not have complete digestion with some enzymes.

Probes P1-7 and P1-8 were used to probe the DNA of bacterial strains by the dot-blotting method. *P. polymyxa* PKB1 and all strains in *P. polymyxa* could be detected (**Figures. 4.3A** and **4.3B**). This result confirmed that the probes were species-specific.

DNA cloning and sequencing

DNA fragments of four probes were cloned and sequenced. The four fragments had the length of 609 bp (P1-7), 713 bp (P1-8), 270 bp (P1-13) and 617 bp (P1-14), respectively. The nucleotide sequences of two probes are presented in **Figures 4.4** and **4.5**. These two sequences could be translated into polypeptides with no interruption stop codons in some reading frames analyzed with the computer software GeneJockey (© P. L. Taylor, 1992-1995). The nucleotide sequence of P1-8 (**Figure 4.4**), in the reading frame 3, had 65% identity with the gene spoOK of a protein required to initiate sporulation in *B. subtilis* (Perego et al. 1991; Rudner et al. 1991). The design and development of specific primers for *P. polymyxa* PKB1 according to the nucleotide sequences in **Figures 4.4** and **4.5** were carried out.

Detection of *P. polymyxa* PKB1 with specific primers

The eight primers (**Table 4.3**) designed had ten primer pair combinations. At 55°C annealing temperature, six primers, designed according to the sequence of P1-8, with eight pairs gave similar results in the PCR tests. The primers amplified only one DNA band (about 500 bp) of *P. polymyxa* PKB1 and the other nine strains of *P. polymyxa,* which had showed inhibitory effects on *L. maculans.* However, PCR products were produced neither in the strains 97-2, 97-3, and 96-17 of the same species, which had no antifungal effects on *L. maculans*, nor in 10 strains of other *Bacillus* spp. (**Figures 4.6A, B, C, E** and **F**). Another pair primer (J3 and JY5) amplified only one DNA band (about 400 bp) of *P. polymyxa* PKB1 and nine strains in the *P. polymyxa*, but not in *P. polymyxa* 96-17, 97-2 and 97-3 in the same species and strains of other *Bacillus* spp. at 68°C annealing temperature (**Figure 4.6D**). DNA of two strains (97-4 and 96-9), that had inhibitory effects on *L. maculans*, *Rhizoctonia solani* and other pathogens (Lange et al. 1994), also could be amplified with these designed primers. The results showed that these primers were specific for

detecting *P. polymyxa* PKB1 and strains of *P. polymyxa* having antifungal activity against *L. maculans* and could be used for detection of this biocontrol agent during field studies.

To further distinguish these two groups of *P. polymyxa* (the Vegreville strains, and strains 97-4 and 96-9), the DNA fragments (513 bp) of 96-1 (lane 2 in Figure 4.6A) and 97-4 (lane 12 in Figure 4.6A) from the same PCR reaction, amplified with the designed primers J1 and JY1, were purified and sequenced in a DNA sequencer (Applied Biosystems/Peskin Elmer 373). DNA fragments of 96-1 (*P. polymyxa* PKB1) and 97-4 amplified with J1 and JY1 differed by only one base (**Figure 4.7**).

RFLP analysis of the PCR product

RFLP of 10 bacterial strains with three restriction enzymes showed no difference in the nucleotide sequences of these two groups (the Vegreville strains and strains 97-4 and 96-9). Rsa I gave three DNA bands, Mse I gave two DNA bands, and Sau3A I had four bands (**Figure 4.8**).

The complete restriction map was generated for the DNA fragment of *P. polymyxa* PKB1 and 97-4 based on the known nucleotide sequences, respectively, by using DNA Strider™ 1.2 computer software. These two DNA fragments differed by one restriction endonuclease site (TthIII II). Because this enzyme is not commercially available at the present time, the RFLP procedure could not be conducted. However, this enzyme should prove to be a useful marker for differentiating *P. polymyxa* PKB1 from other *P. polymyxa* strains based on the RFLP pattern, once it becomes commercially available.

Detection of bacteria in compost inoculated with *P. polymyxa* PKB1

Bacterial isolates from composts with and without inoculation of *P. polymyxa* PKB1 showed inhibitory effect against the fungal pathogens *L. maculans, Rhizoctonia solani* and *Sclerotinia sclerotiorum* of canola (**Figure 4.9**). Isolates showing an inhibitory effect were selected and purified. DNA was extracted and amplified with OPA-07 in a PCR reaction. Two isolates (CB-F and CB-C in **Figure 4.10**) from inoculated compost had the same DNA amplification pattern as *P. polymyxa* PKB1 (96-1 and 97-1). Isolates C1 – C4 were from uninoculated compost showing an inhibitory effect against fungal pathogens. These four isolates had different DNA patterns from that of *P. polymyxa* and were identified as *B. licheniformis* (C1 – C3) and *B. subtilis* (C4) by Microbiology Lab, ARC, Vegreville. Two isolates from uninoculated compost

(C-C and C-O) showing no biocontrol effect had completely different DNA pattern compared with those of other *Bacillus* isolates.

Discussion

Biocontrol agents must be genetically stable and compatible after introduction into the environment and should be able to survive and maintain themselves for adequate biocontrol activity. Careful evaluation of biocontrol agents and their environmental impacts are essential. This research was aimed towards developing rapid and accurate molecular tools to detect *P. polymyxa* PKB1 in the biological control field, especially to distinguish this strain introduced as an inoculant from other field isolates. Amplified DNA profiles were highly reproducible and suitable for tracking the individual strains that were released in the environment (Migheli and Cavaliarin, 1994). Other tracking techniques have been developed for antagonistic *Fusarium oxysporum,* such as the use of polyclonal antibodies, β-d-glucuronidase (GUS)-marked strains (Eparvier and Alabouvette, 1994), RFLP analysis, and PCR-based fingerprinting (Edel et al., 1995). In this study, the DNA patterns of *P. polymyxa* PKB1 were distinguishable from those of the other strains of *P. polymyxa* that had no antifungal activity against *L. maculans* and strains of other *Bacillus* species. The RAPD-PCR technique can thus allow unequivocal recognition of specific strains.

DNA probes made from RAPD fragments were first used for the construction of a genetic map in *Glycine max* (Williams et al., 1990). Later, Dig-labelled probes were used on some pathogenic fungi for diagnostic purposes (Koopmann et al., 1994; Schafer and Wostemeyer, 1994; Sharma et al., 1996; Sharma and Tewari, 1998). In the current study, four DNA fragments were selected and Dig-labelled as probes. All probes could hybridize with the digests of all 13 strains of *P. polymyxa* but not with those from other *Bacillus* spp. These probes were, therefore, species-specific and could be used to detect strains of *P. polymyxa.* The main advantages of these probes are that they are non-radioactive and safe to use, and can be used in both *in vitro* and *in situ* hybridization without any additional facilities required for maintaining radioactive materials. Once the specific probe is developed, the dot-blot method is very simple and easy to use in the detection of this bacterium. This method makes the detection process simple and fast.

P1-7 and P1-8 hybridized with the Hind III and Eco RI digests and polymorphic bands were obtained among the isolates. It seemed that strains 96-17, 97-2 and 97-3 had similar patterns (**Figure 4.3 A** and **4.3E**).

Strains 96-9 and 97-4 had similar patterns and belonged to the same group (**Figure 4.3D** and **4.3E**), and all Vegreville strains showed the same patterns except that 96-2 did not have complete digestion with some enzymes. The results showed that the intra-specific variation could be detected among strains within *P. polymyxa* by the RFLP technique. However, the RFLP patterns obtained from the genomic DNA did not give the same groupings with different restriction enzymes. The RAPD markers could distinguish genetic variations that could not be detected by the RFLP analysis. Unknown bacterial strains isolated from compost and canola stubble could not be detected by any of the probes. It is possible that the applied bacterium might not have survived one year after application under field conditions.

In the present study, the designed primers from P1-8 were partially strain-specific for *P. polymyxa* PKB1 and related strains. All strains from Alberta could be amplified by these primers and showed one DNA band, whereas the other three strains from United States (97-3 and 96-17) and Japan (97-2) could not be amplified. A bioassay with these bacterial strains had shown that the strain 97-2 had less inhibitory effect on *L. maculans* compared with that of *P. polymyxa* PKB1, and strains 97-3 and 96-17 had no inhibitory effect on the growth of *L. maculans* (Chapter 2). Thus, the designed primers could detect strains with strong antifungal activity against *L. maculans* within the species *P. polymyxa*. Further experiments will be needed to verify the value of these specific primers by using additional strains of other *Bacillus* spp. and to detect the *P. polymyxa* PKB1 applied to the environment. The DNA sequence of the PCR product generated by the designed primers was highly homologous to the spoOK locus controlling a membrane-associated protein, which is required to initiate sporulation in *B. subtilis* (Perego et al., 1991; Rudner et al., 1991).

Antifungal compounds are increasingly believed to be the dominant factors for biocontrol activities in many systems (Hill et al., 1997). Many studies have demonstrated that restrictive growth conditions, nutrient limitations or entry into the stationary growth phase typically induce the production of antibiotics and secondary metabolites. Studies demonstrated that a membrane-associated antifungal protein was produced by *P. polymyxa* PKB1 and that its production was associated with sporulation in this bacterium (Beatty, 2000). The information obtained here is valuable for further detecting the gene involved in the anti-fungal substance production. It is possible that these DNA fragments (P1-8 and PCR product from designed primers) contain some genetic information relating to the anti-fungal substance production and

may provide some information for further studies on gene regulation and identification. Gene regulation of antibiotic production has been studied in great detail in *Streptomyces* sp. and *Pseudomonas* spp. A two-component regulatory system was described, which consists of a membrane-bound sensor kinase and a transcription activator (Ishizuka et al., 1992). Further studies are required to understand the genetic regulation of sporulation and antibiotic production in *P. polymyxa*.

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Table 4.1. Strains of *Paenibacillus polymyxa* and *Bacillus* species used in this study*

No.	Code	Species	Source
1	96-1	*Paenibacillus polymyxa* (PKB1)	Alberta Research Council (ARC), Vegreville, Dr. P. D. Kharbanda
2	96-2	*P. polymyxa*	ARC, Vegreville
3	96-3	*P. polymyxa*	ARC, Vegreville
4	96-4	*P. polymyxa*	ARC, Vegreville
5	96-5	*P. polymyxa*	ARC, Vegreville
6	96-9	*P. polymyxa*	University of Alberta, Dr. J. P. Tewari
7	96-17	*P. polymyxa* ATCC 842	ARC, Dr. R. Coleman
8	97-1	*P. polymyxa*	ARC, Vegreville
9	97-2	*P. polymyxa* subsp. *colistinus koyama* ATCC 21830	Dr. S. Jensen (University of Alberta)
10	97-3	*P. polymyxa* NCIB 8648	Dr. S. Jensen (University of Alberta)
11	97-4	*P. polymyxa*	ARC, Mr. R. Lange
12	R1	*P. polymyxa*	ARC, Vegreville, selection of PKB1, Resistant to rifampicin (100 mg/L)
13	R2	*P. polymyxa*	ARC, Vegreville, selection of PKB1, Resistant to rifampicin (200 mg/L)
14	96-8	*B. subtilis*	Dr. J. P. Tewari, University of Alberta
15	96-19	*B. subtilis* ATCC6051	ARC, Dr. R. Coleman
16	96-10	*B. megaterium*	Dr. J. P. Tewari, University of Alberta
17	96-11	*B. alvei* ATCC6344	ARC, Dr. R. Coleman
18	96-12	*B. cereus* ATCC14579	ARC, Dr. R. Coleman
19	96-13	*B. macerens* ATCC120	ARC, Dr. R. Coleman
20	96-14	*B. thuringiensis* ATCC106	ARC, Dr. R. Coleman
21	96-15	*B. thuringiensis* ATCC107	ARC, Dr. R. Coleman
22	96-16	*B. thuringiensis* ATCC10792	ARC, Dr. R. Coleman
23	96-18	*B. sphaericus* ATCC14577	ARC, Dr. R. Coleman

* Strains 1 – 6 were identified by Dr. R. Coleman in Microbiology Lab at Alberta Research Council, Vegreville, Canada.

ATCC = American Type Culture Collection, U. S. A.

NCIB = National Collection of Industrial Bacteria, Scotland

Table 4.2. Sources of RAPD bands used for probe synthesis

Probe	Bacterium Strain	Primer	Probe Size (bp)
P1-7	*Paenibacillus polymyxa* PKB1	OPA-07 (5'-GAAACGGGTG)	609
P1-8	*Paenibacillus polymyxa* PKB1	OPA-08 (5'-GTGACGTAGG)	713
P1-13	*Paenibacillus polymyxa* PKB1	OPA-13 (5'-CAGCACCCAC)	270
P1-14	*Paenibacillus polymyxa* PKB1	OPA-14 (5'-TCTGTGCTGG)	617

Table 4.3. Primers designed from nucleotide sequences of two DNA probes

Primer	Source	Sequence
J1 (sense)	P1-8	5'-AGCGTCAACGGATTGGCATAGC
J2	P1-8	5'-GTCAACGGATTGGCATAGCG
JY1 (anti-sense)	P1-8	5'-CTATTCCTGAGCCACCTGCTTATGC
JY2	P1-8	5'-GTTCATCTATTCCTGAGCCACCTGC
JY3	P1-8	5'-AAGTTCATCTATTCCTGAGCCACC
JY4	P1-8	5'-AGTTCATCTATTCCTGAGCC
J3	P1-14	5'-GGCGACATTGAGTTATTTTGCTTTTATCCC
JY5	P1-14	5'-CCATCTGTTTCCAACTGACCTACTTGTTCC

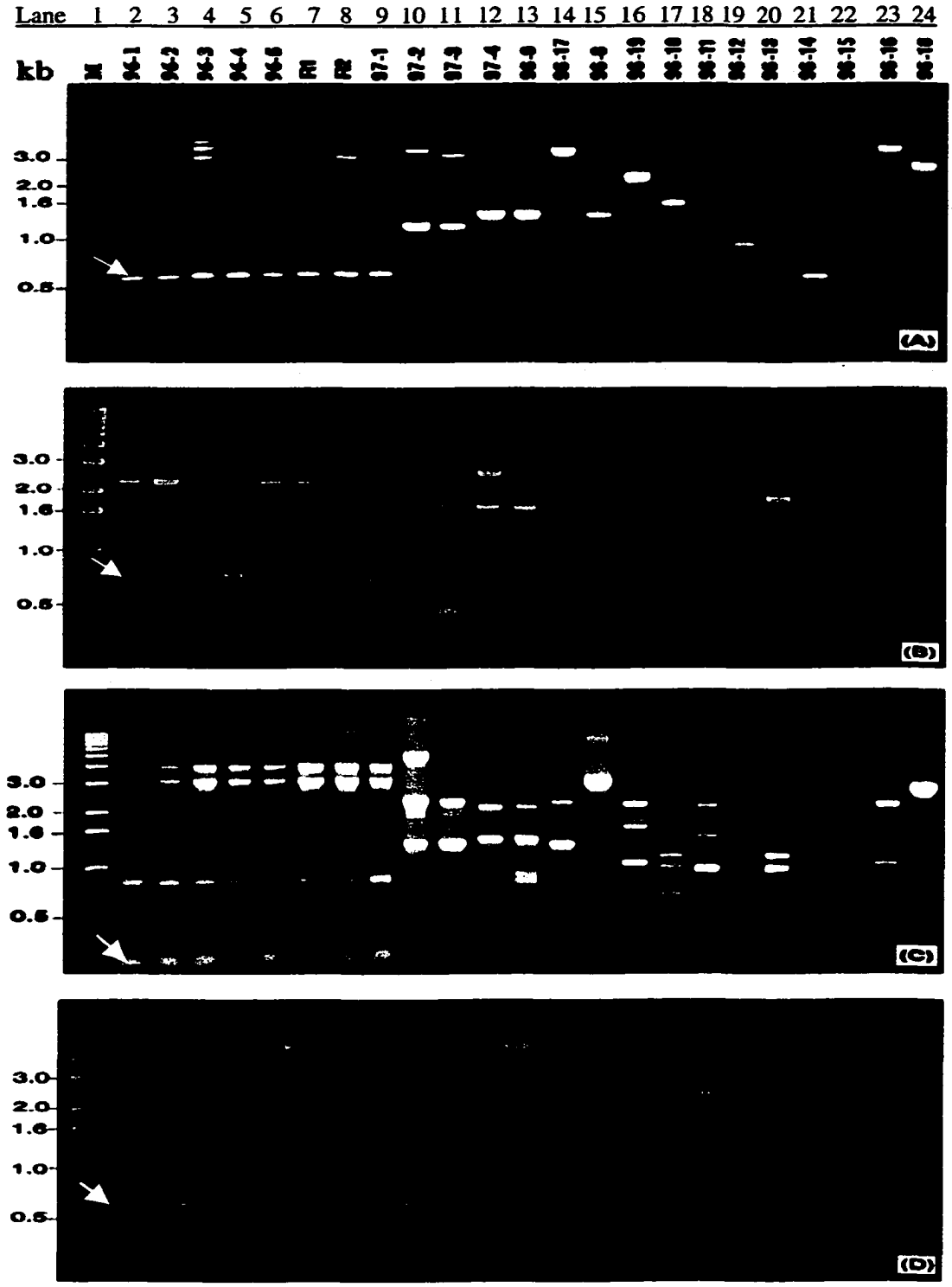

Figure 4.1. DNA amplification patterns of 13 strains of *P. polymyxa* and 10 strains from seven *Bacillus* spp. amplified with primers OPA-07 (A), OPA-08 (B), OPA-13 (C), and OPA-14 (D). Four DNA fragments (indicated by arrows) were used for making probes. M = 1 kb DNA ladder.

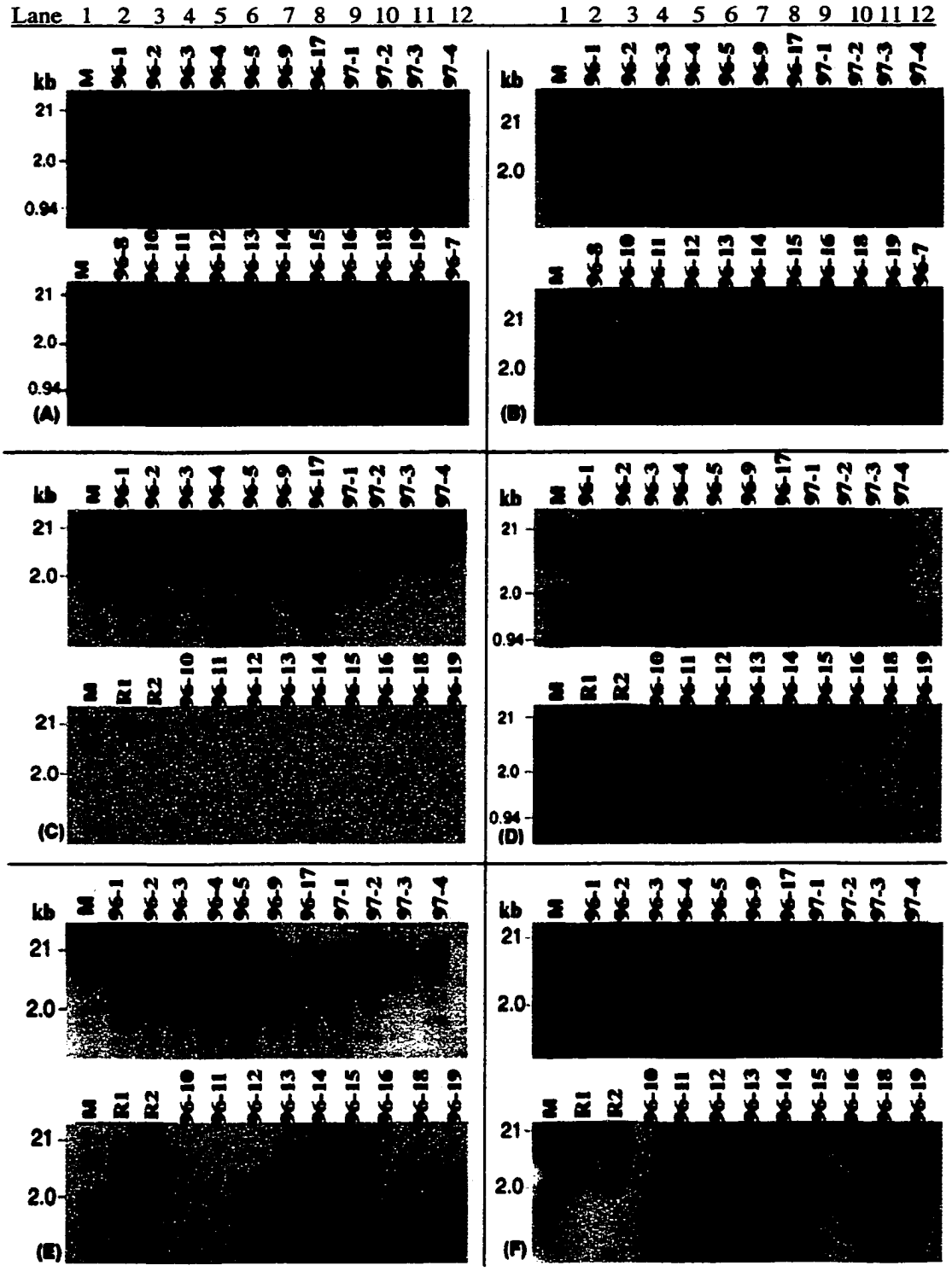

Figure 4.2. Autoradiographs of Southern blots of the DNA of 13 strains of *P. polymyxa* and 10 strains of *Bacillus* spp. digested with Hind III (A, B), Pst I (C) and Eco RI (D, E and F), and hybridized with P1-7 (A, C, and D), P1-8 (E) and P1-14 (B and F). M = 1 kb DNA marker.

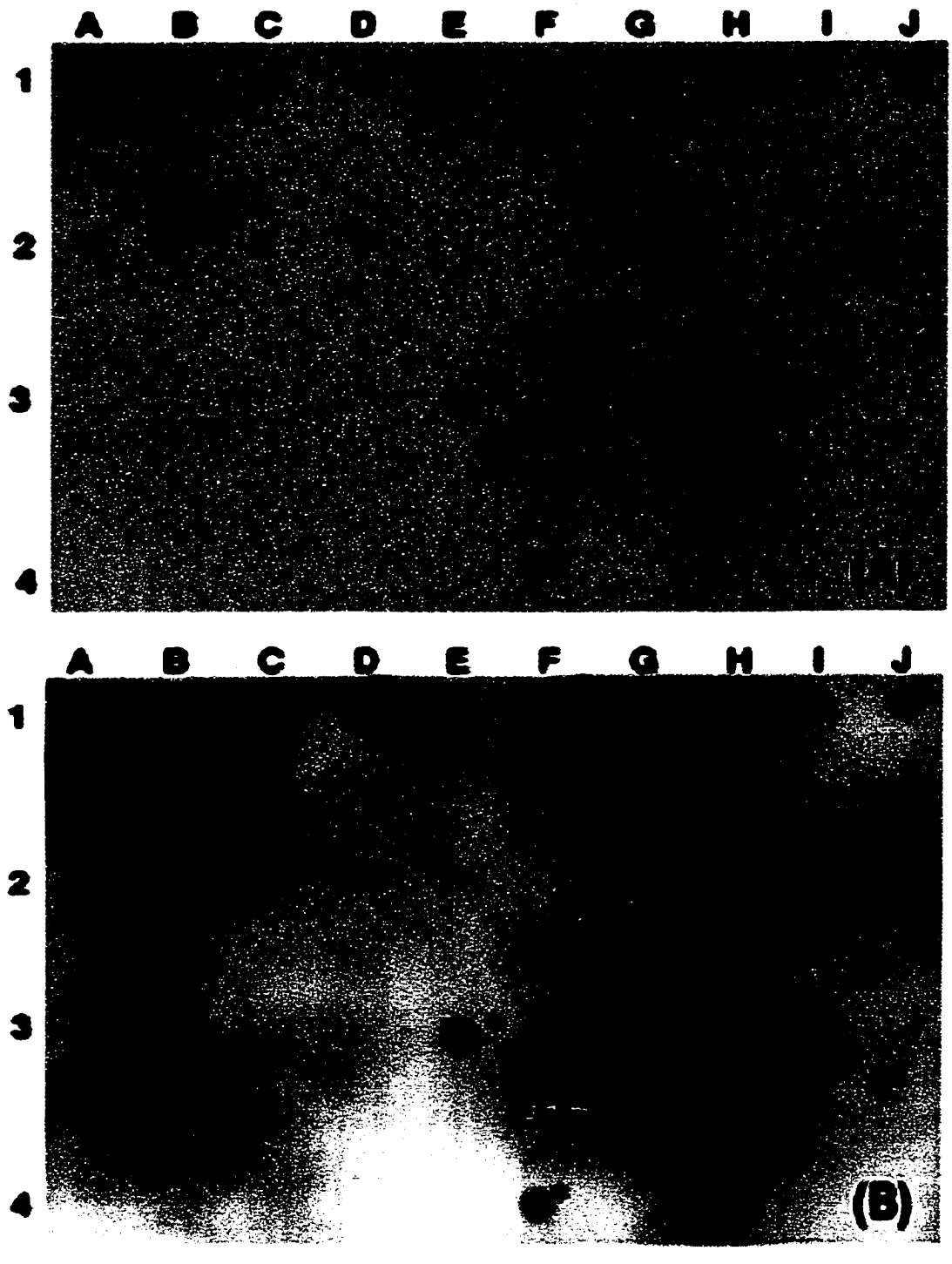

Figure 4.3. Dot-blotting of 13 strains of *P. polymyxa* and 10 strains of *Bacillus* spp. and 10 unknown bacterial strains isolated from compost probed with probes P1-7 (A) and P1-8 (B), B1 = 96-1, C1 = 96-2, D1 = 96-3, E1 = 96-4, F1 = 96-5, G1 = R1, H1 = R2, I1 = 97-1, J1 = 97-2, A2 = 97-3, B2 = 97-4, C2 = 96-9, D2 = 96-17, E2 = 96-8, F2 = 96-19, G2 = 96-10, H2 = 96-11, I2 = 96-12, J2 = 96-13, A3 = 96-14, B3 = 96-15, C3 = 96-16, D3 = 96-18, * DNA of *P. polymyxa* PKB1 used as positive checks.

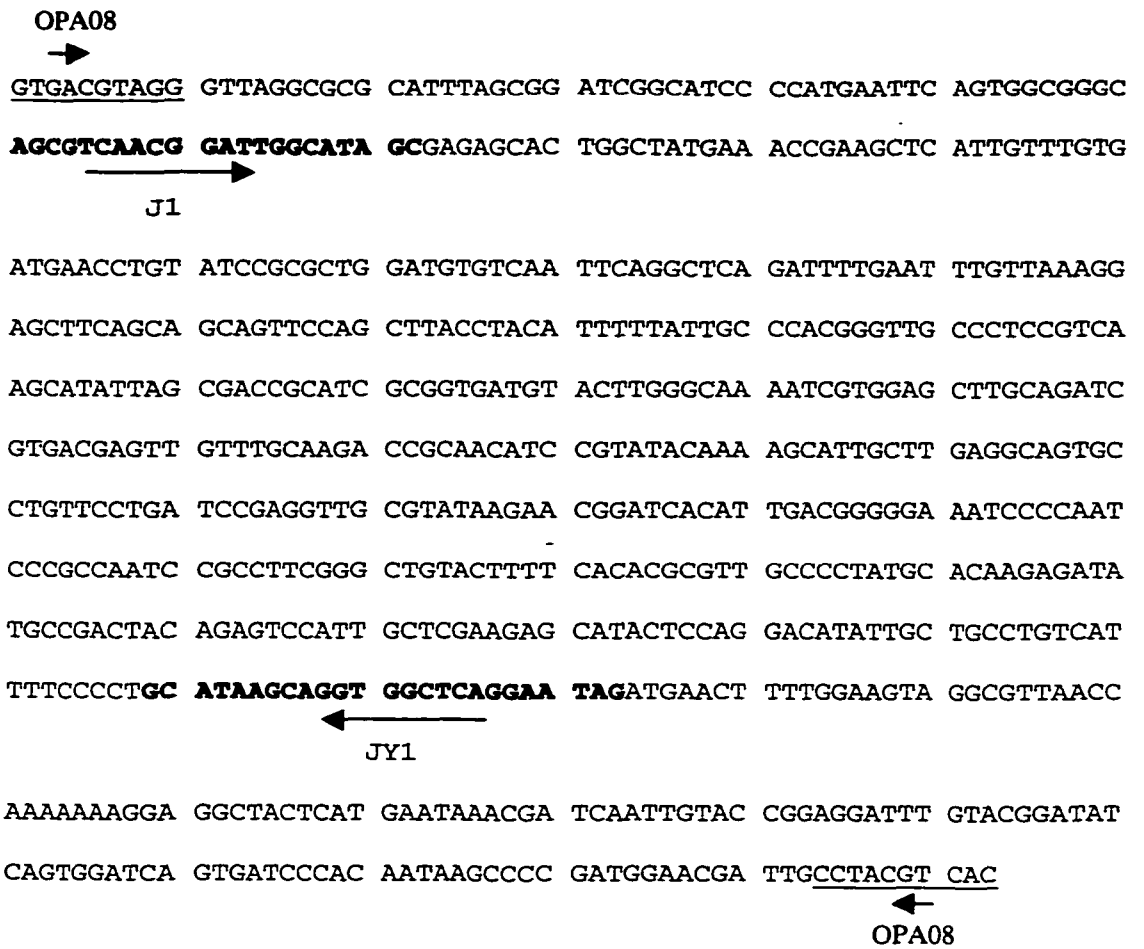

Figure 4.4. Nucleotide sequence of the probe P1-8 (713 bp).

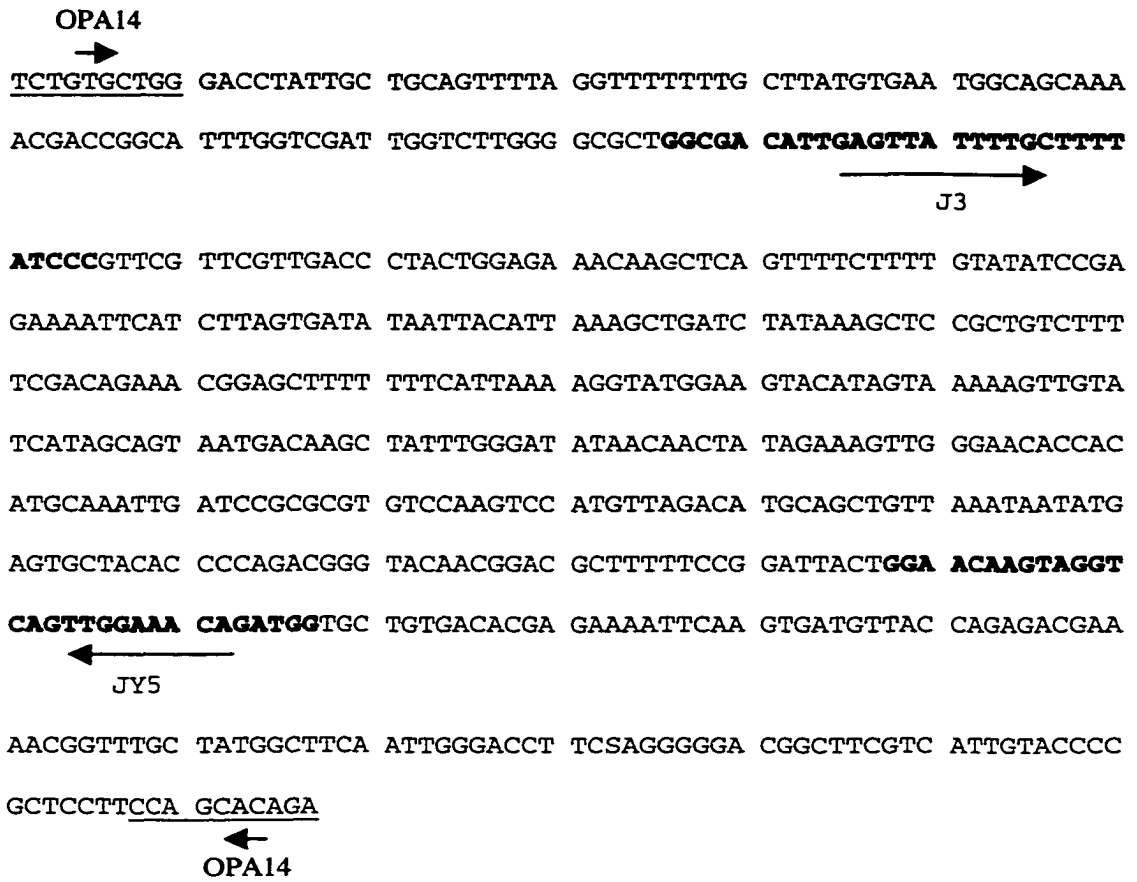

Figure 4.5. Nucleotide sequence of the probe P1-14 (617 bp).

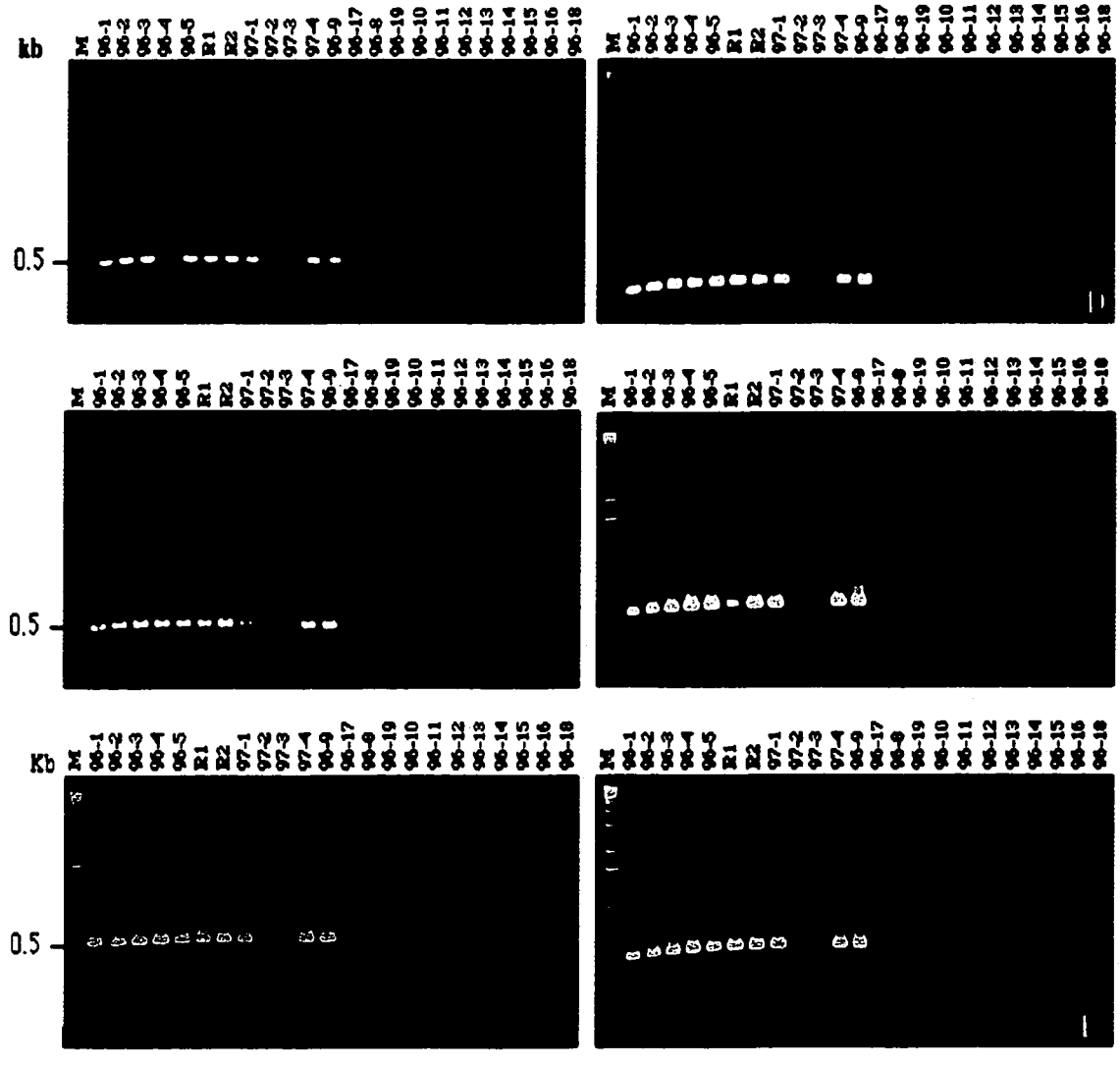

Figure 4.6. PCR products of 13 strains of *Paenibacillus polymyxa*, and 10 strains from seven *Bacillus* spp. amplified with the designed primers. M = 1 kb DNA marker.

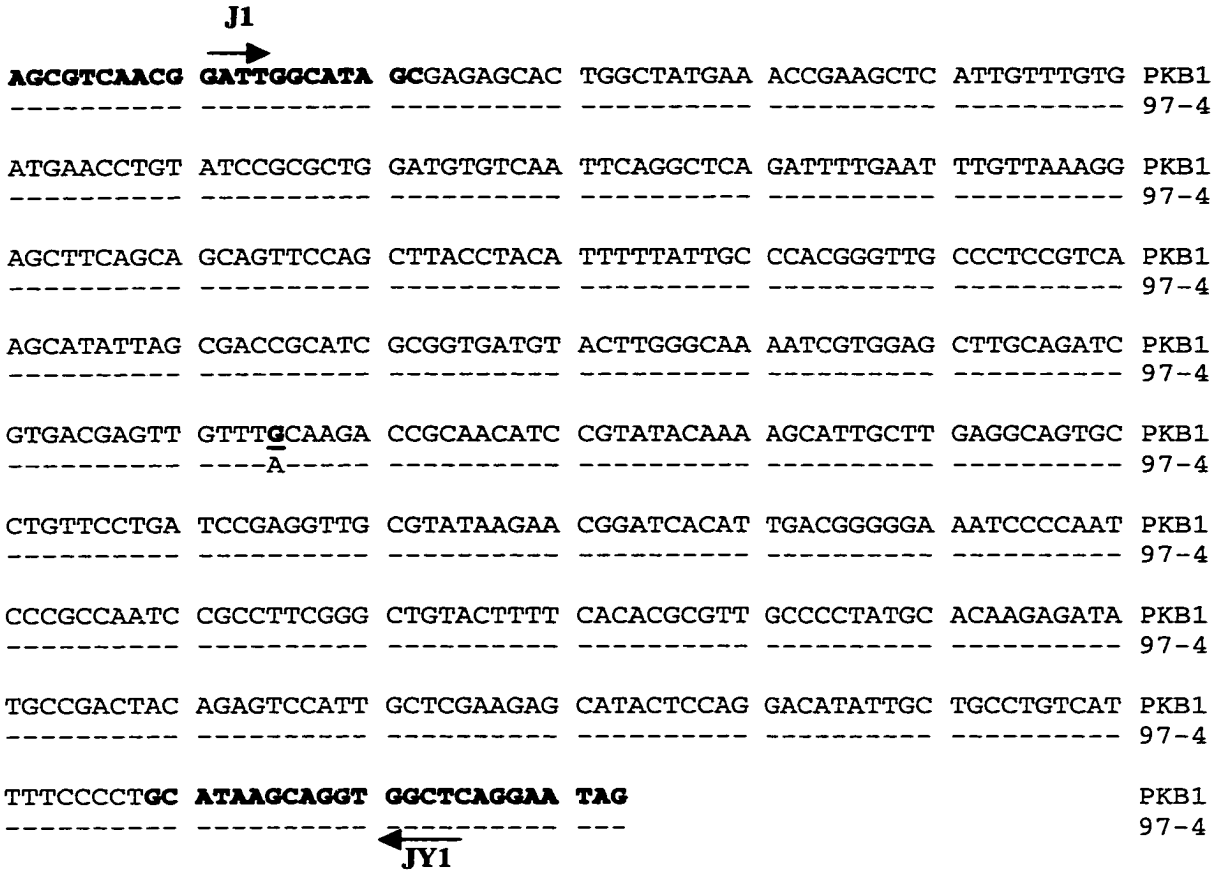

Figure 4.7. Nucleotide sequence of a DNA fragment from *Paenibacillus polymyxa* PKB1 (upper line) amplified with primers J1 and JY1 compared with DNA sequence of *P. polymyxa* 97-4 (lower line, '-' means the same base with the one in the upper line.). Two nucleotide sequences differed at one base (underlined).

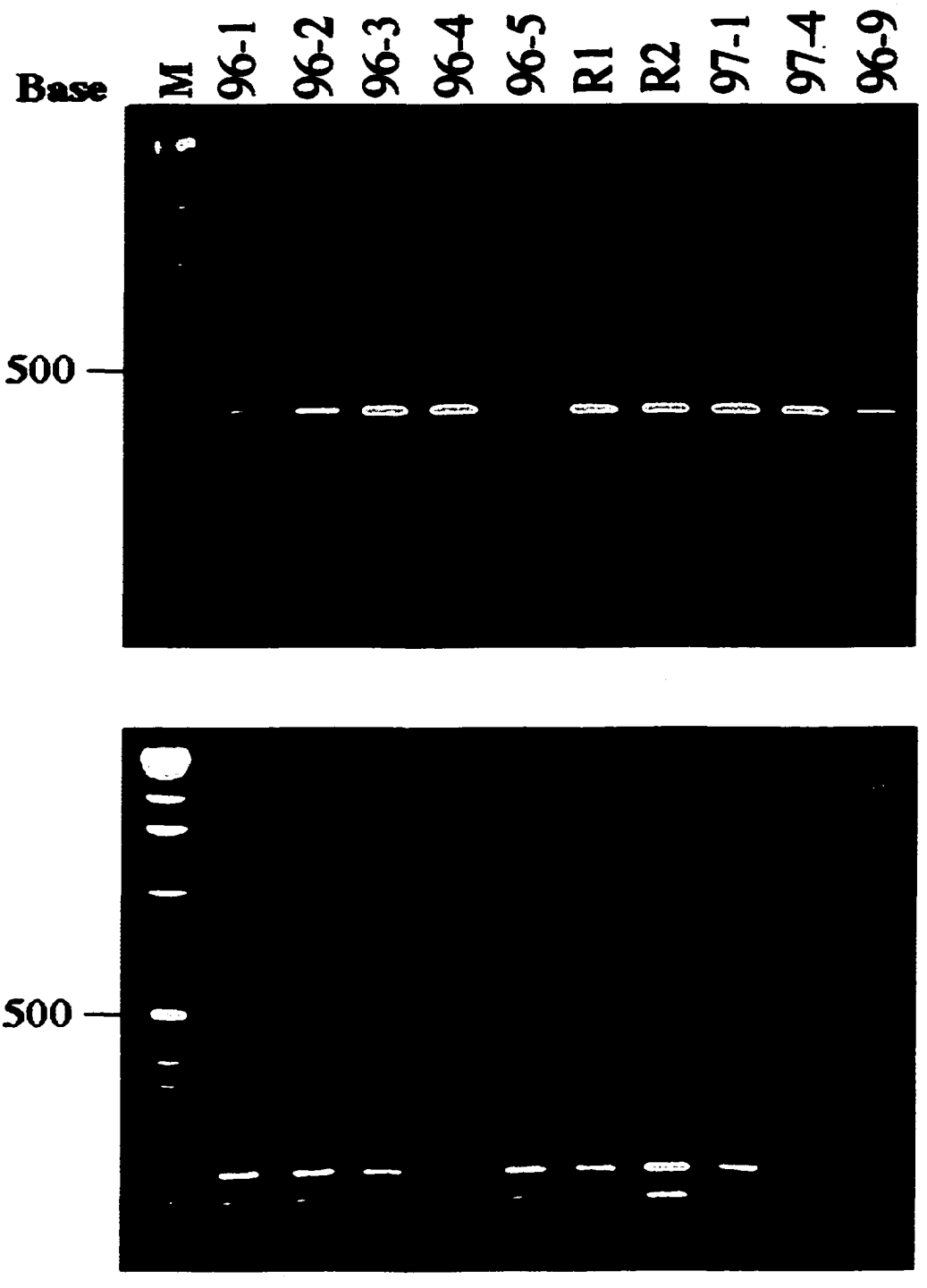

Figure 4.8. RFLP of PCR products, amplified with primers J1 and JY1, of 10 bacterial strains digested with restriction endonuclease Mse I (A) and Rsa I (B).

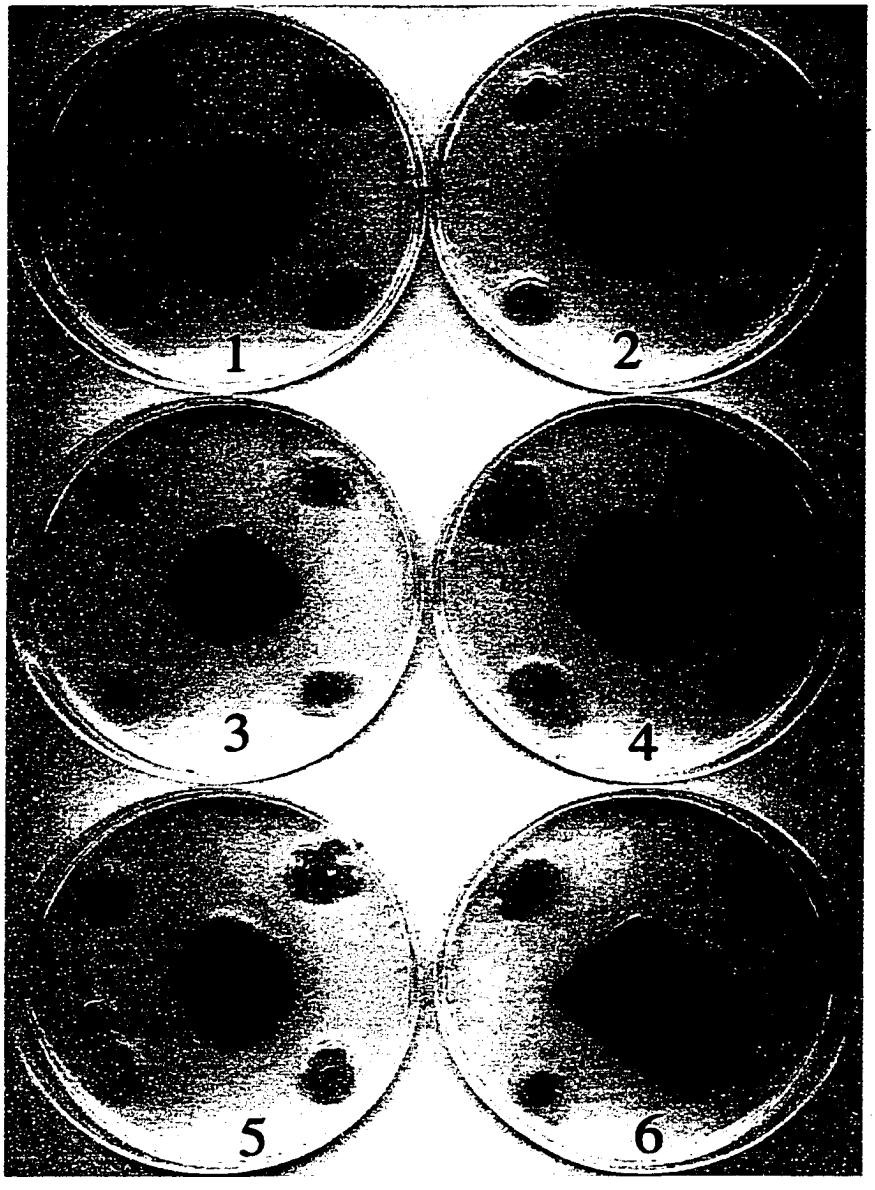

Figure 4.9. Inhibitory effect of different bacteria isolated from compost against a virulent isolate of *Leptosphaeria maculans*. 1 = C1 (*Bacillus licheniformis*); 2 = C2 (*B. licheniformis*); 3 = C3 (*B. licheniformis*); 4 = C4 (*B. subtilis*); 5 = CB-C, isolated from compost inoculated with PKB1; 6 = C-O, isolated from compost without inoculation.

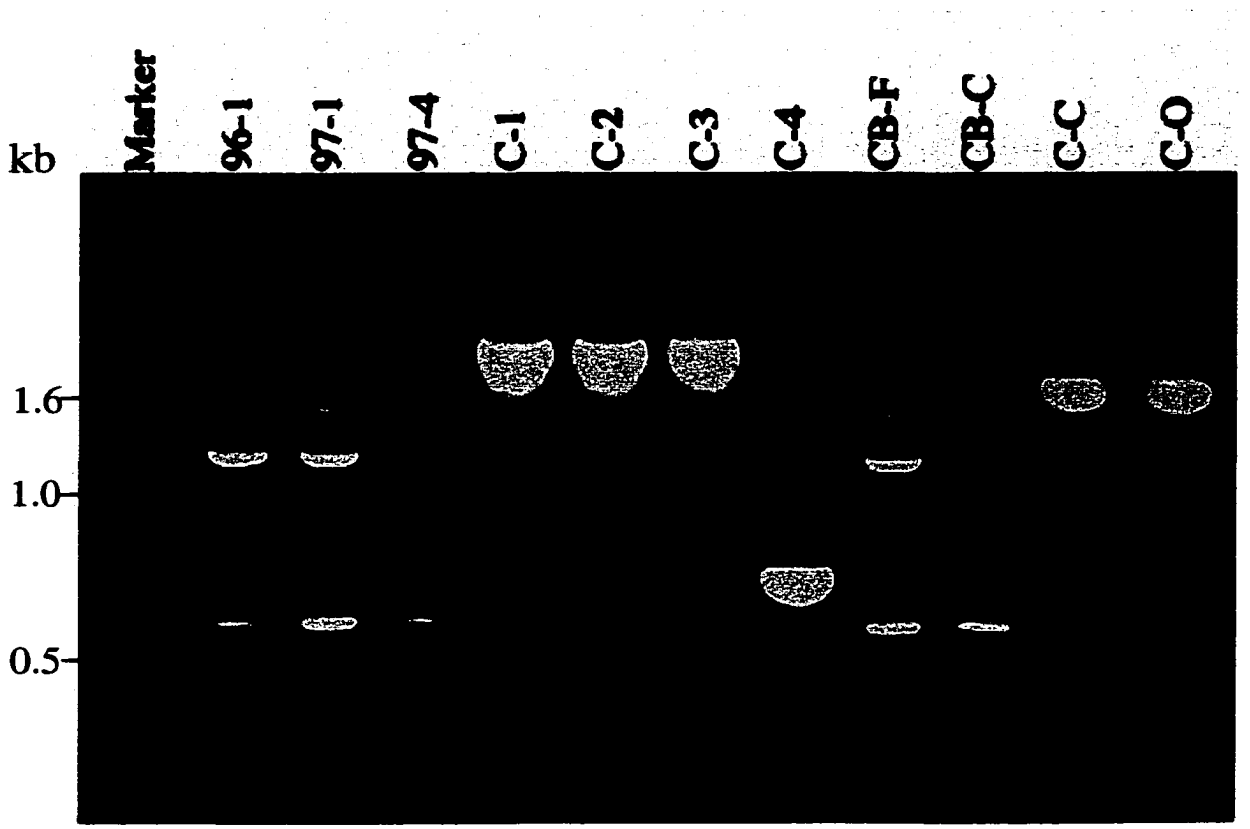

Figure 4.10. DNA amplification pattern of 11 bacterial strains in a PCR amplified with primer OPA-07. Strains 96-1, 97-1 and 97-4 are *Paenibacillus polymyxa*, C1, C2 and C3 are *Bacillus licheniformis*, C4 is *B. subtilis*, CB-F and CB-C are isolates from compost inoculated with *P. polymyxa* PKB1, and C-C and C-O are isolates from compost without inoculation. Marker = 1 kb DNA ladder.

Chapter 5

Molecular Polymorphism among Isolates of *Leptosphaeria maculans* in Western Canada

Introduction

L. maculans exhibits considerable variability in virulence and in cultural morphological characteristics. This variability was observed as early as 1918 (Henderson, 1918). The blackleg isolates are broadly divisible into two groups, virulent and weakly virulent isolates, based on their aggressiveness on *Brassica* (Bonman et al., 1981, Humpherson-Jones, 1983; Koch et al. 1989; McGee and Petrie, 1978). The pathotypes differ in their morphology and virulence. Virulent isolates are strongly pathogenic on *Brassica* spp., relatively slow-growing, and have a short germ-tube length, while weakly virulent isolates are faster-growing, have a long germ-tube length, produce a yellow to brown pigment and are nonstaling in culture (Williams, 1992). Kharbanda (1993) evaluated 350 isolates of *L. maculans* collected in Alberta and found considerable variations in their morphological characteristics, amount of aerial mycelium, production of pycnidia, color of pigmentation in the medium and overall colony appearance. The isolates were classified into 15 groups according to their cultural characteristics. Of the 67 isolates collected in Alberta, Saskatchewan and Manitoba, 67% were grouped as aggressive and the rest as non-aggressive based on a cotyledon test (Lange, 1993). The two pathotypes differed in morphology and pathogenicity (Hanacziwskyj and Drysdale, 1984; Hill et al., 1984; Johnson and Lewis, 1994; Kutcher, 1990), soluble protein and isozyme patterns (Balesdent et al., 1992; Gall et al., 1995; Sippel et al., 1988), polygalacturonase zymograms (Annis and Goodwin, 1997), production of sirodesmin (Koch et al., 1989), glucose phosphate isomerase (Brun et al., 1997; Sippel et al., 1995), cell wall degradation enzymes (Annis and Goodwin, 1996), extracellular enzymes, and fungal surface proteins (Hassan et al., 1991). Immunochemical techniques have also used to differentiate virulent and weakly virulent isolates (Dahiya, 1988; Stace-Smith et al., 1993).

DNA analysis showed considerable genetic variation between the virulent and weakly virulent isolates. DNA polymorphism in *L. maculans* was first described in repetitive DNA sequences (Jonhson and Lewis, 1990). Since then, extensive investigations have been conducted to distinguish these two groups of isolates

by using the analysis of restriction fragment length polymorphisms (RFLP) (Koch et al., 1991; Patterson and Kapoor, 1995), karyotype analysis (Morales et al., 1993b; Plummer et al., 1993; Taylor et al., 1991), random amplified polymorphic DNA-polymerase chain reactions (RAPD-PCR) (Goodwin and Annis, 1991; Hassan et al., 1991; Keri and Rimmer, 1997; Meyer et al., 1992; Plummer et al., 1994; Schafer and Wostemeyer, 1992, 1994), amplified fragment length polymorphism (AFLP) analysis (Pongam et al., 1999), and ribosomal DNA sequences (Balesdent et al., 1998; Morales et al., 1993a, 1995; Xue et al., 1992). Due to the large differences between these two groups of isolates, it has been proposed that they may belong to different species. It has been found that the weakly virulent isolates can also produce a yellow pigment. The secondary metabolite profile and the molecular genetic characteristics studies indicated that the weakly virulent isolate is closely related to *Phoma wasabiae* (Pedras et al., 1995).

Within the virulent group, isolates could be further divided into three pathogenicity groups according to the differential interaction phenotypes expressed on cotyledons, hypocotyls and stems of *Brassica* species (Koch et al., 1991; McGee and Petrie, 1978; Newman, 1984). Koch et al. (1991) have identified a set of four differential *B. napus* cultivars that classify *L. maculans* isolate into four pathogenicity groups (PG1 – PG4). Weakly virulent strains fall into PG1, based on the cotyledon reaction of the susceptible cultivar Westar. Virulent strains are divided into three groups (PG2 – PG4) on the basis of their reaction on the cultivars Westar, Glacier and Quinta. By using three additional *Brassica napus* lines, isolates of *L. maculans* from Europe and Australia were divided into 5 pathogenicity groups based on host-pathogen interaction (Keri and Rimmer, 1997). Based on an extended set of *Brassica* differentials, the virulent isolates were differentiated into six pathogenicity groups (Kuswinnanti et al., 1999).

As more molecular tools become available, more genetic variations among *L. maculans* isolates are being detected worldwide. However, DNA analysis also gave variable results. No relationship between RAPD pattern and PGs was found in 93 highly virulent isolates of *L. maculans* (Mahuku et al., 1997), whereas a consistent match of RAPD pattern and PGs was observed in another study (Keri and Rimmer, 1997). A total of 49 virulent isolates representing three pathogenicity groups (PG2, 3 and 4) from different geographical areas have been tested for the variation by using AFLP analysis (Pongam et al., 1999). It was found that isolates from western Canada, North Dakota and Georgia belonged to one group that is relatively less variable. Isolates from Ontario, Australia, Germany, France and most of UK formed another group

that exhibited greater variation. In another report, several isolates collected in Georgia were identified as pathogenicity groups of 3 and 4 (Philips et al., 1999). Research showed that the genetic variation was related to the geographic areas. Williams and Fitt (1999) indicated that characterization of isolate type is important in seed testing and crop breeding programs.

This paper will report the results of a study on genetic variation among the isolates of *L. maculans* collected in the last 15 years from western Canada, mainly Alberta, by using the RAPD-PCR technique. The same technique was also used to detect if there was any genetic change of subcultures of sectors from a virulent isolate of *L. maculans*. The objectives of this study were to group *L. maculans* isolates on a molecular basis and to determine the inhibitory effect of *Paenibacillus polymyxa* PKB1 on different groups of *L. maculans*.

Materials and Methods

Isolates collection

A total of 162 isolates of *L. maculans* were tested in this study (**Table 5.1**). More than 130 isolates of *L. maculans* were collected from Alberta, Saskatchewan and Manitoba during 1983 - 1994 at the Alberta Research Council and the University of Alberta, and 17 isolates were collected in Alberta in 1998, including two isolates from canola (cv. Quantum, *B. napus*) plants. Three representative isolates of PG2, PG3, and PG4 were provided by Dr. P. H. Williams in the U. S. A., and six isolates from the United Kingdom and one from Germany were also included in this study. All isolates were purified as single-spore cultures and stored in liquid nitrogen as pycnidiospores in 20% (w/v) glycerol solution.

Extraction of genomic DNA

The stored isolates from liquid nitrogen were thawed, and 0.5 ml of the spore suspension of each isolate was spread on V8-Rose Bengal agar [20% (v/v) V8 juice, 0.04% (v/v) Rose Bengal] plates. The plates were incubated under 12 h cool white fluorescent light (150 $\mu E\ s^{-1}\ m^{-2}$) at room temperature for seven days. Genomic DNA was extracted according to the procedure described by Raeder and Broda (1985) with some modifications. The mycelium, pycnidia and pycnidiospores were scraped from the

culture on agar medium and ground with a pre-cooled pestle and mortar to a fine powder in liquid nitrogen. The genomic DNA was extracted with extraction buffer (1:5 w/v ratio) and phenol-chloroform. Crude DNA preparations were digested with RNAse A (Sigma) at 20 mg/ml at 37 °C for 1 h followed by chloroform extraction. The DNA was precipitated with 1 volume isopropanol, washed with 70% ethanol, dried, and dissolved in 100 µl TE buffer (10 mM Tris HCl, pH 8.0, and 0.1 mM EDTA). The amount of DNA was measured with a Gene Quant., RNA/DNA Calculator (Phamacia LKB Biochrom LTD., Cambridge, England). The quality of the DNA was viewed by electrophoresis on 1% (w/v) agarose gel and stained with ethidium bromide.

Detection of DNA polymorphism of *L. maculans* isolates

A total of 142 random primers were used for PCR reactions with three isolates in an initial test. Forty primers were from Operon Technologies Inc., Alameda, CA, U.S.A., 100 from the Nucleic Acid-Protein Service Unit, Biotechnology Laboratory, University of British Columbia, and two were synthesized according to the sequence of P-188 and P-SW1 (Mahuku et al., 1997). Fourteen primers (**Table 5.2**), giving good DNA amplification patterns were selected for further studies.

PCR reactions were carried out in a solution of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT (dithiothreitol), 200 M each of dNTPs, 0.5 M primer, 1.25 U Taq DNA polymerase and 20 ng genomic DNA in 25 µl aliquots by using a thermal cycler (Thermolyne, Temp. Tronic, Barnstead/ Thermolyne Corporation, Dubuque, IA, U.S.A.). The thermal cycling profile consisted of 40 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min. In addition, all profiles were preceded by an initial denaturation at 94°C for 5 min, followed by a final extension at 72 °C for 5 min. Electrophoresis of amplified products were carried out in 1% (w/v) agarose gels, stained with ethidium bromide and photographed under UV light. One kb DNA ladder (Gibco-BRL) was used as a DNA size marker.

Data analysis

The DNA bands obtained for each individual were scored based on their presence (1) or absence (0). Pairwise genetic distances were expressed as the compliment of Nei and Li's *F* statistic. Cluster analysis was done by using POPGENE software (© Dr. F. Ye, University of Alberta).

Inhibitory effect of *P. polymyxa* PKB1 on *L. maculans* isolates from different groups and culture variants of a virulent isolate

Ten isolates of *L. maculans* from different groups, including one cultural variant isolated from a sector of a virulent isolate, based on the cluster analysis, were tested in a bioassay. The bacterium ***P. polymyxa*** PKB1 was grown on nutrient agar (NA, Difco). Strains of *L. maculans* were cultured on V-8 juice (20%)-Rose Bengal (0.04%) agar for 7 days. One 5 mm-diameter plug containing the *L. maculans* mycelium was transferred to the centre of a potato dextrose agar (PDA, Difco) plate, and a loop of bacterial cells was placed at the four places around the agar plug. The plates were incubated under fluorescent light at 25°C for two weeks. Inhibition zones in the plate of each bacterial strain were observed, and the mycelial growth was measured and presented as the radius of the colony.

Detection of DNA polymorphism of 20 subcultures from a cultural variant of a virulent *L. maculans* isolate

Sectors were often formed in cultures of virulent *L. maculans* isolate (**Figure 5.1**). Those variants were morphologically different from the original isolate. A pathogenicity test on cotyledon of canola plants showed that the sector had lost its virulence on the canola plant (Bansal, unpublished data). Twenty single subcultures from a variant of a virulent isolate (3356) of *L. maculans* were purified by single hyphal tip isolation technique and used in a RAPD-PCR test to investigate if any genetic variation was involved in the sectors.

A total of 142 random primers (Operon Technologies Inc., Alameda, CA. U.S.A., and Biotechnology Laboratory, U.B.C., Canada) were used to detect the genetic variation within 20 isolates from a cultural variant. Original isolate 3356 and a weakly virulent isolate (Unity) were also included for comparisons. The DNA extraction and PCR program were the same as those described above.

Results

Detection of DNA polymorphism of *L. maculans* isolates

Forty 10-mer primers of arbitrary nucleotide sequences were tested for PCR amplification of the total DNA of three isolates of *L. maculans*. Fourteen primers (OPA-07, OPA-10, OPA-13, OPX-01, OPX-02, OPX-07, OPX-08, OPX-10, OPX-11, OPX-12, OPX-13, OPX-14) were selected and used for testing all the 160 isolates in the study. Successful amplification of DNA from isolates of *L. maculans* was obtained, and polymorphic bands were observed with all the primers selected. The virulent isolates of *L. maculans* were distinguishable from the weakly virulent isolates, but the variation in DNA amplification patterns was less within virulent isolates (**Figures 5.2, 5.3,** and **5.4**). Most primers used could not distinguish different PG2 (isolate No.69 in **Table 5.1**) and PG3 (isolate No.70 in **Table 5.1**) pathotypes within virulent isolates, but a few primers, such as OPA-13, could detect the differences between PG2 and PG4 (isolate No.71 in **Table 5.1**) pathotypes (**Figures 5.3**).

Analysis of the total data set of 162 isolates obtained from seven primers was performed with POPGENE software. A dendrogram was obtained by using the UPGMA method based on Nei's genetic distance (**Figure 5.5**). There were two distinguishable groups, virulent and weakly virulent isolates. The virulent isolates were grouped into four major groups based on the seven primers used. Most isolates were in the same group with the PG2 standard culture (isolate No.69 in **Table 5.1**). All isolates collected in 1998 and the UK isolates were in the same group. The third group included a standard PG4 isolate (isolate No.71 in **Table 5.1**) and four other isolates from Alberta. The fourth group was a small one and included an isolate from Germany and two other Alberta isolates. All weakly virulent isolates showed wide variations and could be grouped into several groups.

Isolates collected in 1998 had similar DNA amplification patterns to those collected from the United Kingdom. There were few DNA fragments absent in isolates collected in 1998 compared with those collected 10 years ago although their PCR profiles were quite similar.

Inhibitory effect of *P. polymyxa* PKB1 on *L. maculans* isolates from different groups

P. polymyxa PKB1 showed an inhibitory effect against all isolates from different groups of *L. maculans*. *P. polymyxa* PKB1 could inhibit mycelial growth of the virulent isolates and also the weakly virulent isolates (**Figure 5.6**).

Detection of DNA polymorphism of 20 subcultures from a cultural variant of a *L. maculans* isolate

One hundred and forty-two primers were used to detect the genetic variation within 20 isolates from a cultural variant of a virulent isolate of *L. maculans*. The PCR profile of four isolates amplified with five primers is shown in **Figure 5.7**. RAPD-PCR profiles were similar among the 20 isolates. All isolates from the variant had the same DNA amplification patterns as those of the parent isolate (3356) tested by 142 primers. **Figure 5.8** shows the DNA amplification pattern of 22 *L. maculans* isolates with primer OPX-11.

Discussion

Genetic variation between virulent and weakly virulent isolates of *L. maculans* was detected in this study. This finding was in agreement with other investigations that showed these two groups of isolates were distinguishable in morphology, pathogenicity and genetics (Goodwin and Annis, 1991; Hassan et al., 1991; Johnson and Lewis, 1994; Keri and Rimmer, 1997; Kutcher, 1990; Plummer et al., 1994; Pongam et al., 1999; Schafer and Wostemeyer, 1992, 1994). A partial relationship between pathogenic phenotype and RFLP data has been demonstrated by Koch et al. (1991). Results of this study confirmed that western Canadian isolates were divisible into two main groups, virulent PG2 and weakly virulent PG1, based on their reaction on *B. napus* cv. Westar cotyledons and DNA amplification patterns.

RAPD-PCR is a good method to detect the genetic relatedness among isolates. Virulent and weakly virulent isolates were distinguished based on the DNA amplification patterns and gave the same results as those based on their morphology and pathogenicity reported by Kharbanda (1993) and Lange (1993). Identification of pathotypes and variants within isolates of *L. maculans* would be very useful in population studies and disease management. The RAPD technique could provide a fast and reliable assay to fulfil this purpose.

The relatively high similarity among the virulent isolates collected from different provinces in western Canada in this study indicated that the isolates have not geographically evolved. They might have a common ancestor or may have spread quickly long-distance by infected seeds since being introduced into western Canada. This finding was in agreement with those in other reports (Goodwin and Annis, 1991;

Kharbanda, 1993; Lange, 1993). Geographic origin did not appear to influence clustering of the isolates, suggesting a high degree of phenotypic uniformity among *L. maculans* isolates in western Canada. Results of this study further confirm the suggestion by other researchers that western Canadian *L. maculans* isolates conformed to a two-strain model of population structure, based on pathogenicity profiles and morphological characteristics.

It is important to monitor, from one year and geographic area to the next, the genetic variation in the population of *L. maculans*, especially in the virulent isolates. Genetic variation has significant effects on breeding for disease resistance and on disease control. The observed lack of diversity in PCR profiles reflects a corresponding lack of diversity in genotypes among virulent isolates of *L. maculans* in western Canada. This uniformity implies that screening for resistance of *B. napus* germplasm against a single isolate should produce breeding lines resistant to blackleg across the western provinces. The simple pathotype structure of the virulent *L. maculans* population simplifies introgression of resistance, since resistance against multiple physiological races of the pathogen need not be incorporated. In recent years, the resistant cultivar Quantum (*B. napus*, Stringam et al., 1995) has been widely grown in Alberta. Two *L. maculans* isolates were isolated from Quantum plants in 1998 and tested in this RAPD-PCR study. No major genetic difference between these two isolates and the other virulent isolates were observed. This finding suggests that the wide-spread use of this resistant cultivar has so far not led to the production of new virulent race(s) in Alberta. However, the blackleg-resistant *B. napus* cultivars currently registered in western Canada were probably not screened against multi-pathotypes such as PG3 or PG4 during the selection process. Disease resistance could be lost through a new virulent race resulting from genetic change at a single virulent locus. This genetic change may be induced by a high degree of host homogeneity. Continuous monitoring the genetic variation within *L. maculans* population could provide useful information for the disease control. Knowledge of the virulence characteristics of this fungus could lead to the development of better disease management strategies.

In this study, most virulent isolates were grouped into PG2. This grouping was in agreement with the results of the greenhouse test with the cotyledon-inoculation technique (Kharbanda, 1993; Lange, 1993). Studies have shown that virulent isolates of *L. maculans* from western Canada belong to PG2 only (Kutcher et al., 1993; Mengistu et al., 1991; Rimmer and van den Berg, 1992) and isolates from Ontario have been

placed in PG4 (Mahuku et al., 1997). Based on the primers used in this study, differences between PG2 (isolate 69) and PG3 (isolate 70), as reported by Keri and Rimmer (1997), could not be distinguished. Williams (1992) suggested that methodology was a limiting factor for conclusion of virulence in the pathogenicity test. The purity of the differential host, optimum temperature and amount inoculum are essential and may affect the result of the tests. Kharbanda (1993) tested 350 isolates including representative isolates from the University of Wisconsin and found that the representative isolates did not give the reaction as reported. It was suggested that the cultivars used in Kharbanda's study did not prove to be truly differential hosts. In the present molecular study, the use of additional primers might have shown more genetic variations among the virulent isolates.

Isolates collected in western Canada in 1998 have similar DNA amplification patterns to those from the United Kingdom. The German isolate and two Canadian isolates form a small group within the virulent isolates. A total of 49 virulent isolates representing three pathogenicity groups (PG2, 3 and 4) from different geographical areas have been tested for the variation by using AFLP analysis (Pongam et al., 1999). It has been found that isolates from western Canada, North Dakota and Georgia belong to one group which is relatively less variable. Isolates from Ontario, Australia, Germany, France and most of UK form another group, which exhibits greater variation. In other report (Philips et al., 1999), several isolates collected in Georgia were identified as pathogenicity groups of 3 and 4. Populations of *L. maculans* from Poland consist of non-aggressive species while those from Western Europe, Canada, and Australia consist of aggressive species causing blackleg of rapeseed (Jedryczka et al., 1999).

Subcultures from a sector of a virulent isolate did not show any genetic variation compared to the parent isolate based on the primers used in the test although the sector had changed virulence. The sector had lost the virulence to *B. napus*, and the second sector formed from the first sector gained the virulence back (Bansal, unpublished data). The mechanisms involved in this phenomenon are not known. Change of morphology in culture and loss of virulence in the virulent isolate were also observed (Lange, 1993). It was possible that the genetic change was very subtle, and that the primers used in this study could not detect the change. An unusual repetitive element (LMR1) from highly virulent isolates of *Leptosphaeria maculans* was identified, cloned and sequenced (Taylor and Borgmann, 1994). This element could be transferred to weakly virulent isolate naturally (rare case) and cause the isolate to become a virulent type.

It was suggested that some gene or genes encoding pathogenicity factors of the virulent strain were coincidentally attached to the transposable element that was transferred. It was unknown if this transposable element hypothesis applies to the virulence changes in the sectors of virulent isolates. Further work needs to be conducted to reveal the reasons/causes for this phenomenon.

Isolates of blackleg pathogen of canola, *L. maculans*, from different groups and sectors showed reduced mycelial growth on the PDA plates when the bacterium *P. polymyxa* PKB1 was present. This result indicates that *P. polymyxa* PKB1 has a wide antifugal spectrum and has a potential as a biocontrol agent. In natural conditions, more than one pathotypes of *L. maculans* are present, and these should be controlled by the bacterium after application of the biocontrol agent to the agricultural soil.

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Table 5.1. Isolates of *Leptosphaeria maculans* collected in western Canada and tested in this study *.

No.	Code	Isolate	Source	Virulence
1	LM1	B2	Sedgewick, Alberta	Virulent (V)
2	LM 2	B3	Sedgewick, Alberta	V
3	LM 3	C	Sedgewick, Alberta	V
4	LM 4	1.3	Peace River, Alberta	V
5	LM 5	4.2	Peace River, Alberta	V
6	LM 6	4.3	Peace River, Alberta	V
7	LM 7	1.6.8	Brooks, Alberta	V
8	LM 8	3.2.4	Brooks, Alberta	V
9	LM 9	3.2.5	Brooks, Alberta	V
10	LM 10	3.8.8	Brooks, Alberta	V
11	LM 11	4.9.1	Brooks, Alberta	V
12	LM 12	4.10.6	Brooks, Alberta	V
13	LM 13	8.8.5	Fairview, Alberta	V
14	LM 14	163	Fairview, Alberta	V
15	LM 15	1347	Fairview, Alberta	V
16	LM 16	1415	Fairview, Alberta	WeaklyVirulent (WV)
17	LM 17	1418	Fairview, Alberta	V
18	LM 18	1425	Fairview, Alberta	V
19	LM 19	1450	Fairview, Alberta	WV
20	LM 20	1451	Fairview, Alberta	V
21	LM 21	1452	Fairview, Alberta	V
22	LM 23	1455	Fairview, Alberta	V
23	LM 24	1457	Fairview, Alberta	WV
24	LM 25	1469	Fairview, Alberta	WV
25	LM 26	1470	Fairview, Alberta	WV
26	LM 27	1475	Camrose, Alberta	V
27	LM 28	OS95-6	Camrose, Alberta	WV
28	LM 29	OS95-9	Alberta	WV
29	LM 30	OS96-16	Alberta	V
30	LM 31	OS562	Alberta	V
31	LM 32	OS569	Alberta	V
32	LM 33	OS601	Alberta	V
33	LM 34	OS602	Alberta	V
34	LM 35	OS633	Alberta	V
35	LM 36	OS641	Alberta	V
36	LM 37	OS652	Alberta	V
37	LM 38	OS654	Alberta	V
38	LM 39	OS656	Alberta	V
39	LM 40	OS657	Alberta	V
40	LM 41	OS659	Alberta	V
41	LM 42	OS660	Alberta	V
42	LM 43	OS670	Alberta	V
43	LM 44	OS675	Alberta	V
44	LM 45	OS676	Alberta	V
45	LM 46	OS683	Alberta	V
46	LM 47	OS696	Alberta	V
47	LM 48	OS742	Alberta	V
48	LM 49	OS749	Alberta	V
49	LM 50	OS753	Alberta	V
50	LM 51	OS754	Alberta	V
51	LM 52	OS765	Alberta	V
52	LM 53	OS805	Alberta	V
53	LM 54	OS806	Alberta	V

Table 5.1. Isolates of *Leptosphaeria maculans* collected in western Canada and tested in this study* (Cont'd).

No.	Code	Isolate	Source	Virulence
54	LM 55	OS831	Alberta	V
55	LM 56	OS833	Alberta	V
56	LM 57	WW-I	Alberta	V
57	LM 58	WW-ii	Alberta	V
58	LM 59	WW-4	Alberta	V
59	LM 60	WW-5	Alberta	V
60	LM 61	WG-1	Alberta	V
61	LM 62	BRV	Brooks, Alberta	V
62	LM 63	BRWV	Brooks, Alberta	WV
63	LM 64	OS684	Alberta	V
64	LM 65	OS677	Alberta	V
65	LM 66	OS669	Alberta	V
66	LM 67	OS672	Alberta	V
67	LM 68	BLA124	Alberta	V
68	LM 73	WV151	Alberta	WV
69	LM 74	PG2	U.S.A.	V
70	LM 75	PG3	U.S.A.	V
71	LM 76	PG4	U.S.A.	V
72	LM 77	OS95-152	Alberta	WV
73	LM 78	BLB	Alberta	V
74	LM 79	BLF	Alberta	V
75	LM 80	BLP	Alberta	V
76	LM 81	BLN	Alberta	V
77	LM 82	BLS	Alberta	V
78	RL 1	LMM89-1	Riding Mountain, Man.	WV
79	RL 2	LMM89-2	Westbourne, Man.	WV
80	RL 3	LMM89-3	Neepawa, Man.	V
81	RL 4	LMM89-4	Headingly, Man.	V
82	RL 5	LMM89-5	Elgin, Man.	V
83	RL 6	LMM89-6	Pilot Mound, Man.	V
84	RL 7	LMM89-7	Darlingford, Man.	V
85	RL 8	LMM89-8	Deloraine, Man.	V
86	RL11	LMS89-11	Choiceland, Sask.	WV
87	RL12	LMS89-12	Codette, Sask.	WV
88	RL14	LMS89-14	Prince Albert, Sask.	V
89	RL17	LMS89-17	Alvena, Sask.	V
90	RL18	LMS89-18	Wakaw, Sask.	V
91	RL19	LMS89-19	Lake Lenore, Sask.	V
92	RL22	LMS89-22	Leroy, Sask.	V
93	RL24	LMS89-24	Norquay, Sask.	WV
94	RL25	LMS89-25	Wynyard, Sask.	V
95	RL26	LMS89-26	Mikado, Sask.	V
96	RL27	LMS89-27	Stockholm, Sask.	V
97	RL29	LMS89-29	Lashburn, Sask.	V
98	RL30	LMS89-30	Cutknife, Sask.	V
99	RL31	LMS89-31	North Battleford, Sask.	V
100	RL32	LMS89-32	Marsden, Sask.	V
101	RL33	LMS89-33	North Battleford, Sask.	V
102	RL35	LMS89-35	Rockhaven, Sask.	V
103	RL36	LMS89-36	Aberdeen, Sask.	V
104	RL37	LMM89-37	Dauphin, Man.	WV
105	RL40	LMA89-40	Legal, Alta.	WV
106	RL41	LMA89-41	Sherwood Park, Alta.	WV

Table 5.1. Isolates of *Leptosphaeria maculans* collected in western Canada and tested in this study* (Cont'd).

No.	Code	Isolate	Source	Virulence
107	RL42	LMA89-42	Vegreville, Alta.	V
108	RL43	LMA89-43	Vegreville, Alta.	V
109	RL44	LMA89-44	Vegreville, Alta.	V
110	RL45	LMA89-45	Vermillion, Alta.	WV
111	RL48	LMM89-48	Oakner, Man.	V
112	RL49	LMM89-49	Star City, Man.	V
113	RL51	LMS89-51	Cudworth, Sask.	V
114	RL52	LMS89-52	Cudworth, Sask.	V
115	RL53	LMS89-53	Meacham, Sask.	V
116	RL54	LMS89-54	Kelvington, Sask.	WV
117	RL55	LMS89-55	Hazel Dell, Sask.	V
118	RL56	LMS89-56	Rapid View, Sask.	V
119	RL58	LMS89-58	Glaslyn, Sask.	WV
120	RL60	LMS89-60	Marshall, Sask.	V
121	RL61	LMS89-61	Cutknife, Sask.	V
122	RL64	LMA89-64	Innisfree, Alta.	V
123	RL65	LMA89-65	Mannville, Alta.	V
124	RL77	LMA89-77	Andrew, Alta.	V
125	RL78	LMA89-78	Andrew, Alta.	WV
126	RL79	LMA89-79	Mundare, Alta.	WV
127	RL81	LMA89-81	Sedgewick, Alta.	V
128	RL82	LMM89-82	Hamiota, Man.	V
129	RL84	VBL89-14	AEC, Alta.	V
130	RL90	BL87-10	AEC, Alta.	V
131	RL92	AVBL89-23	AEC, Alta.	WV
132	RL96	BL88-22 (4)	AEC, Alta.	WV
133	RL104	BL86-27V	AEC, Alta.	V
134	RL108	OS90-IV	AEC, Alta.	V
135	RL114	SASK87-IV	Sask.	V
136	VB1	LMA90-3356	U. of A., Dr. Bansal	V
137	VB2	3356S1	U. of A., Dr. Bansal	sector of 3356
138	VB3	3356S2	U. of A., Dr. Bansal	sector of 3356S1
	Isolates collected in Alberta in 1998			
139	LM98-1	98-c-s-1	Fort Saskatchewan, AB.	V
140	LM98-2	P98-14	Hanna, AB.	V
141	LM98-3	120	Brooks, AB.	V
142	LM98-4	P98-26	Peace Region, AB.	V
143	LM98-5	VB98-8	Three Hills, AB.	V
144	LM98-6	VB98-4	Consort, AB.	V
145	LM98-7	87-1	Bonnyville, AB.	V
146	LM98-8	29-4	Sedgewick, AB.	V
147	LM98-10	VB98-9	Viking, AB.	V
148	LM98-12	Quantum	Viking, AB.	V
149	LM98-18	VB98-5	Consort, AB.	V
150	LM98-19	W-4	Willindon, AB.	V
151	LM98-20	T-1	Tofield, AB.	V
152	LM98-22	8-0513	BioVision Seed Ltd.	V
153	LM98-27	8-3487	BioVision Seed Ltd.	V
154	LM98-28	2544	Peace Valley Seed Ltd.	WV
155	LM98-29	8-5214	BioVision Seed Ltd.	V

Table 5.1. Isolates of *Leptosphaeria maculans* collected in western Canada and tested in this study* (Cont'd).

No.	Code	Isolate	Source	Virulence
	Isolates obtained from United Kingdom			
156	UK8		United Kingdom	V
157	UK9		United Kingdom	V
158	UK16		United Kingdom	V
159	UK20		United Kingdom	V
160	UK30		United Kingdom	V
161	UK32		United Kingdom	V
	Isolates obtained from Germany			
162	RL115	LMA89-42	Germany	V

* Isolates 1 to 77 were provided and the virulence was determined using plate and cotyledon methods by Dr. P. D. Kharbanda (1993) at the Alberta Research Council, and isolates 78 to 136 were provided by Dr. J. P. Tewari, University of Alberta, and the virulence was determined by using the cotyledon method by Mr. R. Lange (1993) at the University of Alberta. Isolates 139 to 155 were collected, and the virulence was determined by J. Yang by using the Blackleg Alert Kit tested at the Alberta Research Council.

Table 5.2. Some random primers used in RAPD-PCR study of *Leptosphaeria maculans* isolates collected in western Canada.

	Primer	Oligonucleatide
1	OPA-07	5'- GAAACGGGTG
2	OPA-08	5'- GTGACGTAGG
3	OPA-10	5'- GTGATCGCAG
4	OPA-13	5'- CAGCACCCAC
5	OPA-14	5'- TCTGTGCTGG
6	OPA-20	5'- GTTGCGATCC
7	OPX-01	5'- CTGGGCACGA
8	OPX-07	5'- GAGCGAGGCT
9	OPX-08	5'- CAGGGGTGGA
10	OPX-11	5'- GGAGCCTCAG
11	OPX-12	5'- TCGCCAGCCA
12	OPX-13	5'- ACGGGAGCAA
13	OPX-14	5'- ACAGGTGCTG
14	OPX-19	5'- TGGCAAGGCA

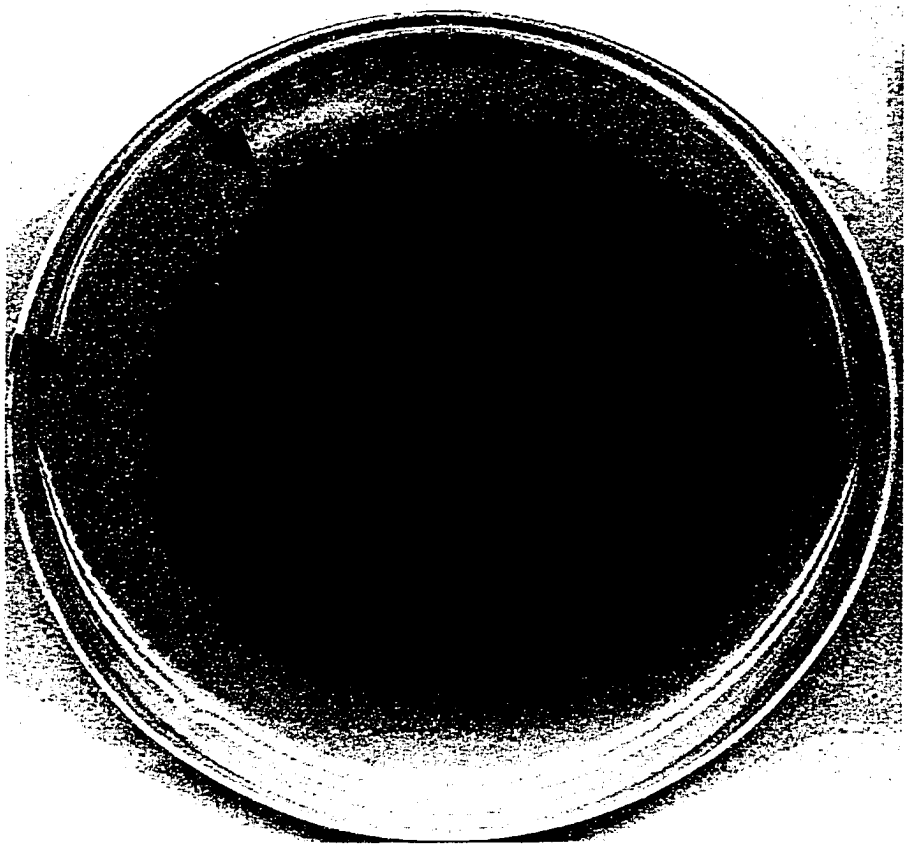

Figure 5.1. *Leptosphaeria maculans* culture growing on V-8 juice agar for 10 days. A sector (arrow) is formed.

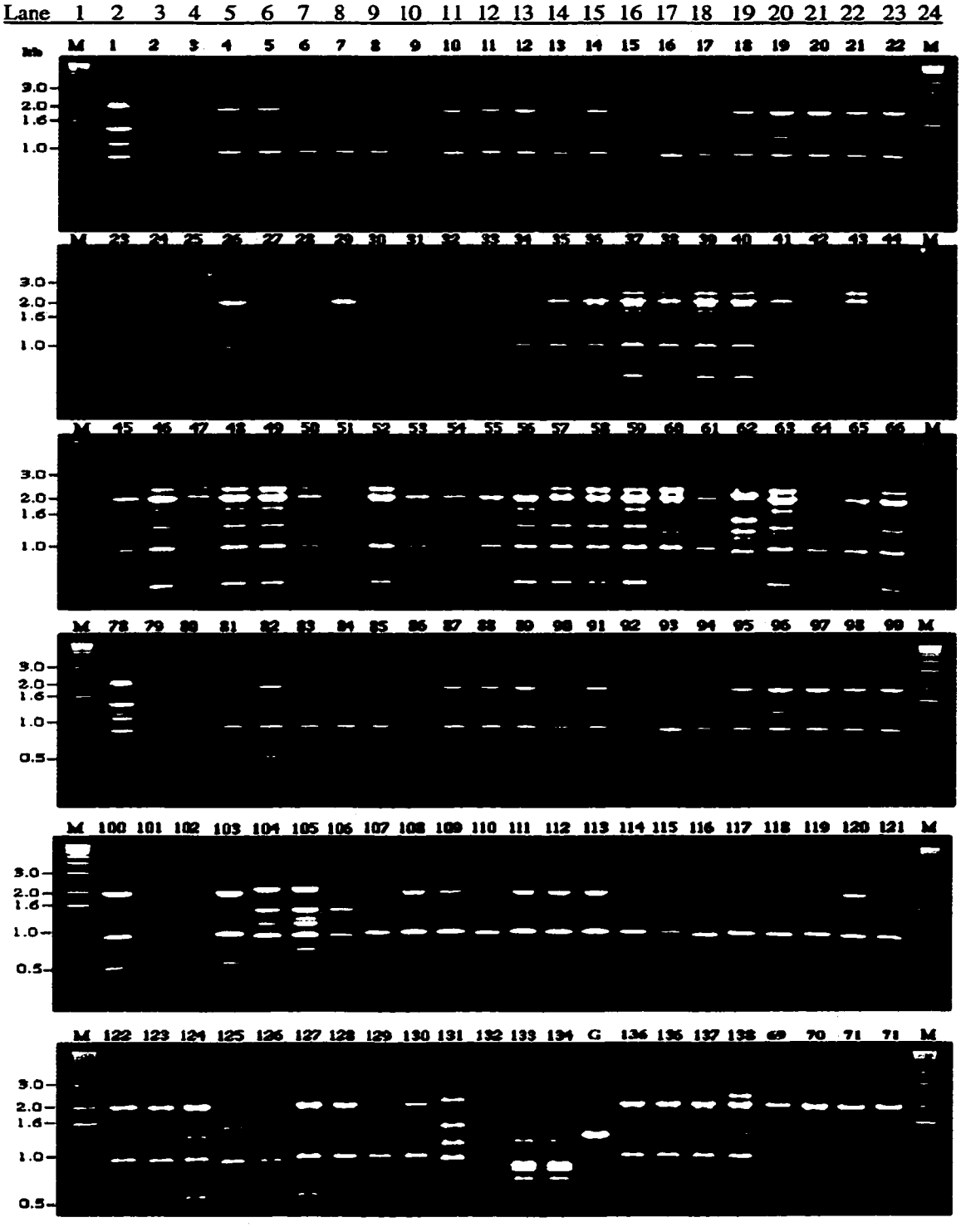

Figure 5.2. RAPD-PCR profile of *Leptosphaeria maculans* isolates amplified with primer OPA-10, M = 1 kb DNA marker.

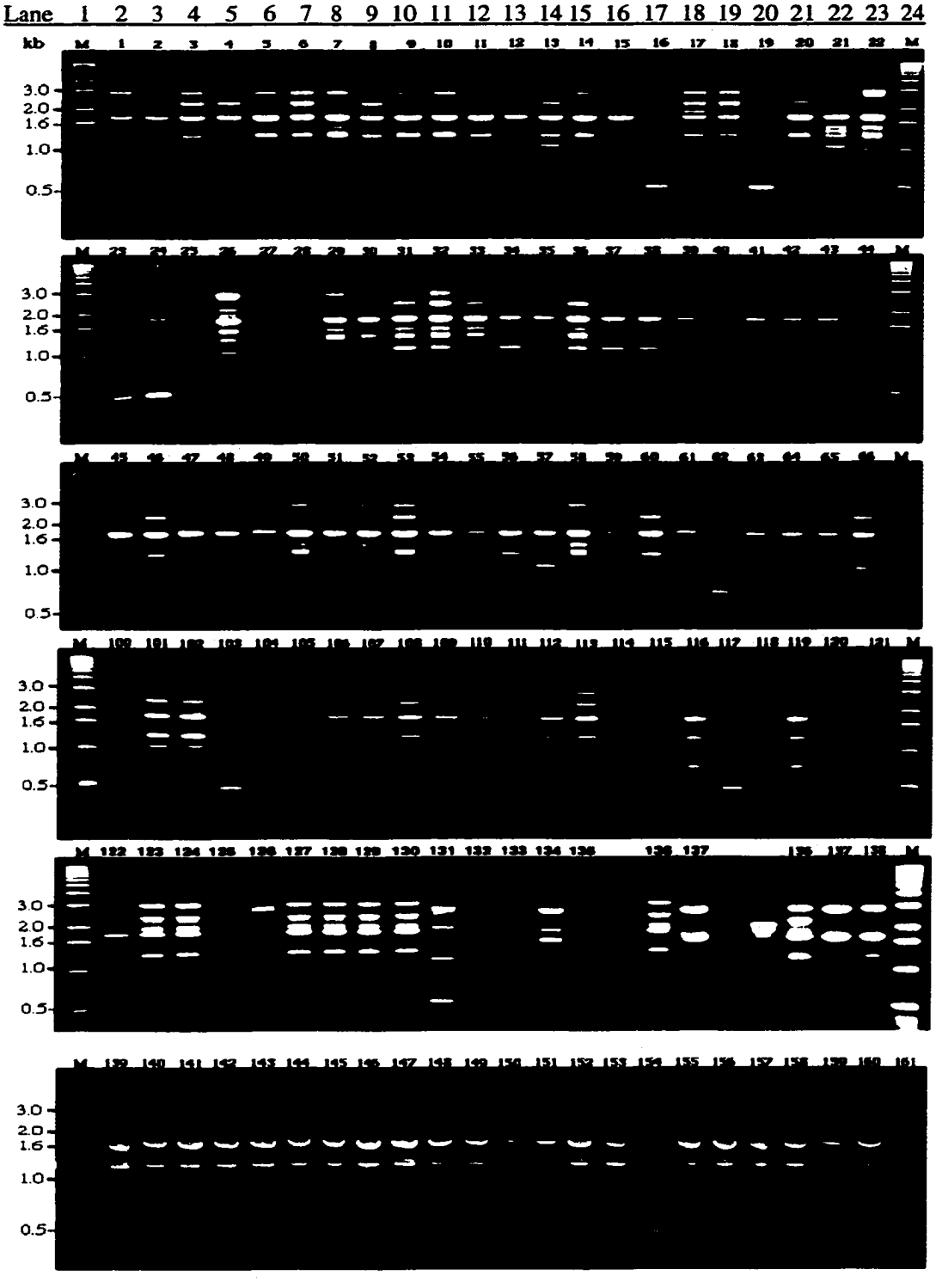

Figure 5.3. RAPD-PCR profile of *Leptosphaeria maculans* isolates amplified with primer OPA-13, M = 1 kb DNA marker.

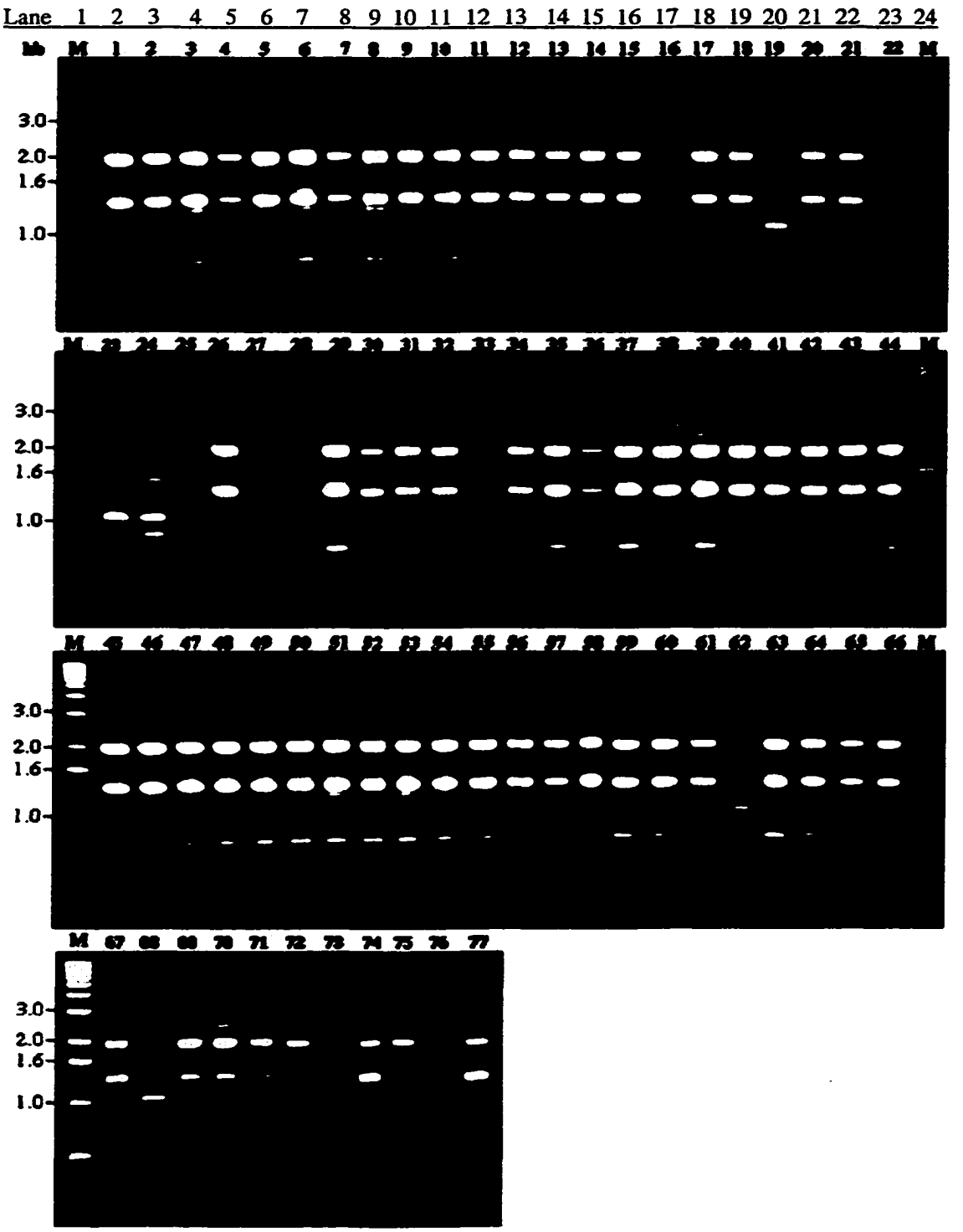

Figure 5.4a. RAPD-PCR profile of *Leptosphaeria maculans* isolates amplified with primer OPX-12, M = 1 kb DNA marker.

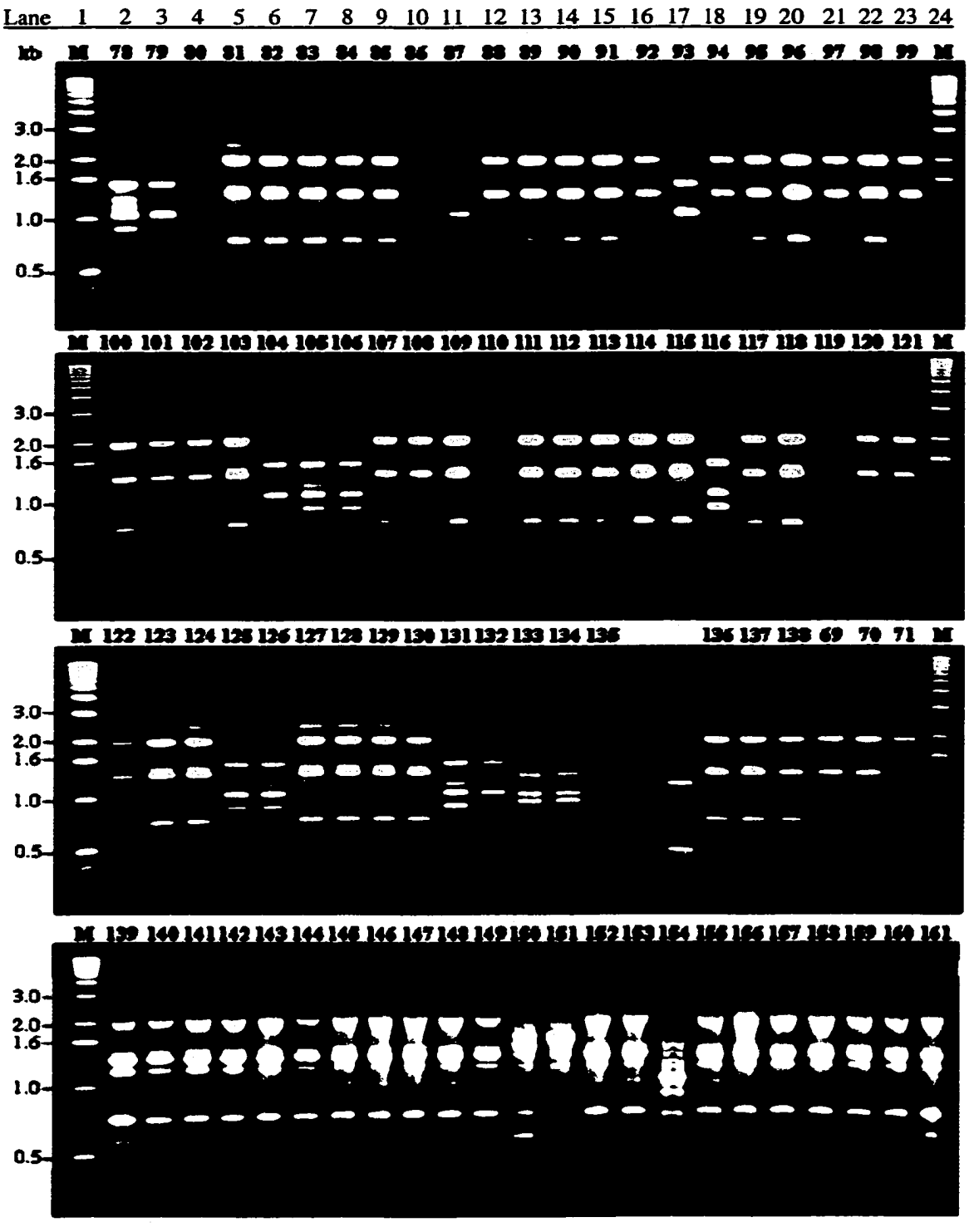

Figure 5.4b. RAPD-PCR profile of *Leptosphaeria maculans* isolates amplified with primer OPX-12, M = 1 kb DNA marker.

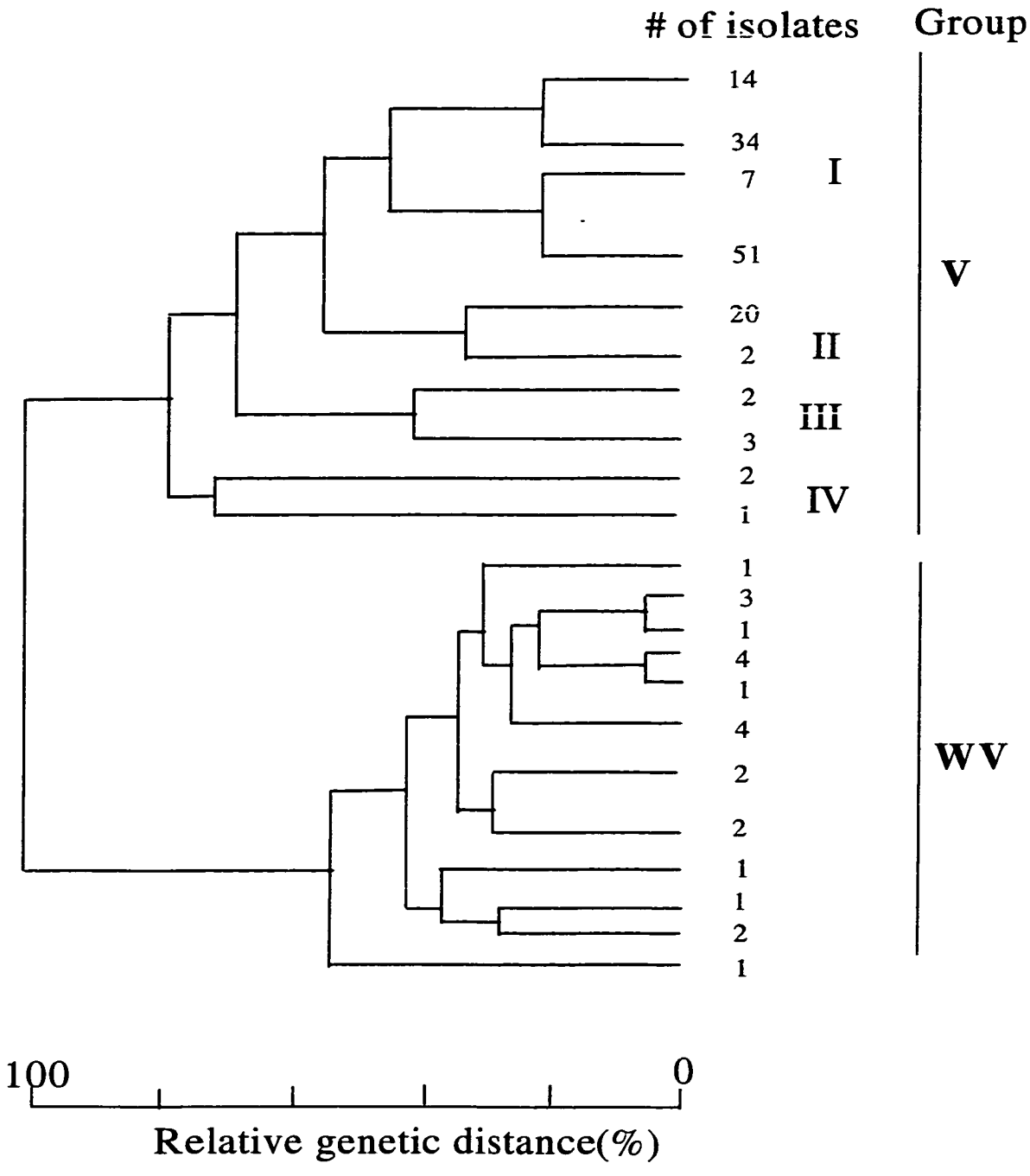

Figure 5.5. Cluster analysis of PCR products based on seven primers used on 159 isolates of *Leptosphaeria maculans*.

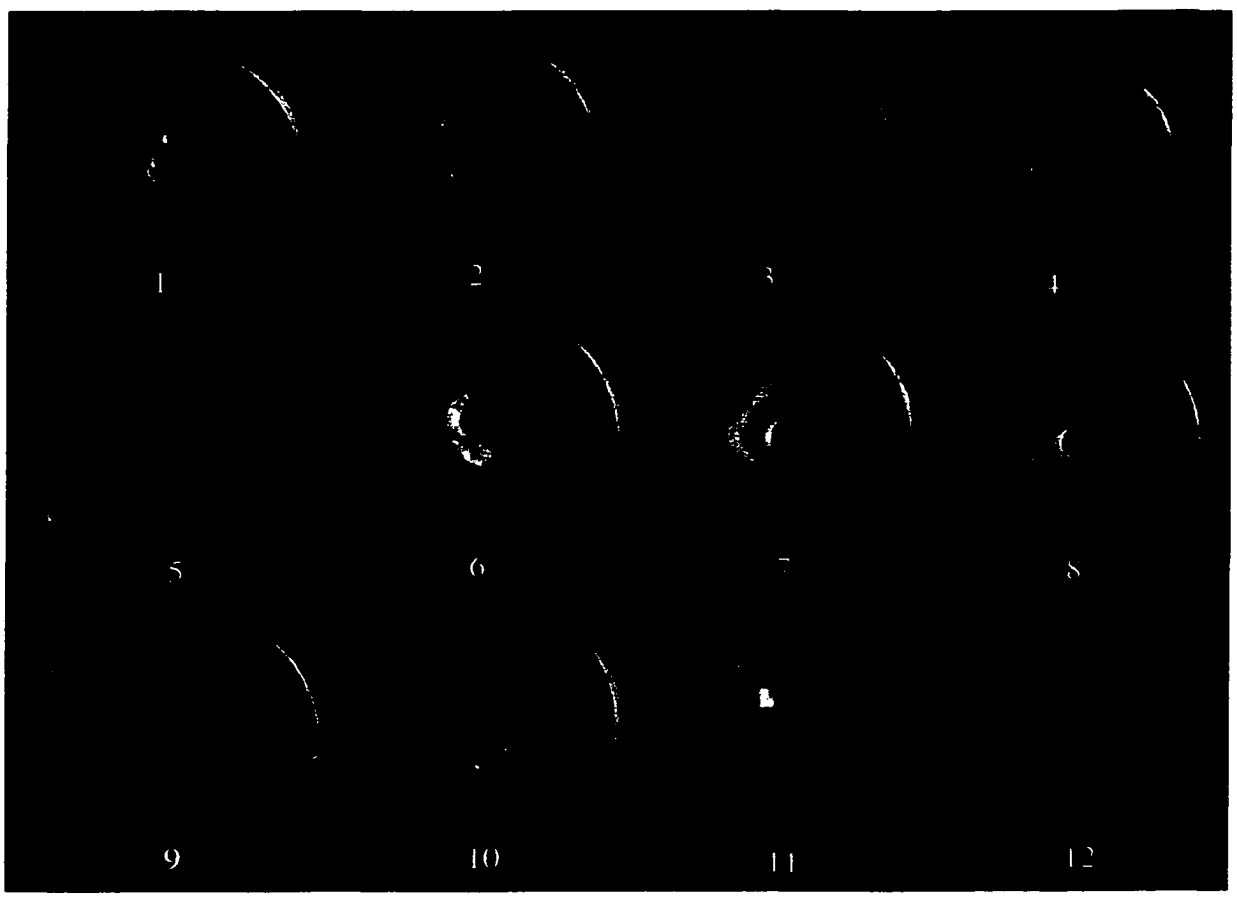

Figure 5.6. Inhibitory effect of *Paenibacillus polymyxa* PKB1 to different isolates of *Leptosphaeria maculans* in a petri plate test. 1 = BLA, 2 = BLB, 3 = BLN, 4 = BLP, 5 = CK1, BLA without bacterium, 6 = LM74, 7 = LM75, 8 = LM76, 9 = CK2, plain PDA plate, 10 = sector of BLA, 11 = 98-1, 12 = WV151.

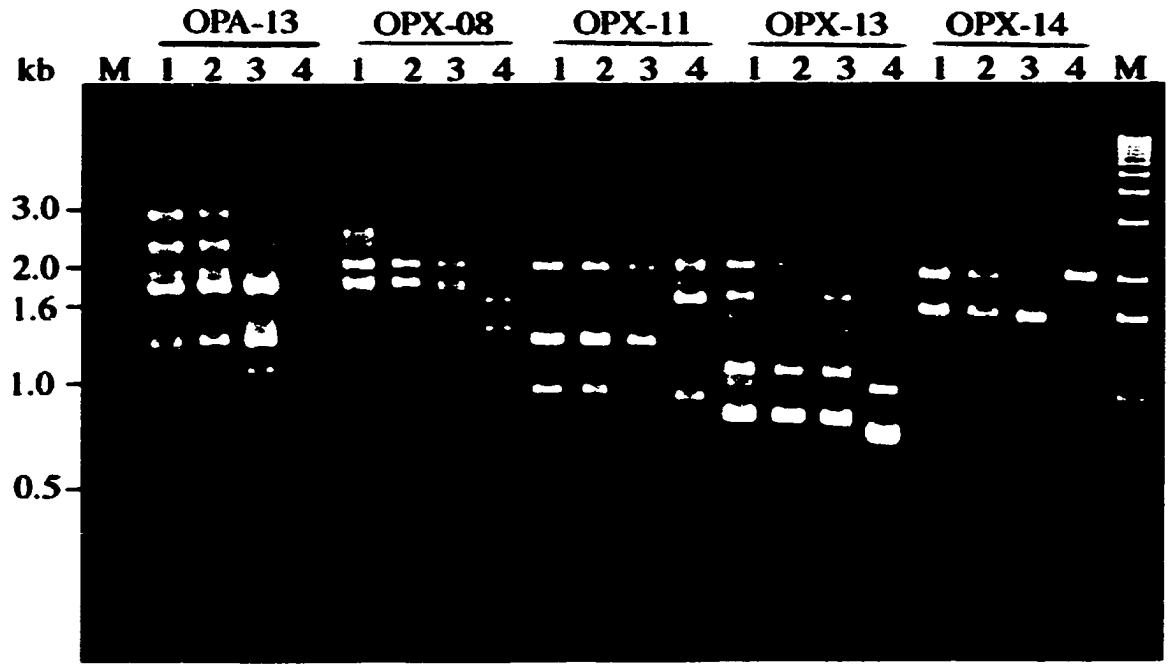

Figure 5.7. PCR profile of four *Leptosphaeria maculans* isolates amplified with five random primers, OPA-13, OPX-08, OPX-11, OPX-13 and OPX-14. 1 = 3356, 2 = 3356S1, sector of 3356, 3 = 3356S2, 4 = weakly virulent isolate Unity, M = 1 kb DNA marker.

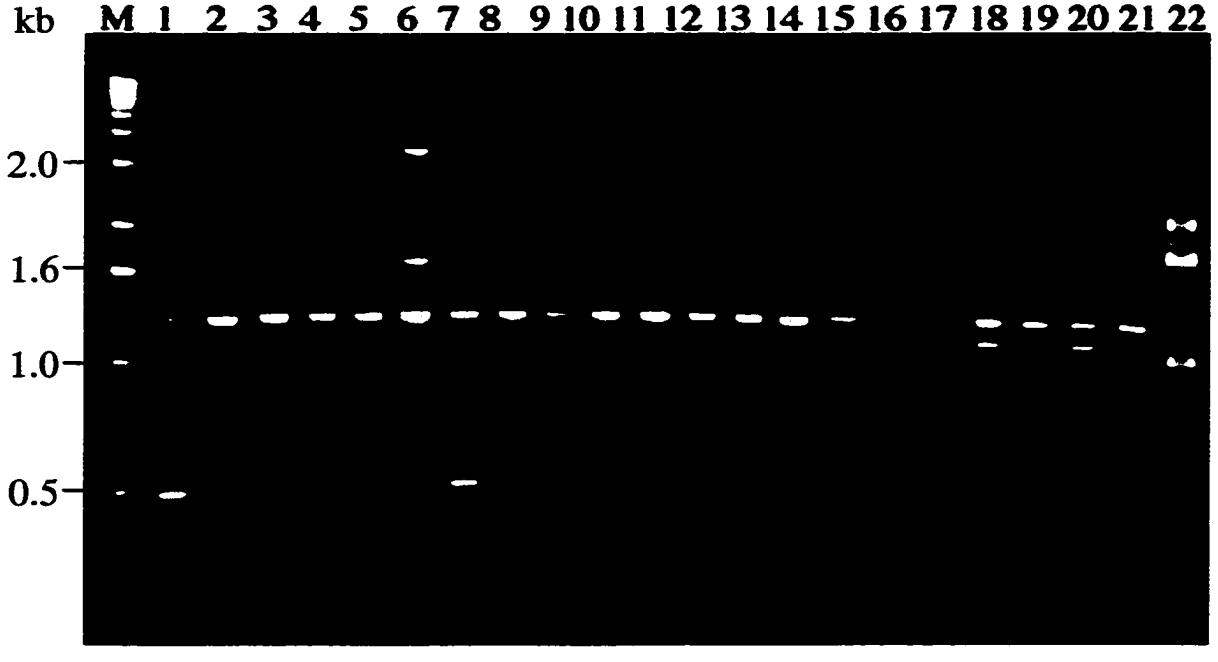

Figure 5.8. PCR profile of 20 cultural variants of *Leptosphaeria maculans* virulent isolate 3356, compared with 3356 and Unity (weakly virulent), amplified with primer OPX-11. 1-20 = sectors of 3356, 21 = 3356, 22 = Unity, M = 1 kb DNA marker.

Chapter 6

General Discussion and Summary

Some of the widely used biocontrol formulations in the world include the bacterial genera *Paenibacillus* and *Bacillus*. In particular, isolates of *Paenibacillus polymyxa* and *Bacillus subtilis* are used against soil- and seed-borne diseases and *B. thuringiensis* on insect pests of economically important crops. The wide range of applications result from the various antagonistic mechanisms enabling these isolates to function as potent biocontrol agents on many different crops. The bacterium strain *Paenibacillus polymyxa* PKB1 isolated from canola stubble was found to inhibit the growth of *Leptosphaeria maculans*, the causal agent of the blackleg disease on canola. The bacterial spore suspension significantly reduced the pycnidiospore germination and germ-tube length of *L. maculans* observed under light, fluorescence, scanning electron, and confocal laser scanning microscopes in culture and on the canola leaf surface. *P. polymyxa* PKB1 could grow and multiply rapidly on the leaf surface on detached leaves in a plate test. More experiments should be conducted to quantify the reduction of fungal growth under natural conditions. When specimens were examined under a fluorescent microscope and a confocal laser-scanning microscope, fungal spore viability in the presence of *P. polymyxa* PKB1, was observed to be significantly reduced. When bacterial cells were added to *L. maculans* spore culture, the number of viable pycnidiospores was significantly reduced. The value of this bacterium as a potential biocontrol agent was demonstrated and proved in the laboratory experiments. More studies are needed to evaluate the efficacy of control of the canola blackleg disease under field conditions.

There were no obvious morphological changes in the pycnidiospores treated with the bacterial suspension and examined under a scanning electron microscope. This result suggested that the mechanism of the inhibitory effect of *P. polymyxa* PKB1 against *L. maculans* was chemical and not mechanical. Research by Beatty (2000) demonstrated that the inhibition effect by the bacterium was due to the production of an antifungal metabolite prior to, or at the onset of, bacterial sporulation.

The bacterium could inhibit the growth of several isolates from different groups of *L. maculans*. It also successfully inhibited the growth of several other pathogenic fungi *in vitro*. A wide inhibitory spectrum of *P. polymyxa* PKB1 indicated its possible usefulness as a potential biocontrol agent in controlling other diseases as well. Significant effect of *P. polymyxa* PKB1-inoculated compost on the germination of sclerotia of *S.*

sclerotiorum was tested and demonstrated (Yang et al., 1999). Further work also needs to be done to demonstrate the use of *P. polymyxa* PKB1 in control of the other diseases of canola, such as Sclerotinia stem rot and Rhizoctonia root rot under field conditions.

None of the fungicides or herbicides tested was deleterious to the growth of the bacterium on nutrient agar and on potato dextrose agar. The results suggest that *P. polymyxa* PKB1 has potential value as a biocontrol agent in an integrated disease control of the blackleg of canola, as the bacterium can be used together with chemicals.

Investigation of integrated control of blackleg with *P. polymyxa* PKB1, fungicide Tilt® and compost was conducted in a growth chamber and in the field. Bacterial suspension, inoculated on intact canola leaves together with a *L. maculans* spore suspension, significantly suppressed blackleg disease development on stems. Tilt® significantly reduced the number of pycnidia of *L. maculans*, whereas the bacterium significantly reduced viability of the fungus in the infected canola stubble in a growth chamber study. Temperature and burial treatments had a significant effect on *L. maculans* development on canola stem (**Tables 3.4** and **3.6**).

In field experiments, application of *P. polymyxa* PKB1 alone had no significant effect on reducing formation of ascospores of *L. maculans*. However, application of Tilt®, *P. polymyxa* PKB1 and compost in combination significantly reduced the formation of pseudothecia and ascospores, as well as survival of the fungus, on infected canola stubble. It was demonstrated that the integrated control method, chemical and biocontrol, could be effective in managing the blackleg disease of canola. The advantage of combining plant protection chemicals with biocontrol agents in integrated treatments is that the pathogen can be controlled under climatic conditions beyond the effective range of the biocontrol agent. As well, the biocontrol agent reduces both environmental pollution and the danger of the pathogen developing fungicide resistance if the dosage of fungicide treatments is reduced.

Compost contains some beneficial microorganisms, such as *Bacillus subtilis, B. licheniformis, Trichoderma* spp. and inhibits growth of soil-borne and stubble-borne pathogens. Although, compost without inoculation of *P. polymyxa* PKB1 has showed significant effect on reducing ascospore formation in field conditions, inoculation of *P. polymyxa* PKB1 into the compost could ensure the disease-suppression effect of the compost for field application. *P. polymyxa* PKB1 could establish itself in the environment, thus providing a persistent disease control effect. Variability in suppression of compost to soil-borne diseases such as

Rhizoctonia damping-off and Fusarium wilt has been observed (Kuter et al., 1988). The reason is partially due to a random conlonization of compost by an effective biocontrol agent after heating. The problem could be solved by inoculating composts with specific biocontrol agents to induce consistent levels of suppression on pathogens (Hoitink et al., 1997). The present study showed that the percentage of beneficial bacteria was significantly increased in compost with *P. polymyxa* PKB1 amendment compared to that of compost without inoculation.

P. polymyxa PKB1 has many advantages as biocontrol agent. First, it grows fast and can be propagated on a wide range of substrates for biomass production. The optimum substrate and suitable growth conditions for fermentation of *P. polymyxa* PKB1 have been studied (Beatty, 2000). Thus, it is easy to produce the type of biomass appropriate to the intended application. Second, *P. polymyxa* PKB1 has a wide range of environmental-condition tolerance due to the production of endospores. Endospores of *P. polymyxa* PKB1 can survive at high temperature that enables the bacterium to be applied to the compost, therefore enhancing the biocontrol effect of compost. Also, *P. polymyxa* PKB1 is tolerant to most fungicides and herbicides used in the commercial canola production. It should therefore possible to use *P. polymyxa* PKB1 in an integrated control system. Finally, *P. polymyxa* PKB1 can form endospores in a fermenter in 4-5 days at suitable conditions (Beatty, 2000). The final product of the biocontrol agent will be easy to store, transport, and will have a long shelf life.

The genetic polymorphism in 13 strains of *P. polymyxa* and 10 strains of various *Bacillus* spp. were determined by randomly amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR). When comparing the DNA amplification patterns with those of other strains of *Bacillus* spp., *P. polymyxa* strains were clearly distinguishable. The development of methods for the detection and correct identification of applied biocontrol agents is essential in the biocontrol studies. When *P. polymyxa* PKB1 was introduced into the soil environment, it was expected that the bacterium could colonize the subterranean portions of plants. A reliable and fast technique was needed to predict the colonization behavior of the introduced *P. polymyxa* PKB1. The RAPD-PCR technique was used to detect *P. polymyxa* PKB1 inoculated into compost, and it was found that bacteria re-isolated from compost had similar DNA amplification pattern compared to that of *P. polymyxa* PKB1. The molecular detection of *P. polymyxa* PKB1 showed that *P. polymyxa* PKB1 could survive in the compost.

DNA polymorphism within *P. polymyxa* isolates made possible the development of molecular probes to detect the biocontrol agent. DeParasis and Roth (1990) explained that ".....the ideal DNA probe would be based on known nucleic acid sequences that taxonomically define the target and are present in high copy number". Four DNA fragments of the PCR products obtained, which were specific for *P. polymyxa*, were excised, purified and digoxigenin-labelled. Probes P1-7, P1-8, P1-13 and P1-14 were species-specific and hybridized with only the DNA of *P. polymyxa* PKB1 and other strains in *P. polymyxa* but not with that of other *Bacillus* spp. The specificity of probes was confirmed by detecting the bacteria isolated from compost that was inoculated with *P. polymyxa* PKB1. These probes provided direct diagnostic assays, based on southern hybridization or dot blotting methods, on soil and compost samples. The information on colonization and population of *P. polymyxa* PKB1 in the rhizosphere could be correctly identified. Such information is required for effective biocontrol applications. Genetic approaches for analysis of beneficial bacterial colonization in the rhizosphere have been applied to other biocontrol studies (Hartung, 1997; Roberts et al., 1997). Molecular biotechnology is having a revolutionary effect on biological control. This technology provides relatively simple methods to gain an understanding of the genetic basis for microbial behaviour and microbes' activity as biocontrol agents. With *P. polymyxa* PKB1, four peptides appear to be responsible for the anti-fungal effect, and DNA fragments, corresponding to portions of peptide synthetase modules, were amplified from the *P. polymyxa* PKB1 genome (Beatty, 2000).

The four probes were cloned and sequenced. Eight primers were designed, synthesized and tested with 13 strains from *P. polymyxa* and 10 from *Bacillus* spp. The designed primers were strain-specific and amplified only one band of *P. polymyxa* PKB1 and related strains of *P. polymyxa*. The specific primers were very useful in the detection of the biocontrol agent. A similar technique was used for detection of spore-forming bacteria and proved to be very effective (Arcuri et al., 1999). The identification of the biocontrol agent is necessary for making a rapid advancement in the biocontrol program. Knowledge of such molecular probes and specific primers should enable the development of effective screening programs to identify biocontrol agents. Also, strain improvement of antagonists will be possible and probable by using molecular biology techniques.

The genetic variation of 161 isolates of *L. maculans* collected in western Canada was tested with the RAPD-PCR technique. The DNA-amplification patterns showed polymorphism among these isolates.

Virulent and weakly virulent isolates of *L. maculans* were clearly distinguishable by all the primers used in the RAPD-PCR test. Three major groups were identified within virulent isolates based on RAPD data. *P. polymyxa* PKB1 showed an inhibitory effect on the isolates from different groups. Results suggested that *P. polymyxa* PKB1 has a wide biocontrol spectrum and can inhibit virulent isolates from different groups of *L. maculans* collected in western Canada.

No genetic changes were detected in 20 sectors of a virulent isolate of *L. maculans*. *P. polymyxa* PKB1 can inhibit the growth of the sectors of a virulent isolate of *L. maculans* as well.

Future Work

Application of *P. polymyxa* PKB1 to control blackleg of canola under field conditions is still in an early stage. Further investigations need to be conducted to understand the effect of environmental conditions on the bacterial antagonistic effects as well as on *P. polymyxa* PKB1's growth and survival. Much emphasis should focus on learning more of the ecological effects of this biocontrol agent. Such studies should involve many different characteristics such as those relating to general rhizosphere competence and ability to survive during unfavorable environmental conditions.

Application of *P. polymyxa* PKB1 to control other pathogens of different field and greenhouse crops, and turf grass has some potential in the area of integrated pest management (IPM) systems. The current study demonstrates that *P. polymyxa* PKB1 has a wide inhibitory spectrum against various fungal pathogens. Further understanding of this bacterium in disease control under controlled and natural conditions need to be carried out.

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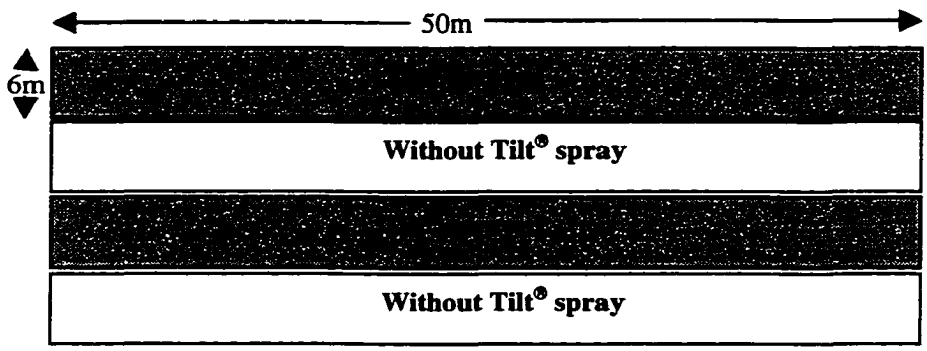
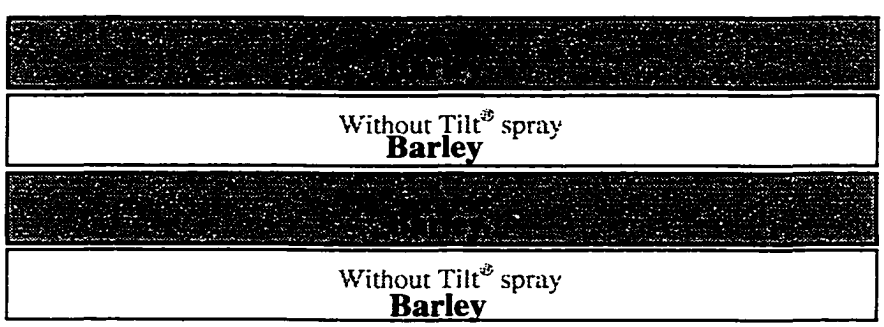
Appendix 1

Experimental design for the field test of Post-harvest application of Tilt® to control blackleg disease of canola in Wainwright, Alberta, 1993 - 1995:

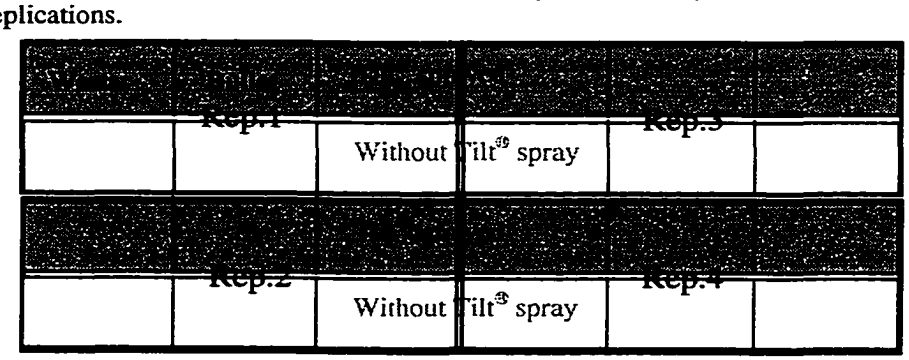
In the fall of 1993, field plots were established and Tilt® was sprayed to canola stubble in two of four 50 x 6m blocks.



In the summer of 1994, barley was grown in all four blocks.

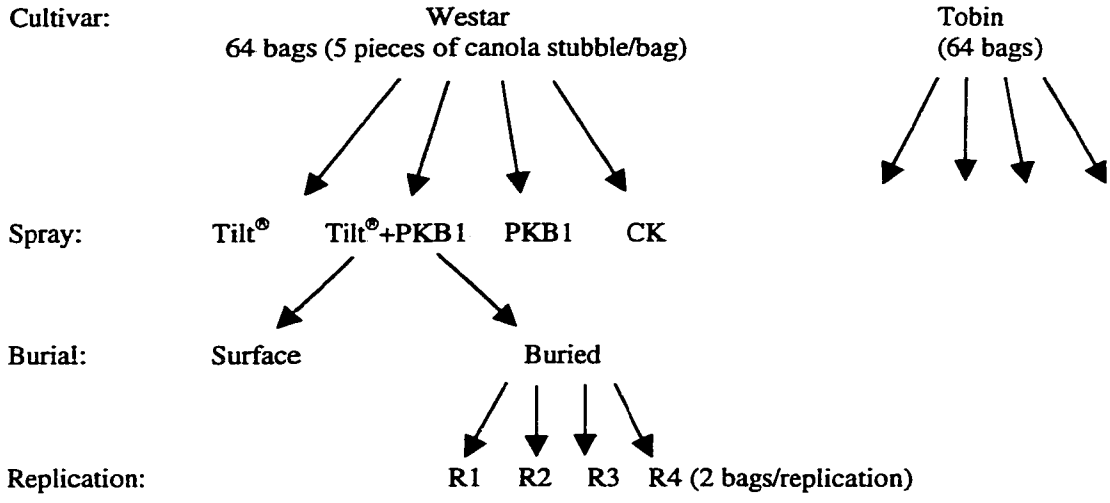


In the summer of 1995, three canola cultivars were grown in the plots arranged in a split-plot design with four replications.

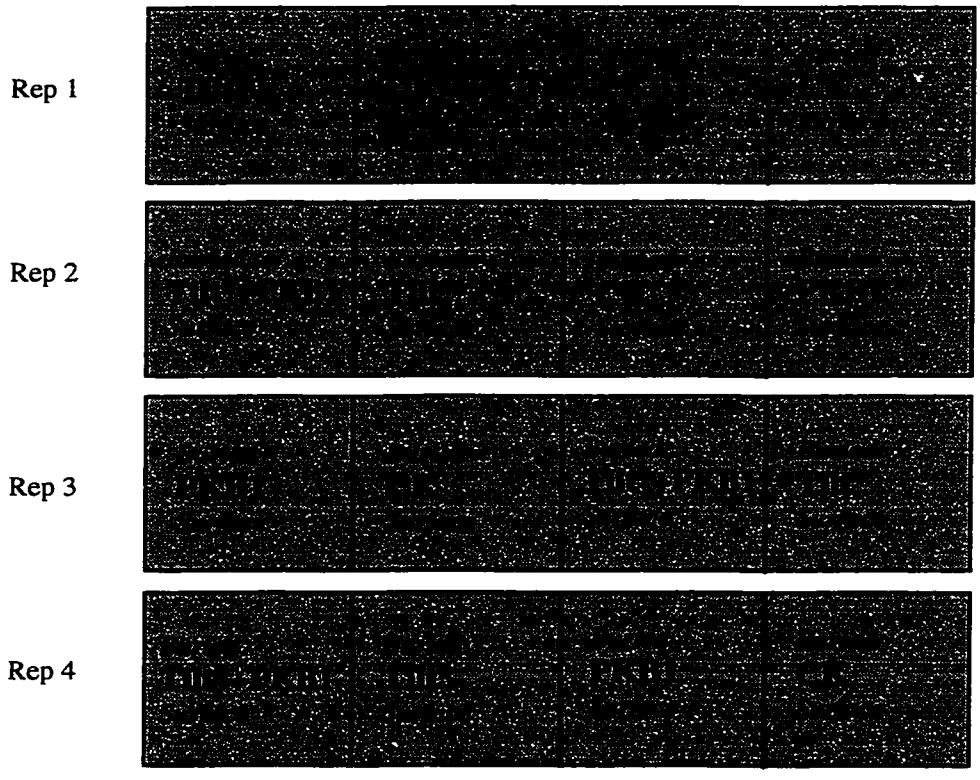


Appendix 2

Experimental design for the field test of effect of Tilt® and *P. polymyxa* PKB1 against *L. maculans* in Vegreville, Alberta, 1995 – 1996:

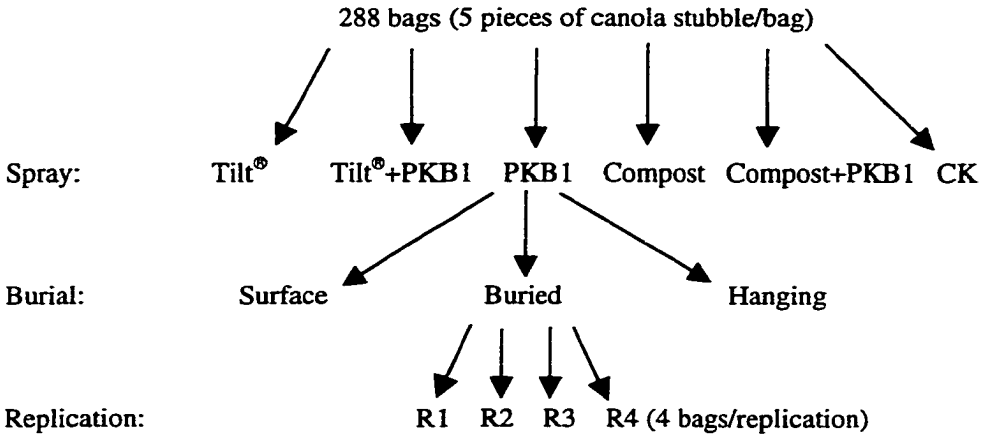


Field plot layout:



Appendix 3

Field experimental design for the test of Tilt® and *P. polymyxa* PKB1 against *L. maculans* in Vegreville, Alberta, 1996 – 1998:



Field plot layout: