BIOCONTROL OF SCLEROTINIA STEM ROT OF CANOLA BY BACTERIAL ANTAGONISTS AND STUDY OF BIOCONTROL MECHANISMS INVOLVED

by
Yilan Zhang

A Thesis

Submitted to the Faculty of Graduate Studies

In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Plant Science

University of Manitoba

Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES
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Biocontrol of Sclerotinia Stem Rot of Canola by Bacterial Antagonists and Study of Biocontrol Mechanisms Involved

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ACKNOWLEGEMENTS

Fist of all, I would like to express my appreciation to my supervisor Dr. Dilantha Fernando for his guidance in my experiments, and support given at conference presentations and applying for several travel awards. Only with your patience, kindness and encouragement I could grow as a student and plant pathologist. I also appreciate the help of my co-supervisor Dr. Fouad Daayf, for the advice and support for my project during my Masters program. I would also like to thank both of you for your great input on my thesis writing. To two of my other committee members, Dr. Annemieke Farenhorst and Dr. Georg Hausner, thank you very much for your great effort on advising me in my project and thesis.

Special thanks to Paula Parks, Alvin Iverson and Lorne Adam for your technical support. Also a big thank you to my lab mates for your continuous support and help. Special thanks to Clinton Jurke for providing breeder tents and some of the pictures. A very special thank you to Dr. Teresa de Kievit and Chrystal Berry for the work on southern blots. I thank Dr. Tim Paulitz for giving me the idea to investigate bacterial antibiotic-related genes using PCR. I also appreciate the staff and fellow graduate students in Plant Science who helped me on my project and shared the fun at BBQ's and parties…

I would like to thank my parents for their never changed love and support. The mountains and oceans would never separate our hearts being close to each other. Only when I live far away from you, I realize how much you sacrificed in making me an independent person.

I also would like to thank the life itself. I had fully experienced the interesting, exciting and frustrations in life in Canada in the past two years. I survived and I did a good job! Nothing to regret!

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ABSTRACT

Zhang, Yilan. M.Sc., The University of Manitoba, July, 2004. Biocontrol of Sclerotinia stem rot of canola by bacterial antagonists and study of biocontrol mechanisms involved. Advisor: W.G.D. Fernando; co-advisor: F. Daayf.

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is an economically important pathogen on canola (*Brassica nanups* L.). Due to the increasing public concerns of fungicide usage and the limitations of cultural methods, biological control is emerging as a promising alternative to control *S. sclerotiorum* in an environmentally-friendly way.

Several bacterial strains, mainly *Bacillus* spp., were isolated from canola and wheat plants. Eight Gram-positive bacterial strains were inhibitory against mycelial growth of *S. sclerotiorum in intro*. Three *Bacillus* spp., one *Staphylococcus* spp. and one *Pseudomonas* spp. (control) were tested in whole plant assays, and all of them achieved significant ($P<0.05$) disease suppression. An experiment in the greenhouse to investigate the optimal time of application showed that the highest disease suppression was achieved when *B. amyloliquefaciens* BS6 was inoculated at the same time as the pathogen. The field experiment (Trial two) in 2003 showed a significant ($P<0.05$) reduction of disease incidence and severity in bacteria-pre-treated plots, compared to control plots, even though the survival of bacterial populations of *Pseudomonas chlororaphis* PA-23 and *B. amyloliquefaciens* BS6 were low on the petals' surface. The bacterial strains were effective against *S. sclerotiorum* under laboratory, greenhouse, and field conditions, and these results also suggest that antibiosis and plant induced resistance might be involved in the disease suppression.

To identify antibiotic-related genes in these potential biocontrol bacterial agents, polymerase chain reaction (PCR) with specific primers was used. The sequencing of PCR products and BLAST search in the gene bank showed that *P. chlororaphis* PA-23 contains biosynthetic genes for phenazine-1-carboxylic acid, pyrrolnitrin and probably 2,4-diacetylphloroglucinol, and that *B. thuringiensis/cereus* BS8, *B. cereus* L and *B. mycoides* S contain zwittermicin A self-resistant gene. The significance of the presence of these genes is discussed.

Induced resistance mediated by *S. sclerotiorum* and *B. amyloliquefaciens* BS6 was evaluated in a greenhouse study followed by analysis of the phenolic compounds from canola leaf extracts by high performance liquid chromatography (HPLC). Pre-treatment with bacteria significantly ($P<0.05$) reduced the disease symptoms on canola plants. The HPLC results indicated that the disease suppression from the bacterial pre-treatment was related to the induction of secondary metabolites in canola leaves. The HPLC results also showed that the inoculum of *S. sclerotiorum* was also associated with the induction of these compounds in canola leaves.

The knowledge accumulated during this study has shown that the bacterial biocontrol agents tested have a great potential in controlling sclerotinia stem rot of canola. The understanding of the biocontrol mechanisms involved in the disease suppression would help optimize their biocontrol efficiency. Therefore, further studies on the role of bacterial antibiotics in disease suppression, and better understanding of plant induced resistance mediated by pathogen and bacterial agents are needed.

FOREWARD

This thesis is written in a manuscript style, with each manuscript having its own abstract, introduction, materials and methods, results and discussion. There is a general introduction and review of the literature prior to the manuscripts, followed by a general discussion and conclusions and the references cited.

1.0 INTRODUCTION

Canola is a crop derived from rapeseed, which was cultivated as early as 5000 B.C. in China (Yan 1990). To be called "canola", the oil component must contain less than 2% erucic acid, and the solid component of the seed less than 30 μmol/g total glucosinolates (Canola Council of Canada 2001e). Canola is now widely used as edible oil in human consumption and an important source of proteins for animal feed. It is now the most important oilseed crop in Canada, and is second only to soybean as the most important source of vegetable oil in world production (Raymer 2002). The harvested area of canola in 2003 was 11,587,200 acres in Canada, and the cash receipts totalled $483.6 million in 2002 (Manitoba Agriculture 2003; Canola Council of Canada 2004).

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de Bary is one of two most economically important diseases on canola in Canada, and losses ranging from 5-100% were reported in Western Canada (Manitoba Agriculture 2002). *Sclerotinia sclerotiorum* is an ubiquitous pathogenic fungus that is known to infect over 400 plant species (Boland and Hall 1994). There are more than 100 species of plants reported to be the hosts for *S. sclerotiorum* in Canada (Boland and Hall 1994). The pathogen infects canola mainly through its petals, which are infected by *Sclerotinia* ascospores (Turkington and Morrall 1993)**.** Canola cultivars currently registered in Canada have little or no resistance to *S. sclerotiorum* (Kharbanda and Tewari 1996), and only limited partially resistant cultivars are available in the world (Fuller et al. 1984; Wang et al. 2003; Kharbanda and Tewari 1996). The control of sclerotinia stem rot of canola is mainly achieved by using fungicides, due to the difficulty of using cultural methods, such

as rotation and tillage to reduce the inoculum of the pathogen. However, public concern of environmental pollution and pathogen resistance has facilitated research on alternative ways such as biological control to control this disease (Bardin and Huang 2001).

Biological control is a method that is economical and most important, sustainable, to achieve the goal of increasing crop yield (Cook and Baker 1983d). The biocontrol agents used to control *S. sclerotiorum* are mainly parasitic fungi used in soil applications, such as *Coniothyrium minitans* Campbell (Huang 1980). However, many field studies failed to consistently control this pathogen (Hedke et al. 1999; McQuilken et al. 1995), due to the fact that *Sclerotinia* ascospores can disperse long distance (Venette 1998) and that even a reduced number of sclerotia in a field can cause significant infection and yield loss (Davies 1986). Therefore studies on foliar applied biocontrol agents are worthwhile (Boyetchko 1999).

Bacterial biocontrol agents against *S. sclerotiorum* are rarely studied (Boyetchko 1999). Some bacterial strains have shown antifungal activity to *S. sclerotiorum*, such as *Erwinia herbicola* (Godoy et al. 1990c) and *Bacillus* spp. (Tu 1997; Godoy et al. 1990c; Huang et al. 1993); however, most of the studies did not consistently achieve disease suppression under field conditions (Yuen et al. 1992; Boland 1997). Savchuck (2002) screened several bacterial antagonists against *S. sclerotiorum* in the lab and greenhouse, and *Sclerotinia* control by *Pseudomonas chlororaphis* strain PA-23 and *Pseudomonas* spp. strain #41 was evaluated under field conditions in 2001. A double application of *P. chlororaphis* PA-23 showed significantly ($P<0.05$) lower disease incidence of *Sclerotinia* on canola stem under field conditions in 2001, but biocontrol

mechanisms involved in the disease suppression were unclear. In order to continue the field study of *Pseudomonas chlororaphis* PA-23 and *Pseudomonas* spp. #41, a similar field study was carried out in 2002, but the experiment failed simply because of lack of pathogen inoculum (ie. ascospores) at flowering.

An understanding of biocontrol mechanisms would benefit the utilization of biocontrol agents. Several biological mechanisms might be involved in disease suppression, which include parasitism, competition, antibiosis and plant induced resistance, etc. (Handelsman and Stabb 1996).

The first objective of this study was to screen bacterial biocontol agents, mainly *Bacillus* spp. in the lab and greenhouse, and evaluate their biocontrol abilities along with *Pseudomonas chlororaphis* PA-23 and *Pseudomonas* spp. #41 under field conditions. Methods of *Sclerotinia* inoculation and disease evaluation in the greenhouse and the field will be developed, and bacterial population dynamics will be investigated.

The second objective was to study the biocontrol mechanisms involved in disease control. Due to preliminary observations made during this study, antibiosis and plant induced resistance in biocontrol will be investigated. The presence of antibiotic biosynthetic genes or self-resistant genes will be studied by polymerase chain reaction (PCR) and subsequent sequencing of the PCR-products. The plant induced resistance will be studied in a greenhouse experiment by comparing the phenotypic changes among different treatments, and the accumulation of phenolic compounds from leaf extracts will be investigated over time.

2.0 LITERATURE REVIEW

2.1 Canola

2.1.1 Crop History

Canada plays a lead role in large-scale production of rapeseed-canola; however, she is relatively new to the rapeseed cultivation. The earliest proof of rapeseed cultivation has been found back to 5000 B.C. in China based on archaeological discoveries (Yan 1990). It was recorded in use in India about 2000 B.C., and was introduced to Japan from China or the Korean Peninsula over 2000 years ago (Bell 1982). The rapeseed comes from three species: *Brassica campestris*, *B. napus*, and *B. juncea* (L.) Czernj. &Cosson (Shahidi 1990). *Brassica campestris* was introduced to Canada by a Polish immigrant Fred Solvonik in 1936 (Bell 1982) and *B. napus* was introduced by the Canada Department of Agriculture prior to World War II (Busch et al. 1994).

The shortage of marine lubricant in the World War II led the Canadian government to encourage the rapeseed planting through a price support program; large quantities of rapeseed were grown in the prairie provinces of Saskatchewan, Manitoba, and Alberta. The production of rapeseed fell in late 1940s when the government support ceased, however, Canada was far from self-sufficient in edible oil production at that time (Busch et al. 1994). Therefore, the question of transforming rapeseed oil to largely used edible oil was raised. The erucic acid and glucosinolates in rapeseed were the two major concerns. High erucic acid diet has been found to have several detrimental effects on rats, such as formation of high level of fat in heart, formation of fibrous tissue in the heart muscle, growth retardation, reductions in mitochondrial oxidation of glutamate and in the

rate of ATP synthesis in the heart muscle (cited inGriffiths et al. 1998). Glucosinolates are the sulphur compounds that give mustard their sharp taste, and several reports have shown that high concentrations of glucosinolates in diet have adverse effects on pigs, rats, laying hens, ducks, geese, quail and turkeys, which included detrimentally affecting their growth, food intake, thyroid size or hormone levels (cited inGriffiths et al. 1998).

In 1967, Jan Krzymanski from Agriculture Canada Research Station of Saskatoon discovered a cultivar, Bronowski, with low levels of glucosinolates (Busch et al. 1994). In 1974, Baldur Stefansson of the University of Manitoba developed world's first zero-erucic, low glucosinolate *B. napus* cultivar, Tower (Bell 1982). Later Sid Pawlowski of Saskatoon station successfully crossed two species, *B. napus* and *B. campestris*, and introduced a third species, *B. juncea* (Busch et al. 1994). In 1977, a new cultivar, Candle, was released, which became the world's first zero erucic acid low-glucosinolate, *B. campestris* cultivar (Bell 1982).

In the late 1970s, the term "canola" was registered by the Western Canadian Oilseed Crushers' Association, which is now called the Canadian Oilseed Processor Association (Canola Coucil of Canada 2001). Present official definition of canola requires an oil that contains less than 2% erucic acid, and the solid component of the seed contains less than 30 micromoles of any one or any mixture of 3-butenyl glucosinolate, 4-pentenyl glucosinolate, 2-hydroxy-3 butenyl glucosinolate, and 2-hydroxy-4-pentenyl glucosinolate per gram of air-dry and oil-free solid (Canola Coucil of Canada 2001).

Currently, Canada has approximately 110 registered *B. napus*, and 28 *B. rapa* spring varieties, 3 *B. napus* winter varieties, 3 high erucic acid rapeseed (HEAR)

varieties, 76 herbicide-resistant varieties and another 12 varieties registered in Eastern Canada only (Canola Council of Canada 2003).

2.1.2 Crop usage

Canola is now widely used as an edible oil. In January 1985, canola oil was granted Generally Recognized as Safe (GRAS) status in use of foods by the U.S. Food and Drug Administration. Canola oil has lower levels of saturated fats than any other widely used edible oil. It is also rich in monounsaturated fatty acids and has an intermediate level of polyunsaturated fatty acids. The three key nutrients of canola oil – oleic acid, alpha-linolenic acid (ALA) and vitamin E help protect blood vessels against early, damaging events in the development of artherosclerosis, which is an inflammatory disease in which lipids build up within the artery wall, thickening the blood vessel wall and restricting blood flow (McDonald 2001; Morris 2002). In addition, canola oil also performs well in food processing and fast food industry due to its desirable properties (Busch et al. 1994).

Canola also serves as an important source of protein for animal feed. Canola meal is widely used in pig diets around the world. Current data showed that canola meal, when properly formulated in pig diet, will support high levels of feed intake and efficient performance (Canola Council of Canada 2001b).

Canola is well adapted to cool, short-season conditions found on the Canadian prairies and northern Great Plains border states of the U.S.A. (Johnston et al. 2002). Its role in double-cropping and rotation has been extensively studied under different systems

worldwide, including effects on soil-borne diseases (Wilson and Phillips 1987 ; Nielsen et al. 2003; Gossen and Derksen 2003; Turkington et al. 2000), crop production (Arshad et al. 2002; Wilson and Phillips 1987; Pullins and Myers 1998; Maali and Agenbag 2003; Soon and Clayton 2002), soil microbial biomass (Lupwayi et al. 2001), weed community (Blackshaw et al. 2001) and mineral levels (Soon and Clayton 2002; Soon and Arshad 2002).

2.1.3 Transgenic canola

Transgenic canola varieties have been introduced to Western Canada since 1995. In 2000, over 80% of growers in Western Canada chose transgenic varieties and planted them on approximately 55% of the 12 million canola acres (Canola Council of Canada 2001a). The registered transgenic canola in Canada are RoundUp Ready (glyphosate (N-(phosphonomethyl) glycine) tolerant), Liberty Link (glufosinate (2-amino-4-(hydroxybenzonitrile) butanoic acid) tolerant) and the Navigator varieties (bromoxynil (3,5-Dibromo-4-hydroxybenzonitrile) tolerant) (Stringam et al. 2003). The key benefit and motivator for growers to adopt transgenic canola is due to more efficient weed control and higher yield comparing to conventional canola varieties. Data in 2000 showed that averagely transgenic varieties resulted in a 3 bu/acre or 10% yield and $5.80/acre increase in net return advantage over conventional varieties (Canola Council of Canada 2001a). Since the early 1990's, growers in Western Canada have been reducing their tillage operations for soil conservation benefits. Before the introduction of transgenic canola varieties, growers use tillage or incorporating herbicides to control weed prior to seeding a crop. With transgenic herbicide tolerant varieties, weed control

can be done “in-crop” and thereby tillage was reduced and soil conservation was achieved (Canola Council of Canada 2001a).

In addition to herbicide resistance, transgenic canola was studied in various systems. *Bacillus thuringiensis* (Bt) cry 1Ac gene was introduced on canola for insect control (Stewart et al. 1996; Ramachandran et al. 1998a; Halfhill et al. 2001; Ramachandran et al. 1998b). Other transgenic varieties or study on canola include resistance to *Leptosphaeria maculans*, a causal agent of blackleg of canola (Kazan et al. 2002), kanamycin resistance (Arnoldo et al. 1992), metal tolerance (Nie et al. 2002; Anoop et al. 2003) and enhanced expression of nutrients, such as methionine (Altenbach et al. 1992), lysine (Falco et al. 1995), and phytase (Ponstein et al. 2002).

The common concern of commercial release of transgenic canola into cropping system is the risk of gene flow from the genetically modified (GM) canola and the risk of them becoming volunteer weeds (Hall et al. 2000; Kwon and Kim 2001). Studies have so far not been able to give a clear answer for the risk of using transgenic canola (Rieger et al. 2002; Andow 2003; Baker and Preston 2003; Kwon et al. 2001; Kwon and Kim 2001).

2.1.4 Production and economic importance

Canola is the most important oilseed crop in Canada. The harvested area of canola in 2003 was 11,587,200 acres, and Saskatchewan had almost always the largest harvest acreage among all the provinces in Canada. The average yield of canola in Canada is 0.567 tonnes/acre (25.4 bushels/acre) in 2003 (Canola Council of Canada 2004). Annual canola cash receipts rose from $197.1 million in 1992 to a record of

$622.3 million in 1998, but totalled $483.6 million in 2002. Manitoba exported rapeseed and canola seed valued at $205.5 million in 2002. Approximately 99% of canola exports went to Japan, Mexico, the U.S.A. and China (Manitoba Agriculture 2003).

Canola is also produced extensively in Europe, Asia, Australia, and to a more limited extent in the United States. During the past 20 years, this crop has passed peanut, sunflower and, most recently, cottonseed in worldwide production, and become second only to soybean as the most important source of vegetable oil (Raymer 2002).

2.1.5 Growth stages and condition

Canola growth stages consist of pre-emergence (germination), seedling, rosette, bud, flowering and ripening. The length of each growth stage is greatly influenced by temperature, moisture, light, nutrition and variety. Canola is a relatively cool season crop, and its best growth occurs above 12 ºC and below 30 ºC. The optimum temperature for maximum canola growth and development has been estimated at just over 20 ºC (Canola Council of Canada 2001d). Soil moisture is one of the major factors controlling canola germination. Canola seed requires a high percentage of its weight in water before germination can begin. Vegetative and root growth result in a gradual increase in water use, and the peak use period occurs during the flowering stage. As the crop ripens, its ability to transmit water from the soil declines and water use decreases. Canola plants require a threshold amount of water before any yield is obtained. Beyond that threshold increasing amounts of water will result in higher yields. Usually 25 mm of water will result in about 150 kg/ha. (2.75 bu/ac.) of yield with good growing conditions and adequate fertility (Canola Council of Canada 2001c).

2.1.6 Diseases on canola

Canola diseases are mainly caused by fungal pathogens except herbicide injury, sulphur deficiency and Aster yellows caused by mycoplasma like organisms (Martens et al. 1994). Fungal diseases of canola and pathogen names are shown in Table 2.1.

Seedling stand establishment is a widespread problem of canola production. Poor stand establishment may be caused by a seedling disease complex, which is caused primarily by *Rhizoctonia solani*, but *Fusarium* spp. and *Pythium* spp. may also be present. The symptoms of this complex appear during the four weeks following seeding, or up to the fourth leaf stage. The complex exhibits several symptoms called seed decay, pre- and post-emergence damping-off, seedling blight and seedling root rot (Martens et al. 1994).

Blackleg caused by *Leptosphaeria maculans* and sclerotinia stem rot caused by *Sclerotinia sclerotiorum* are the two most economically important diseases on canola in Canada. Blackleg is becoming an increasing problem in the Canadian prairies. Based on interaction phenotypes on the differential canola cultivars: Westar, Glacier, and Quinta, the blackleg isolates can be ranged into four pathogenicity groups (PGs): PG1, PG2, PG3 and PG4. PG1 isolates are weakly virulent, and PG2-4 isolates are highly virulent. PG2 strain is now widely spread in Canada. PG3 was recently reported in Western Canada by Fernando and Chen (2003).

Sclerotinia stem rot will be described in detail in the following chapter, as this is the focus of the thesis.

Table 2.1 Fungal diseases and pathogens on canola (modified from Martens et al. 1994).

Name of Disease	Pathogen
Seedling blight	*Rhizoctonia solani*
	Pythium spp., *Fusarium* spp.
Foot rot (basel stem rot)	*Fusarium* spp.
	R. solani
Root rot (brown girdling root rot)	*R. solani* and associated fungi
White rust (staghead)	*Albugo candida*
Black spot	*Alternaria brassicae*
	A. raphani
Downy mildew	*Peronospora parasitica*
White leaf spot and gray stem	*Pseudocercosporella capsellae*
Sclerotinia stem rot (stem blight)	*Sclerotinia sclerotiorum*
Blackleg	*Leptosphaeria maculans* (*Phoma lingam*)
Fusarium wilt	*Fusarium oxysporum* f.sp. *conglutinans*

2.2 *Sclerotinia sclerotiorum*

2.2.1 Introduction

Sclerotinia sclerotiorum is the causal agent of sclerotinia stem rot on canola (Martens et al. 1994). *S. sclerotiorum* was first described as *Peziza sclerotiorum* by Madame M. A.Libert in 1837, and later renamed as *S. libertiana* Funkel in 1870 (Purdy 1979). This binomial was accepted until it was demonstrated as inconsistent with the

international Rules of Botanical Nomenclature, so the name *S. libertiana* was changed to *S. sclerotiorum* (Lib.) Massee (Duncan 2003b; Wakefield 1924). However, it was later found that de Bary had used this Latin name earlier, so the proper name was established as *S. sclerotiorum* (Lib.) de Bary (Purdy 1979).

2.2.2 Taxonomy

Sclerotinia sclerotiorum, *S. trifoliorum*, and *S.minor* belong to the Sclerotiniaceae (Whetzel 1945 cited in Duncan 2003b) a family of the Class Ascomycotina. The early taxonomy of the three species was based on the size and general characteristics of the sclerotium, host range, and dimensions of ascospores and asci (Willetts and Wong 1980). However, several studies (Purdy 1955; Price and Colhoun 1975; Grogan 1979) showed that this system was inadequate and that a number of species, originally thought to be unique, were actually all members of the *S. sclerotiorum* species.

2.2.3 Host range

S. sclerotiorum is an ubiquitous pathogen, which has an extremely wide host range. The earliest record of the *S. sclerotiorum* host range is from a thesis by Dickson (1930), of 172 species, in 118 genera and 37 families (cited in Willetts and Wong 1980). It was updated by Boland and Hall (1994) to 75 plant families, 278 genera, 408 species, and 42 subspecies, with most of them present in the Dicotyledonae subclass of Angiospermae. The wide host range limits the crop rotations (Boland and Hall 1994). Flax (*Linum usitatissimum* L.) used to be regarded as a non-susceptible host crop that could be used in

rotation, but a recent study by Rashid (2000) found that *S. sclerotiorum* could successfully infect flax in Manitoba and Saskatchewan (Duncan 2003b).

2.2.4 Economic importance

Sclerotinia stem rot is one of the two most devastating diseases of canola in Western Canada, losses ranging from 5-100% in individual fields (Manitoba Agriculture 2002). In central Manitoba, 52% and 76% of canola fields were affected by *Sclerotinia* in 2002 and 2001 respectively (Duncan 2003a). It was reported that yield loss from sclerotinia stem rot in Minnesota and North Dakota was $16,768,955 in total in 2001 (Lamey et al. 2001).

S. sclerotiorum also cause damage to various vegetables (Willetts and Wong 1980). Losses on many crops caused by *S. sclerotiorum* in North America, Europe, and Asia were reported (Willetts and Wong 1980).

2.2.5 Disease cycle, infection and symptomology

A general life cycle for *S. sclerotiorum* on canola is shown in Figure 2.1. Ervio, Halkilahti and Pohjakallio (1964) found that sclerotia, the overwintered structure, can survive in the soil for up to 4 years (Willetts and Wong 1980). However, Duncan (2003b) suggested that sclerotial longevity was often overestimated, since his study showed that the sclerotinia viability reduced to 57.5%, 12.5% and only 2.5% at the 0 cm (soil surface), 5 cm and 10 cm depth, respectively, after buried for 12 months. When conditions are appropriate, sclerotia can germinate either carpogenically (sexual stage) or myceliogenically (asexual stage) (Willetts and Wong 1980). A cold conditioning period at

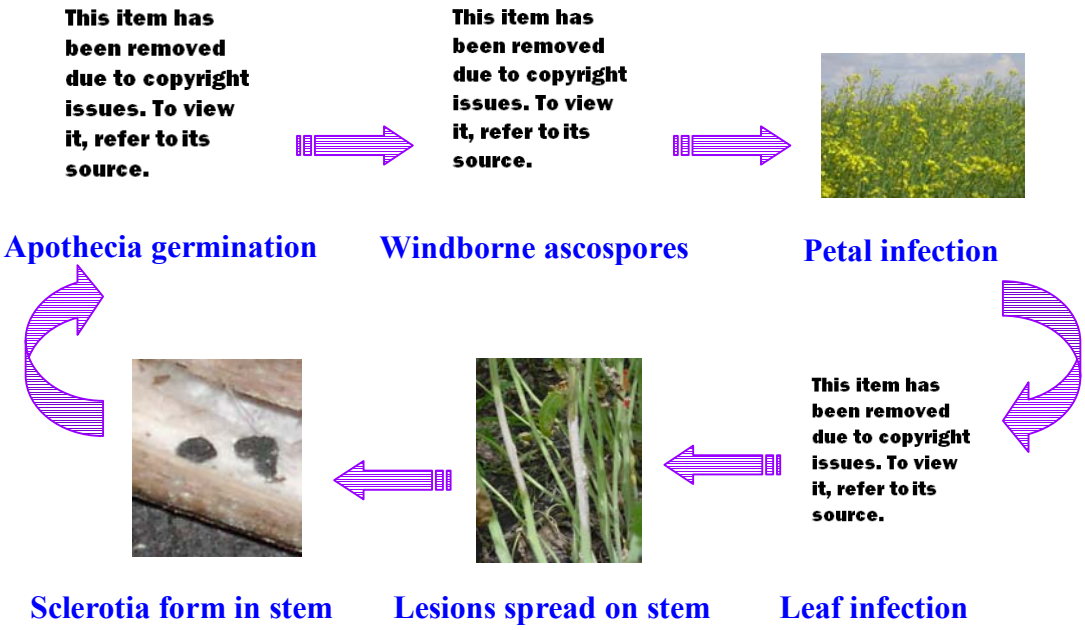

Figure 2.1 Disease cycle of *Sclerotinia sclerotiorum* (Lib.) de Bary on canola.

10 ˚C (Huang and Kozub 1989; Huang and Kozub 1991b) or 4 ˚C (Smith and Boland 1989) is usually required for carpogenic germination when sclerotia are produced at temperatures higher than 20 ˚C, except for when sclerotia produced on potato dextrose agar (Huang and Kozub 1993).

The infection occurrs mainly by ascospores, which are discharged from apothecia into the air. The sclerotia germinate in the summer, producing mushroom-like structures. These release wind-borne spores that travel up to 1 km (Manitoba Agriculture 2002) or several miles (Venette 1998).

Interestingly, senescing blossoms are the most important nutrient source for ascospore germination (Turkington and Morrall 1993). *Sclerotinia* ascospores can remain viable for years under laboratory conditions, but spores in the field can generally only survive for limited periods (Willetts and Wong 1980), although it was reported that they could survive up to several months under dry conditions (Willetts and Wong 1980). It takes a few hours for ascospores to germinate on the petals and at least 24 hours for petals to infect canola plants, providing the petals were wet (McCartney et al. 2001). The ascospore germination is optimum at temperatures of 20-25 ºC (Venette 1998). Chupp and Shef (1960) stated that *Sclerotinia* spp. could infect susceptible hosts over a range of temperatures from 0-25 ºC, with optimum at 15 to 20 ºC . However, McCartney et al. (2001) stated that under close saturated humid conditions, lesions were initiated in 2-4 days at temperatures 20-25 ºC and continuous high humidity was needed between 24-48 hours to initiate lesion formation from ascospores on petals placed on leaves. Several studies showed that humidity and leaf wetness played an important role in oilseed rape/canola

infection via petals (Venette 1998; McCartney et al. 2001). *S. sclerotiorum* can also spread through plant-to-plant contact by mycelium, but this form of infection is rare (Venette 1998).

Mechanical pressure seems to be of major importance in initial penetration of the host plant, but subsequent colonization occurs mainly by enzymic tissue dissolution (Willetts and Wong 1980). Echandi and Walker (1957) showed that *S. sclerotiorum* produced pectin methylesterase and polygalacturonase on wheat bran, and Hancock (Hancock 1966) showed that polygalacturonase could break down pectic substances in sunflower and tomato tissues. Hemicellulolytic enzymes (Hancock 1967) and cellulolytic enzymes (Lumsden 1969) produced by *S. sclerotiorum* were found to be capable of degrading araban and galactan in sunflower hypocotyls and degrading native cellulose from bean cell wall, respectively. Maxwell and Lumsden (1970) found that oxalic acid (OA) was produced by *S. sclerotiorum* and it appeared to act synergistically with pectic and cellulolytic enzymes for destruction of host tissues. OA deficient mutants of *S. sclerotiorum* were found non-pathogenic in bioassays using bean plants though harbouring a full complement of degradative enzymes (Godoy et al. 1990a); therefore, OA is an essential pathogenicity determinant (Dickman and Chet 1998). The accumulation of oxalic acid offers a lower pH environment, which is a better condition for the activities of other enzymes (Dutton and Evans 1996).

The sclerotinia stem rot symptoms begin on canola usually as a soft, watery rot on leaves or stems. The plants then develop pale-grey to white lesions, at or above the soil line and on upper branches and pods. When a stem is completely girdled, the plant wilts and

dies. The diseased stems usually appear to shred and hard black sclerotia form in the hollow centre of the stem. Thomas and Evans (1981) in Alberta demonstrated that there was a close relationship between *Sclerotinia* incidence (percent infected stems) and disease loss (Lamey 1998).

2.2.6 Epidemiology

S. sclerotiorum is currently found across Canada (Bardin and Huang 2001) as well as in countries such as U.S.A. (Koike 1999), Norway, Australia (Kohli and Kohn 1996) and China (Zhao and Meng 2003). The pathogen is mainly transported and initially infected canola as ascospores, and ascopores were reported to be able to travel up to several miles by wind (Venette 1998). Although mycelial infection by plant contact is limited, but it could be greatly favoured when plant lodging happens. Stelfox et al. (1978) reported that *S. sclerotiorum*-contaminated rapeseed pollen grains transported by honey bees were involved in the spread of sclerotinia pod rot of rapeseed (Bardin and Huang 2001).

Sclerotinia stem rot is favoured by cool temperatures and prolonged periods of precipitation. In dry weather, disease progression can be slowed or stopped, but resumes when extended periods of plant wetness favour fungal growth (Venette 1998). Several factors may contribute towards plant wetness, such as closed canopies, narrow rows, restricted wind patterns, irrigation, prolonged dew, and frequent rains.

2.3 Management of *S. sclerotiorum* on canola

2.3.1 Host resistance

Canola cultivars currently registered in Canada have little or no resistance to *S. sclerotiorum* (Kharbanda and Tewari 1996). Plant resistance is very difficult to attain for this pathogen, as it is controlled by additive gene action as demonstrated in bean (Fuller et al. 1984). Krűger and Stoltenberg (1983) have developed cultivars less susceptible to *S. sclerotiorum*, but lower yield was found in these cultivars in sclerotinia-free conditions (cited in Kharbanda and Tewari 1996). Recently, a few partial resistant cultivars were reported in China, including Zhongyou 821 and Zhongshuang No.9. Zhongshuang No. 9 was found to be resistant to *S. sclerotiorum* and lodging, and contains only 0.23% erucic aicd and 22.69 μmol/g glucosinolates in commercial seeds and also has high yield and extensive adaptability (Wang et al. 2003).

2.3.2 Cultural management

Several cultural methods can be used to help control sclerotinia stem rot on canola. Seed without sclerotia contamination should be used. Studies by William and Stelfox (1980) and Morral and Dueck (1982) showed a 3- to 4-year rotation did not reduce the incidence of the disease on canola. Nelson (1998) showed that even when the sclerotia population is as low as 1 sclerotium/800cm of soil, 5 to 6 years rotation were needed and even such a long rotation did not guarantee the elimination of *S. sclerotiorum*. Since *S. sclerotiorum* has an extremely wide host range, it attacks crops such as beans, sunflowers, mustard, sweet clover, and potatoes; therefore, it also limits the crops that can be used in a rotation to manage the disease.

Tillage can be regarded as an effective method of reducing the disease by burying the sclerotia (Gulya et al. 1997). However, Kurle et al. (2001) found carpogenic germination is more probable within the upper 5 cm soil profile, and this result is consistent with the study from Duncan (2003b). A significant negative relationship was found between sclerotial viability and depth of burial, and between sclerotial viability and populations of colonizing bacteria under zero-tillage condition (Duncan 2003b). These results suggest proper tillage might help reduce the disease, but what kind of tillage should be employed has not been clearly answered.

Larger plant spacing will help prevent pathogen from spreading through plant-to-plant contact, and also decrease the relative humidity, which will slow down the disease progression. Using lodging resistant cultivars will also help reduce the disease.

2.3.3 Disease forecasting

The current sclerotinia forecasting is mainly based on weather forecasting and petal test that is available by using a commercial test kit. To help canola growers predict outbreaks of sclerotinia stem rot, several disease forecasting systems have been developed in Canada, such as Alberta's online forecasting system (Government of Alberta 2004) and Agrometeorological Centre of Excellence in Carman, Manitoba (ACE 2001).

The petal test was developed to predict the disease based on a significant relationship between disease incidence and the level of infestation of rapeseed petals by *S. sclerotiorum* ascospores at early bloom stage (Turkington et al. 1991a). This method suggested petals should be sampled in the afternoon, due to the diurnal variation of the

petal infestation level (Turkington et al. 1991b). Several factors, such as canopy density, rain fall and sample size, were shown to influence the relationship between disease incidence and petal infestation (Turkington et al. 1991b; Turkington and Morrall 1993; Turkington et al. 1988).

2.3.4 Chemical control

The main method of control of *Sclerotinia* diseases is achieved by applying fungicides, like Benlate® (benomyl), Ronilan® (vinclozolin), Rovral® (iprodione) and Quadris® (azoxystrobin) (Minister of Agriculture 2001; Morrall et al. 1985). The production of Benlate® has already been ceased in 2001 due to the public heath concerns and the claiming of crop damage by farmers (Gilmour 2001). Fungicides should be applied before symptoms of stem rot are visible, at the 20-30% bloom stage of the crop.

2.3.5 Biological control

Biological control is a promising method of control of *Sclerotinia* diseases (Bardin and Huang 2001). The concept of biological control, biocontrol mechanisms and application in control of *Sclerotinia* will be discussed in the following chapter, as it is the focus of this thesis.

2.4 Biological control

2.4.1 Introduction

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 1983a). The first direct application of biocontrol antagonists to control plant pathogen was made by C. Hartley (1921) inoculating soil with 13 antagonistic fungi in an attempt to control damping-off of pine seedlings (Cook and Baker 1983d). There has been a remarkable increase of interest and research on biological control since 1960s, because of the concern about environmental pollution, development of pesticide resistant in pathogens, lake of adequate or reliable resistance in crops to many important pathogens, and the trend towards more intensive farming with less crop rotation (Cook and Baker 1983d)

Antagonists are biological agents with the potential to interfere in the life processes of plant pathogens, such as fungi, bacteria, nematodes, protozoa, viruses, viroids, and seed plants (e.g., trap plants) (Cook and Baker 1983b). Biological mechanisms involved in disease suppression are diverse and include parasitism, competition, antibiosis and plant induced resistance (Handelsman and Stabb 1996).

2.4.2 Biocontrol mechanisms

2.4.2.1 Antibiosis

2.4.2.1.1 Introduction

W. Roberts (1894) was the first to note antibiotic action in cultures and introduced the term antagonism into microbiology (Cook and Baker 1983c). One remarkable example of application of antibiosis in the early study of S.Y.Yin et al. (1957) is that a *Streptomyces* spp. was selected from among 4000 isolates of actinomycetes from roots of cotton and alfalfa based on *in vitro* antibiosis to *Rhizoctonia solani* and

Verticillium albo-atrum, and that the strain was used on 6 million hectares of cotton over 30 years, giving increased crop growth in China (cited in Cook and Baker 1983c). There are numerous reports on the evidence of antibiosis in biocontrol of plant pathogens. Some of the recent studies include the antagonistic effects of *Streptomyces violaceusniger* strain G10 on *Fusarium oxysporum* f. sp. cubense race (Getha and Vikineswary 2002), the biocontrol of fire blight which caused by *Erwinia amylovora* by *Pantoea agglomerans* strain Eh252 in orchards (Stockwell et al. 2002) and the biocontrol of Pythium damping-off of pea by *Burkholderia cepacia* (Heungens and Parke 2001).

There are numerous reports that antifungal metabolites produced by bacteria *in vitro* are involved in disease suppression *in vivo*. These antifungal metabolites include phenazine-1-carboxylic acid (PCA) (Mavrodi et al. 1998; Delaney et al. 2001), 2, 4-diacetylphloroglucinol (2,4-DAPG) (Nowak Thompson et al. 1994; Mavrodi et al. 2001), pyoluteorin (Plt) (de Souza and Raaijmakers 2003; Nowak Thompson et al. 1999; Brodhagen et al. 2004), pyrrolnitrin (Prn) (de Souza and Raaijmakers 2003; Chernin et al. 1996), zwittermicin A (Raffel et al. 1996), butyrolactones (Gamard et al. 1997), hydrogen cyanide (Flaishman et al. 1996), kanosamine (Milner et al. 1996a), oligomycin A (Kim et al. 1999), oomycin A (Howie and Suslow 1991), etc.

2.4.2.1.2 Major bacterial antibiotics in biological control

The ability to produce phenazines is limited almost exclusively to bacteria and has been reported in *Pseudomonas*, *Streptomyces*, *Nocardia*, *Sorangium*, *Brevibacterium*, and *Burkholderia* spp. (Turner and Messenger 1986). Phenazines are produced by a considerable number of *Pseudomonas* biocontrol strains (Chin-A-Woeng et al. 1998;

Anjaiah et al. 1998; Tambong and Hofte 2001; Thomashow et al. 1990; Thomashow and Weller 1988). Delaney et al (2001) stated that despite the phenazine biosynthetic locus being highly conserved among fluorescent *Psedomonas* spp., individual strains differ in the range of phenazine compounds they produce. Phenazine-1, 6-dicarboxylic acid is the first phenazine formed, and it is thought to be converted to PCA, a key intermediate in the synthesis of other phenazines by fluoresent pseudomonads (Delaney et al. 2001). The mode of action of phenazines has been studied extensively and several strains have already been developed into commercial biocontrol products (Chin-A-Woeng et al. 2003).

The antibiotic 2, 4-DAPG is a major determinant in the biocontrol activity of the plant growth-promoting rhizobacteria (McSpadden Gardener et al. 2001). Numerous studies have demonstrated that 2, 4-DAPG producing *Pseudomonas* spp. can suppress a wide variety of plant pathogens including fungi, bacteria and nematodes (Cronin et al. 1997a; Cronin et al. 1997b; Fenton et al. 1992; Duffy and Defago 1997; Keel et al. 1992). Antifungal action of *P. fluorescens* CHA0, *P. fluorescens* Q2-87 and *P. fluorescens* F113 against *Thielaviopsis basicola*, *Gaeumannomyces graminis* var. *tritici* and *Pythium ultimum is* due to the production of 2, 4-DAPG (Fenton et al. 1992; Harrison et al. 1993; Keel et al. 1992; Shanahan et al. 1992)

Plt and Prn were reported to be isolated from several *Pseudomonas* and *Burkholderia* spp., and both of them play an important role in the suppression of multiple plant diseases (de Souza and Raaijmakers 2003). Plt production has also been

documented for strains of *Enterobacter agglomerans*, *Myxococcus fulvus*, *Corallococcus exiguous*, *Cystobacter ferrugineus* and *Serratia* spp. (Hammer et al. 1999).

Zwittermicin A is an aminopolyol antibiotic produced by *B. cereus* and *B. thuringiensis* (Stabb et al. 1994; He et al. 1994; Raffel et al. 1996; Stohl et al. 1999b). A worldwide survey of *Bacillus* spp. soil insolates showed that there were 10^4 cfu of zwittermicin A producers in every gram of soil tested (Stabb et al. 1994; Raffel et al. 1996). Zwittermicin A does not only inhibit diverse eukaryotes and prokaryotes, but also interact synergistically with Bt toxin to enhance the insecticidal activity of *B. thuringiensis* (Broderick et al. 2000; Silo Suh et al. 1994).

Interestingly, some bacteria can produce more than one antibiotic. Several *P. fluorescens* strains produce both Prn and Plt (de Souza and Raaijmakers 2003). *Pseudomonas* spp. strain PHZ48 produces both phenazine and pyrrolnitrin (de Souza and Raaijmakers 2003). *P. cepacia* strain 5.5B, a biocontrol agent of *Rhizoctonia solani* was also reported to produce both phenazine and pyrrolnitrin (Cartwright et al. 1995). *P. fluorescens* Pf-5 was reported to produce 2,4-DAPG, pyoluteorin and pyrrolnitrin ((Nowak Thompson et al. 1994;de Souza and Raaijmakers 2003). *B. cereus* UW 85 was reported to produce both zwittermin A and kanosamine (Milner et al. 1996a; Silo Suh et al. 1994). Biocontrol agents with ability of producing multi-antibiotics might have greater potential for broad-spectrum disease suppression.

2.4.2.1.3 Identification and characterization of antibiotic-related genes/gene clusters

The application of molecular methods expands our knowledge of antibiotic production. Several genes or gene clusters responsible for major antibiotics, such as phenazine, 2, 4-DAPG, plt, prn and zwittermicin A have been isolated and characterized. Phenazine biosynthetic locus was found to contain a seven-gene core operon, designated *phzABCDEFG* (Mavrodi et al. 1998) from *Pseudomonas fluorescens* 2-79, and *phzXYFABCD,* from *P. aureofaciens* 30-84 (Mavrodi et al. 1998; Delaney et al. 2001), respectivey. This biosynthetic operon is responsible only for synthesis of PCA. A gene *phzO* which located immediately downstream of the core biosynthetic operon of strain 30-84 was not found accordingly in strain 2-79, and *phzO* was found to associate with the biosynthesis of 2-hydroxylated phenazine compounds in strain 30-84 (Delaney et al. 2001). Six open reading frames (*PhlEDBCAF*) responsible for the biosynthesis of 2,4-DAPD were identified, and *phlD* is responsible for the production of monoacetylphloroglucinol (MAPG). *PhlA*, *PhlC* and *PhlB* are necessary to convert MAPG to 2,4-DAPG, and they may also function in the synthesis of MAPG (Bangera and Thomashow 1996; Bangera and Thomashow 1999). The pyoluteorin biosynthetic gene cluster was identified and characterized for *P. fluorescens* Pf-5 (Kraus and Loper 1995; Nowak Thompson et al. 1999), and there are ten genes (*plt*) required for the biosynthesis of plt. Hammer et al. (1997) found that four genes (*prnABCD*) are required for prn production, and the function of each gene product was further described (Kirner et al. 1998). Hammer et al. (1999) also found that the *prnABCD* gene cluster was highly conserved among six pyrrolnitrin-producing strains, including *Pseudomonas*, *Burkholderia* and *Myxococcus* spp.. Milner et al. (1996a) showed that a 1.2-kb DNA

fragment defined an open reading frame referred to as *zmaR* which encoded a zwittermicn A resistance determinant. Further study by Stohl et al. (1999b) suggest that *zmaR* is necessary for high-level resistance to zwittermicin A, but it is not required by but temporally associated with zwittermicin A production.

The characterization of these antibiotic-related genes has facilitated the primer design (Raaijmakers et al. 1997) and the use of the polymerase chain reaction (PCR) method to rapidly detect these genes. Using PCR method with specific primers to detect antibiotic-producing bacterial strains has so far been mainly used for screening biocontrol bacterial agents in soil (Raaijmakers et al. 1997; de Souza and Raaijmakers 2003; Landa et al. 2002; McSpadden Gardener et al. 2000; Picard et al. 2000). Studies of genotypic and phenotypic analysis of zwittermicin A-producing strains of *B. cereus* by Raffel et al (1996) showed that PCR with specific primers for *zmaR* is a reliable method to identify zwittermicin A producers.

2.4.2.2 Plant induced resistance

2.4.2.2.1 Introduction

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated by microorganisms or environmental stress (van Loon et al. 1998). Signalling pathways and the defence reactions or responses are the two main research areas in induced resistance. The defence reactions could happen either local or both local and in remote. The pathogen-related systemic acquired resistance (SAR) and rhizobacteria-mediated induced systemic resistance (ISR) are the two major

components of plant induced resistance (Pieterse and van Loon 1999; Bakker et al. 2003). The mechanisms of SAR and ISR has been widely integrated into biological control of plant pathogens (Bakker et al. 2003; Chin-A-Woeng et al. 2003; van Loon et al. 1998). Unlike animal immunization, plant induced resistance is generally non-specific against plant pathogens (van Loon 1997). In cucumber, for example, a primary inoculation with the fungus *Colletotrichum lagenarium,* the causal agent of anthracnose, induces SAR against several diseases caused by different pathogens (Sticher et al. 1997). In addition, the induced resistance usually persists for a relatively long time in plants, even the level may change after initial elicitation (Kuc 2001). Therefore protection of plants through plant induced resistance has a great potential in biocontrol of a wide spectrum of plant pathogens.

2.4.2.2.2 Induced systemic resistance

The induced systemic resistance (ISR) was described as the mode of action of disease suppression by non-pathogenic rhizosphere bacteria (Peer et al. 1991; Wei et al. 1991). Bacterial determinants of ISR include salicylic acid (SA), siderophores, antibiotics, and lipopolysaccharides (LPS) (van Loon et al. 1998). The involvement of ISR in disease suppression has been found in a wide range of biological control microorganisms and is a powerful mode of action in the biocontrol of both soilborne and aerial plant diseases (Bakker et al. 2003). These plant-defence-inducing bacteria also commonly enhance plant growth and are referred to as plant growth-promoting rhizobacteria (PGPR). The role of ISR in the biocontrol of plant diseases is focused on non-pathogenic rhizosphere-colonizing *Bacillus* and *Pseudomonas* spp. (Whipps 2001).

ISR of cucumber to *Colletotrichum orbiculare* by some PGPR strains indicate that some PGPR strains can induce systemic resistance to foliar pathogens when used as a seed treatment (Wei et al. 1991). Ongena et al. (1999) found the induced resistance by flurorecent pseudomonads can protect cucumber against Pythium root rot, and two of the tested strains were found to increase cucumber growth.

2.4.2.2.3 Systemic acquired resistance

Systemic acquired resistance (SAR) refers to the plant resistance triggered by necrotizing pathogens (Bakker et al. 2003). SAR requires accumulation of salicylic acid (SA) in the plant (Sticher et al. 1997), while ISR is dependent on intact responses to ethylene and jasmonic acid (JA) (Pieterse et al. 1998). SAR is also associated with the pathogenesis-related proteins (PRs) (van Loon 1997), but it was suggested that SA is not the only signal involved in the induction of PRs, but ethylene and JA might also be involved (Pieterse and van Loon 1999).

2.4.2.2.4 Plant defence-related secondary metabolites

Plant secondary metabolites are now being revealed as essential components of defence reactions in determining the fate of many host-pathogen interactions (Grayer and Kokubun 2001; Goodman et al. 1986; Bennett and Wallsgrove 1994; Nicholson and Hammerschmidt 1992). These compounds could be constitutive in plants and/or induced by “attack” or “stress” (Grayer and Kokubun 2001). Plant secondary metabolites include phytoalexins, plant phenolics, plant terpenes, sesquiterpenoids and sterols, cyanogenic

glucosides, non-protein amino acids and glucosinolates, etc. (Bennett and Wallsgrove 1994).

Pytoalexins are low molecular weight, antimicrobial compounds that are both synthesized by and accumulate in plants after exposure to microorganisms and stress (Goodman et al. 1986). Chemically, phytoalexins may belong to polyacetylenes, isoprenes and phenolics, such as flavonoids (Goodman et al. 1986). More than 350 phytoalexins have been chemically characterized from approximately 30 plant families (Kuc 1995). Most of phytoalexins are from dicotyledons, they were found in all plant organs, and some of them have structural specificity (Kuc 1995). The term "elicitor" has been commonly used to refer to compounds that induce phytoalexin synthesis in plants. There are biotic elicitors, such as bacteria, and abiotic elicitors, such as $AgNO_3$ (Kuc 2001; Mert-Turk et al. 2003).

The evidence of phytoalexins as having a role in defence has been studied in several aspects: one approach is to show that they accumulate to inhibitory concentrations at the site of pathogen development (Nicholson and Hammerschmidt 1992). Timing and cellular localization studies have also provided as evidence that supports a role for phytoalexins in the resistance of cotton to *Xanthomonas campestris* (Essenberg et al. 1992; Pierce et al. 1996) and *Verticillium dahliae* (Mace et al. 1989), oats to *Puccinia coronata* (Mayama and Tani 1982), soybean to *Phytophthora megasperma* (Hahn et al. 1985), and carnation to *Fusarium oxysporum* f. sp. *Dianthi* (Niemann et al. 1990). The modes of action of phytoalexins include disruption of membranes, especially plasma

membranes, inhibitory effects on protein synthesis, nucleic acid synthesis and respiration (Smith 1996).

Several bacterial strains have already been reported to induce plants to produce phytoalexins. For example, induced resistance of carnation to fusarium wilt by WCS417 involved phytoalexin production (van Peer et al. 1991). A *P. aureofaciens* strain induced hypersensitive response (HR) on bean cotyledons and induced defence proteins resembling the plant's response to pathogens inducing SAR (Zdor and Anderson 1992).

2.4.2.2.5 Plant phenolics

Phenolic compounds are a group of chemicals composed of one or more aromatic benzene rings with one or more hydroxyl groups (C-OH) (Armstrong 2003). There are three main biosynthesis pathways of plant phenolics: Shikimate, Acetate-melonate, Acetate-mevalonate, which are the same as phytoalexins precursors (Kuc 2001; Goodman et al. 1986). Lots of phenolics are found to be associated with plant defence mechanisms, and the modes of action of these compounds include direct toxic effects (phytoalexin and free radicals formed from lignin precursors) and the active and rapid deposition of barriers such as lignin (Bennett and Wallsgrove 1994). Therefore, lots of plant phenolics also are phytoalexins. The accumulation of phenolics is observed in different cases of disease suppression (Prats et al. 2003; Benhamou and Belanger 1998; Ongena et al. 2000; Daayf et al. 2003; Paul and Sharma 2002; Benhamou et al. 2000). The study by Daayf (2000) showed the accumulation of several phenolic compounds in both susceptible and resistant cultivars of cucumber against powdery mildew was induced by leaf extract of giant knot weed *Reynoutria sachalinensis*. A recent study by

Singh et al. (2003) showed that resistance in chickpea plants induced by *Pseudomonas* strains involed the increased induction of phenolic compounds as well as induced systemic resistance via SA-dependent pathway. In association with phenol biosynthesis, the activity of phenylalanine ammonia-lyase (PAL) and other biosynthetic enzymes might be enhanced, such as tyrosine ammonia-lyase (TAL) (Goodman et al. 1986), cinnamic acid (Shiraishi et al. 1989) and peroxidases (Southerton and Deverall 1990).

2.4.2.3 Plant growth promoting rhizobacteria

As one kind of the biocontrol agents, plant growth promoting rhizobacteria (PGPR) increase plant growth either by indirect suppression of diseases caused by pathogens or by reducing the deleterious effects of minor pathogens (Whipps 2001). Siddiqui and Shaukat (2002) showed three PGPR strains *P. fluorescens* CHA0, *P. aeruginosa* IE-6 S+ and *Bradyrhizobium japonicum* 569Sm(r) not only suppressed root-infecting fungi and root-knot nematodes but also enhanced growth of tomato plants both under glasshouse and field conditions.

PGPR may also increase plant growth in some other ways, for example, by solubilization of nutrients such as P (Whitelaw 2000), releasing phytohormones (Beyeler et al. 1999) and decreasing heavy metal toxicity (Burd et al. 1998).

2.4.2.4 Competition

Competition for niche and/or nutrients is another kind of biocontrol mechanism. A classical example of niche exclusion is the control of leaf frost injury caused by *P. syringae*, which has an ice-nucleation protein on its cell surface. Application of an ice-

nucleation-minus mutant could prevent damage caused by pathogenic wild type strains by competing for the niche with the wild type strains (Chin-A-Woeng et al. 2003; Lindow et al. 1983).

Competition for nutrients, such as carbon, nitrogen and iron, has been demonstrated in several studies to reduce the pathogens' capacity to propagate in the soil (Buyer and Leong 1986; Buyer and Leong 1986; Neilands and Leong 1986; Fernando et al. 1996). Iron is essential for growth for all organisms, but the availability of solubilized Fe^{3+} in soils is limiting at neutral and alkaline pH, leading to Fe^{3+} limitatation (Chin-A-Woeng et al. 2003). Under Fe^{3+} limiting conditions, most organisms will take up ferric iron through high-affinity iron chelators, designated as siderophores. The ability to produce efficient siderophores is sometimes associated with the ability to take up related siderophores from other organisms (Raaijmakers et al. 1995; Koster et al. 1995).

2.4.2.5 Parasitism and predation

Parasitism and predation is a fairly common phenomenon among microorganisms (Cook and Baker 1983b). The study of Fahima et al. (1992) suggested that mycoparasitism of *Verticillium dahliae* microsclerotia by *Talaromyces flavus* hyphae may be involved in the biological control of verticillium wilt disease. The parasitism of macroconidia of *Fusarium culmorum* by several *Pythium* spp. was shown to be involved in disease suppression on barley seedings (Davanlou et al. 1999).

Predation is a phenomenon which appeares more common in microorganisms or mites that feed on insets or grasses (Boulter et al. 2000; Roy and Pell 2000) other than on

plants. One interesting example of predation is *Allothrombium mitchelli* Davis, a large red velvet mite, was found to feed on *Cryptococcus fagisuga* Lindinger, an insect species and a major component of beech scale disease on American beech gaps in the southern Appalachian mountains (Wiggins et al. 2001).

2.4.2.6. Other mechanisms

A variety of other mechanisms of biological control have been studied, some of which include disease reduction by lytic enzymes produced by bacteria and fungi such as β-(1,3)-glucanases (Ruiz Duenas and Martinez 1996), cellulases (Chatterjee et al. 1995), and proteases (Szekeres et al. 2004), and some of which are involved in the breakdown of fungal cell wall (Chin-A-Woeng et al. 2003).

Using oxalic oxidase-producing bacteria in controlling *S. sclerotiorum* is a novel biocontrol method. Oxalic acid (OA) is known to play a critical role in the success of infections caused by *S. sclerotiorum* (Maxwell and Lumsden 1970; Noyes and Hancock 1981; Godoy et al. 1990b). Less susceptible cultivars were found to be able to withstand a higher dose of the compound than those that were known to be more susceptible to *S. sclerotiorum* in white bean (Tu 1985) and sunflower (Noyes and Hancock 1981). Dickman and Mitra (1992) developed a selective plating technique to identify bacterial strains capable of degrading OA. This method was then used to rapidly identify potential biocontrol bacterial strains in several subsequent studies (Dickman and Chet 1998; Savchuck 2002).

2.4.3 Biocontrol of *S. sclerotiorum*

There has been a strong interest in biocontrol of *Sclerotinia* diseases among Canadian researchers in the last few decades, due to the increasing concern over the use of chemical pesticides (Bardin and Huang 2001). Parasitic fungi have been widely studied as biocontrol agents for *S. sclerotiorum*, such as *Coniothyrium minitans* Campbell (Huang 1980), *Talaromyces flavus* (Huang and Erickson 2000), and *Trichoderma* spp. (Huang and Kozub 1991a; Huang and Kokko 1993; Huang 1980). These biocontrol agents were applied to the soil and inhibit the sclerotia carpogenic germination. Recent research on *C. minitans* (Vrije et al. 2001) has led to the development of a commercial biopesticide named "Contans". Hedke et al. (1999) showed 60% disease suppression on oilseed rape by using "Contans" in a 2-year trial, but their experimental design was based on macroplots surrounded by guard areas to prevent major influences of invading external ascopores. Earlier tests in microplots of oilseed rape proved a reduction of soil inoculum; however, this neither led to disease control nor to a yield improvement (McQuilken et al. 1995). The reasons for this are generally attributed to the fact that *S. sclerotiorum* ascospores dispersal occurs over long distances (Venette 1998) and even a reduced number of sclerotia in a field can cause significant infection and yield loss (Davies 1986) thus strongly affecting adjacent plots (Hedke et al. 1999). Therefore studies on foliar applied biocontrol agents are worthwhile (Boyetchko 1999).

Bardin and Huang (Bardin and Huang 2001) suggested the effective colonization of bean flowers by *C. minitans* appears to be an effective mechanism to prevent *Sclerotinia* ascospore infection. However, Huang et al. (2000) showed that the

efficacy of disease suppression on dry bean was not as consistent as fungicide benomyl in the filed when *C. minitans* was applied as a spray. Earlier studies using fungal antagonists screened from growth-room trials did not control white mold of bean consistently in the field (Inglis and Boland 1992).

Bacterial biocontrol agents against *S. sclerotiorum* are rarely studied (Boyetchko 1999). Godoy et al. (1990c) showed the *Erwinia herbicola* and *Bacillus polymyxa* strains could inhibit the *S. sclerotiorum* growth *in vitro*. Bean plants pre-treated by *E. herbicola* in growth chambers were found to have significantly ($P<0.05$) less disease severity than that of control plants. The effective control of white mold of bean under the field conditions by *E. herbicola* was not repeatable in a two-year trial (Yuen et al. 1992). Lyth et al (1993) reported that *Bacillus* spp. could reduce apothecium formation by applying it into the soil. *B. cereus* Frankland and Frankland, strain alf-87A was reported to reduce incidence of basal pod rot of pea caused by ascospore infection of *S. sclerotiorum*, when applied as a spray (Huang et al. 1993). Foliar spray of *B. subtilis* (Ehrenberg) Cohn revealed a reduction in white mold in white bean in a two-year field trial (Tu 1997). However, inconsistent results were found by Boland (Boland 1997) using *B. subtilis* to control white mold of bean between fields. Savchuck (2002) screened several bacterial antagonists against *S. sclerotiorum* in the lab and greenhouse, and *P. chlororaphis* PA-23 had significant disease suppression of sclerotinia stem rot on canola under field conditions in 2001. *P. chlororaphis* PA-23 was shown to produce antibiotic phenazine-1-carboxylic acid in plate assays (W. G. D. Fernando, *unpublished*). *Pseudomonas* spp. #41 was found to inhibit *Sclerotinia* ascospore germination and germ tube growth using microscopic techniques. These results suggest that antibiosis might

play a vital role in the *Sclerotinia* control on canola. Boyetchko (1999) suggest that it was worthwhile to investigate foliar-applied bacteria to control *S. sclerotiorum* through various mechanisms such as antibiosis.

Very few studies have been done on induced resistance to *S. sclerotiorum* in canola/oilseed rape. Systemic resistance to *S. sclerotiorum* by oxalic acid in oilseed rape was the first report of systemic resistance against *S. sclerotiorum* in oilseed rape (Toal and Jones 1999). Oxalic acid is the major pathogenesis factor of *S. sclerotiorum.* Plants treated with oxalic acid were reported to have significantly smaller leaf lesions than the control plants after being challenged by the pathogen (Toal and Jones 1999). No study has been reported on ISR on canola/oilseed rape elicitated by bacterial agents.

Some related studies on plant induced resistance against *S. sclerotiorum were conducted on sunflower (Bazzalo et al. 1985; Prats et al. 2003) and kiwifruit (Reglinski et al. 1997; Reglinski et al. 2001).* Coumarin phytoalexins were found in sunflower to inhibit the growth of *S. sclerotiorum (Urdangarin et al. 1999)***.** Prats et al. (2003) showed the amount of phenolic compounds accumulated after inoculation **with** *S. sclerotiorum* **correlates with the sunflower line, the time after inoculation and the tissue.** Higher constitutive and induced phenolic content as well as phenylalanine ammonia-lyase (PAL) activity were found in the most resistant lines, and these differences correlated with the absence/presence of disease symptoms. Earlier studies also showed that *Sclerotinia*-sunflower interaction directed towards soluble phenolics in infected- and non-infected crop lines and wild relatives, and general lower phenolic accumulation was found in the *Sclerotinia* tolerant varieties (Bazzalo et al. 1985).

Very few studies have been done in induced resistance on canola/oilseed rape. Brassinin, methoxybrassinin, and cyclobrassinin were the first phytoalexins reported from *Brassica* spp. induced by *Leptosphaeria maculans* (Soledade et al. 1992). Study in local and systemic changes in glucosinolates in Chinese and European cultivars of oilseed rape treated with *S. sclerotiorum* suggests that glucosinolates induction may be an important marker of resistance to *S. sclerotiorum* (Li et al. 1999). Pathogen infection, insect feeding and mechanic wounding can also induce the changes in glucosinolates (Doughty et al. 1991; Koritsas et al. 1991; Bodnaryk 1992). These studies indicated that secondary metabolites might play an important role in canola against pathogen *S. sclerotiorum.* However, the current studies on phenolic compounds of rapeseed/canola are mainly for the agronomic purposes other than phytopathological purposes (Naczk et al. 1998). No study has been reported on the relationship between phenolic compounds and induced resistance on canola/oilseed rape against *S. sclerotiorum*.

3.0 EVALUATION OF BIOLOGICAL CONTROL OF *SCLEROTINIA SCLEROTIORUM* ON CANOLA (*BRASSICA NAPUS*) BY BACTERIA THROUGH *IN VITRO* SCREENINGS, GREENHOUSE EXPERIMENTS, AND A FIELD STUDY

3.1 Abstract

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* (Lib.) de bary is found worldwide on canola and rapeseed. Eight Gram-positive bacterial strains isolated from canola and wheat plants were inhibitory against mycelial growth of *S. sclerotiorum* on both PDA and LBA plates. Three *Bacillus* spp., one *Staphylococcus* spp. and one *Pseudomonas* spp. (control) were tested in whole plant assays. Canola petals were pre-treated with bacteria, and then inoculated with *S. sclerotiorum* ascospores (10^5 spores/ml) 24 h later. Seventeen days after inoculation, bacteria and ascospores-treated (BAT) plants had only 0.0-1.0 disease severity (DS) compared to 4.5 in ascospores-treated (AT) plants. Disease incidence (DI) on stems was 0.0-12.5% for BAT plants and 75% for AT plants. DI on leaves was 0.0-50% and 100% for BAT and AT plants, respectively. The BAT plants also had healthy seeds and significantly higher yields than those of AT plants. The time of application experiment in the greenhouse suggested the highest disease suppression was achieved when *B. amyloliquefaciens* BS6 was inoculated at the same time as pathogen. In the 2003 field experiment (Trial two), DI was 5.8-12.5% on stems of bacteria-treated plots and 6.7% in the fungicide-treated (Rovral Flo) plots, which were significantly different ($P < 0.05$) from ascospore-treated control plots (20.4%). The DS on stems was 0.121-0.283 on bacteria-treated plots and 0.167 on fungicide-treated plots

compared to 0.426 in ascospore-treated control plots. The bacterial strains were effective antagonists against *S. sclerotiorum* under laboratory, growthroom, and field conditions.

3.2 Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a pathogen on more than 400 plant hosts, including canola, on which it causes stem rot (Nelson 1998). It is one of the most important diseases of canola in Western Canada (Martens et al. 1994), causing yield losses of up to 70% in Manitoba, Canada (Duncan 2003a). The resistance to *S. sclerotiorum* is very hard to built up on canola, because it is governed by multiple genes (Fuller et al. 1984). Crop rotation is also unrealistic due to the long survival ability of over-wintered structures (sclerotia) in the soil and the vast host range of this pathogen (Nelson 1998). These limitations necessitate the use of fungicides, which have adverse effects on non-target organisms (Campbell 1989) and allow the pathogen to develop resistance. There is also an increasing concern that pesticide residues in soil and water may be harmful to the environment (Huang et al. 2000). Due to the relatively unreliable control attained using cultural methods and due to the public concern over pesticide use, alternative methods of disease control, such as biological control, should be considered.

Research on biocontrol of *S. sclerotiorum* has been primarily focused on the control of carpogenic germination or apothecia production by using hyperparasitic fungal antagonists (Huang 1980; Huang and Erickson 2000; Huang et al. 1997). Recent research on the *Coniothyrium minitans* Campbell (Vrije et al. 2001) has led to the development of a commercial biopesticide named “Contans”. However, the studies by Hedke et al. (1999) and (McQuilken et al. 1995) suggest that the reduction of the number of sclerotia or

apothecia present in the field by *C. minitans* may not preclude infection caused by ascospores from neighbouring fields. In addition, Davies (1986) found that the presence of only a few apothecia in the field might still result in relatively high disease incidence levels. Hence, research on limiting petal infection by ascospores in biocontrol of *S. sclerotiorum* is needed.

Bacterial biocontrol agents against *S. sclerotiorum* are rarely studied (Boyetchko 1999). Early studies by Godoy et al. (1990c) and Yuen (1992) showed that bacterial strains could be used to control white mold of bean caused by *S. sclerotiorum*. Savchuck and Fernando (2004) isolated and screened two Gram-negative bacterial strains (*Pseudomonas chlororaphis* PA-23 and *Psedomonas* spp. #41) against *S. sclerotiorum* in the lab and greenhouse. The field trials in 2001 to evaluate these two strains showed *P. chlororaphis* PA-23 double application significantly reduced sclerotinia stem rot on canola (Savchuck 2002). However, the bacterial survival ability in the field was not clear. During the replication of the field trial in 2002, no disease was caused by inoculating the soil surface with sclerotia. Further replication of the field study, the concerns of using ascospores as disease inoculation and investigating bacterial survival ability are necessary. *Bacillus* spp. are well known for having a better surviving ability under field conditions due to the production of endospores (Boyetchko 1999; Collins and Jacobsen 2003). Therefore screening *Bacillus* spp. to use as phyllosphere biocontrol agents is needed. In addition, using genotypically different bacteria is essential in the screening process of biocontrol agents (Roberts and Lohrke 2003); therefore, other Gram-positive bacteria should also be considered.

The objectives of this study were (i) to isolate *Bacillus* spp. (or other gram-positive bacteria) antagonists against *S. sclerotiorum* by preliminary screening for production of antibiotics, oxalate oxidase and/or volatiles in plate assays, and (ii) to evaluate a select group of potential antagonists from these assays as to the degree of control attainable in the greenhouse and in the field (including strains PA-23 and #41). In the greenhouse study, bacterial agents will be screened by a general biocontrol test and a selected strain will be evaluated on the effect of the time of application on disease control. Two field trials will be conduced using different disease inoculation methods and conditions.

3.3 Materials and methods

3.3.1 *Bacillus* spp. isolation and storage

Bacterial antagonists used in this study were isolated from canola leaves. Samples were collected from a field (Carman Research Centre, University of Manitoba, Carman) that had four years of tillage and rotation practices, and also from a collection from Saskachwan in 2002. Plant material was randomly collected from each field, bagged and then stored in the lab at 4 °C until the isolations were carried out (within one week). A modified method (Kim et al. 1997) to isolate *Bacillus* spp. was employed. One gram of canola leaves was cut into pieces and put into a tube containing 10 ml sterile, distilled water, and the tube was vortexed briefly. After incubating at 80 °C for 20 min, 100 µl solution was spread on both NA (Nutrient Agar, Difco Laboratories, Detroit, Mich., U.S.A.) and PYDA [2 g peptone, 2 g yeast extract, 5 g dextrose, 15 g agar (Difco Laboratories, Detroit, Mich., U.S.A.) L^{-1}] (Brown 2001). Plates were incubated at 30 °C

for 48 h. Typical white colonies were picked up individually and re-streaked on fresh NA plates. Bacteria was stored in Luria Bertani broth [LB broth contains 10.0 g tryptone peptone, 5.0 g yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 5.0 g NaCl L^{-1}] amended with 20% glycerol (v:v) at –80 °C. Isolates used in the plate assays, greenhouse and field trials were all sub-cultured onto Luria Bertani Agar [LBA contains 15.0 g agar technical, 10.0 g tryptone peptone, 5.0 g yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 5.0 g NaCl L^{-1})] plates from storage and assessed for viability and purity before use.

3.3.2 Evaluation of bacterial antagonism through general plate inhibition assays

Inhibition of *S. sclerotiorum* by bacterial strains *in vitro* was assayed on both LBA and PDA (39g Potato Dextrose Agar, Difco Laboratories, Detroit, Mich., U.S.A.) media. One loop of bacteria taken from fresh sub-cultures grown on LBA plates was inoculated into 3ml LB broth and placed on a shaker at 30 °C for 16 hours, or until mid-log phase of growth. Five microliters of the bacterial suspension was pipetted onto either LBA or PDA media in 15×100 mm petri plates at one side of the plate near the periphery, and the bacterial solution was then streaked to a line. The plates were sealed with parafilm® and incubated at 30 °C for 24 h to allow sufficient growth of the colonies. Mycelial plugs, 8 mm in diameter, were taken from actively growing cultures of *S. sclerotiorum* (Canadian isolate Clone 33 from L.M. Kohn, University of Toronto, Canada, was used in all of the biocontrol studies) and placed at the opposite side of each petri plate, and plates were incubated at room temperature. Measurements of radial mycelial growth were taken after 72 h on both bacterial and control plates. Twenty

isolates were assayed for the initial screening on both LBA and PDA using 2 replicates per bacterial treatment for each medium type. Eight gram-positive strains available in the lab, which showed antagonism against other pathogens, were also tested in a similar way. Strains causing inhibition on either PDA or LBA were stored at –80 °C for further study. Strains which showed inhibition zones on both LBA and PDA were re-assayed with 5 replicates, and results were compared by Least Significant Difference (LSD) using Analysis of Variance (ANOVA) in SAS software (Crow 2002). For all general screenings and for the volatile assays, controls were fungal cultures grown without antagonists.

3.3.3 Antagonism through production of volatile compounds

Bacterial inhibition through volatile production was accessed using a modified method used by Fernando and Linderman (1994). All 16 bacterial isolates were streaked onto one half of a divided plate containing tryptic soy agar (TSA, Difco Laboratories, Detroit, Mich., U.S.A.) and the plates were immediately wrapped in parafilm® to seal in the volatiles. Following a 72 h incubation period at 30 °C, 8 mm plugs taken from the periphery of an actively growing culture of *S. sclerotiorum* were placed onto the other side of the plate (containing PDA) and the plates were re-sealed immediately. Strain PA-23 was used as positive control (Savchuck 2002). Measurements of radial mycelial growth were taken after 72 hours on both bacterial and control plates. Three replicates per isolate was conducted for the initial screening, and isolates demonstrating average > 25% inhibition or 100% inhibition in some of plates were retested with 5 replicates, and bacteria were allowed to grow 5 days before the *S. sclerotiorum* inoculation.

3.3.4 Testing for the presence of the oxalate oxidase enzyme

Oxalic acid is known to be the major pathogenesis factor of *S. sclerotiorum* infection (Tu 1985; Noyes and Hancock 1981; Godoy et al. 1990a; Godoy et al. 1990b; Maxwell and Lumsden 1970). To detect the ability of producing oxalate oxidase enzyme, all 16 bacterial isolates were screened twice using the method of Dickman and Mitra (1992) and Savchuck (2002). Bacteria were streaked onto plates containing oxalic acid degradation selective media (1.0 g potassium oxalate, 1.0 g ammonium phosphate, 0.5 g potassium phosphate, 0.2 g magnesium sulphate, 1.0 g sodium chloride, 1.5% agar L^{-1} medium) and incubated at 30 °C in the dark. Plates were scored for the presence or absence of the enzyme (presence of the enzyme being indicated by a clearing in the media around the bacterial growth) following an incubation period of 7 days.

3.3.5 Bacterial identification

The 16 bacteria isolates in the lab which had inhibition against *S. sclerotiorum* on either LBA or PDA plates were Gram-stained (Gram Stain Set, Protocol™, Biochemical Sciences, Inc. Swedesboro, NJ, U.S.A.) and checked for their morphology under the light microscope using the oil lens.

Strains BS6, BS8, H and E16, which were used in the greenhouse study, were further identified using the Biolog© Microplates™ (Microlog, Hayward, CA, U.S.A.) with the accompanying software. Isolates were assayed using GP (Gram positive) Microplates™.

3.3.6 Production of rifampicin-resistant strains and establishing of growth curves

In order to harvest bacteria from the canola petals after inoculation in the greenhouse and field experiments, rifampicin-resistant isolates of #41, BS6, BS8, H and E16 were generated by selective growth of these isolates on LBA amended with rifampicin. Bacteria were grown in LB broth at 30 °C (28 °C for strain #41) for 16 h. One hundred millilitres of bacterial culture was spread on LBA amended with 150 ppm rifampicin (Sigma®, St. Louis, MO, U.S.A.), and wrapped in aluminium foil to avoid light. Plates were incubated at 30 °C (28 °C for strain #41) for 5 days, and individual colonies were picked out and re-streaked on LBA amended with 150 ppm rifampicin. Rinfampicin-resistant strains were stored at –80 °C as previously described and marked as #41-R, BS6-R, H-R, and E16-R. Strain PA-23, used in field experiment was rifampicin-resistant, and the culture is available in the lab. All the bacterial strains used in greenhouse and field study were rifampicin-resistant strains.

The relationship between bacterial growth rate and OD value was established for all the rifampicin-resistant strains of PA-23, #41, BS6, BS8, H and E16 by growing them at 28 ˚C (PA-23 and #41) or 30 ˚C (BS6, BS8, H, and E16) with a rotation speed of 180rpm.

The growth of strain BS6-R on media containing nystatin and rifampicin was also determined. Strain BS6-R was grown in LB broth with or without 100 ppm rifampicin. After incubating at 30 °C for 16 h, a serial dilution was conducted on half NA, half NA amended with both 6.7 ppm nystatin (Sigma®, St. Louis, MO, U.S.A.) and 100 ppm rifampicin, and half NA amended with 100 ppm rifampicin. The plates were

covered by aluminium foil and incubated 30 °C for 1-2 d. The colonies of 20-200 on the plates were counted, and the concentrations of bacterial solution were estimated in each media type.

3.3.7 Evaluation of biocontol agents against sclerotinia on canola in the greenhouse

Canola plants (var. Westar) were grown in the growthroom at 16/ 21 °C and 16 h photoperiod. Plants were inoculated with bacteria and ascospores using a dipping petal method. Plants inoculated with ascospores without bacteria were used as control. Four bacterial isolates BS6, BS8, H and E16 were tested in this experiment, and strain #41 which showed significant ($P<0.05$) disease suppression in the growthroom (Savchuck 2002) was used as the positive control. Eight plants were used in each treatment.

Bacteria were grown at 30 °C for 16 h (strain #41 was grown at 28 ˚C) and concentrations were adjusted to log8 cfu/ml according to the OD values. Petals were sampled and immediately put onto ice. Petals were dipped into bacterial solutions (10^8 cfu/ml), and briefly dried on water agar for 1 h. Petals were then dipped into *S. sclerotiorum* ascospores (10^5 spores/ml), and briefly dried on water agar for another 1 h before the petals were placed onto plant leaves. Plants were kept in the humidity chamber for 3 days (24/16 °C, 16 photoperiod and RH=87%) before being moved to growth cabinet (25/19 °C, 16 photoperiod). 0.02% Tween 20 was used in all inoculum. DI and DS were recorded every 3 days in the following two weeks in the growth cabinet. DI was recorded as 0 (no symptom) and 1 (visual symptom). DS was recorded as 0-7: 0, no

symptom; 1, leaf symptom with no stem symptom; 2-7, stem lesions: 2, 0-20 cm; 3, 21-40 cm; 4, 41-60 cm; 5, 61-80 cm; 6, 81-100 cm, 7, >100 cm or plant death.

The ascospores used in the greenhouse and field studies were purchased from Dr. M.G. Boosalis, Department of Plant Pathology, University of Nebraska, Lincoln, NE, U.S.A..

3.3. 8 Evaluation of the effect of time of application of *Bacillus* spp. BS6 against sclerotinia on canola and bacterial survival in the greenhouse

Plants were grown to 30% bloom stage, and inoculated with *S. sclerotiorum* ascospores (10^4 spores/ml) with/without bacteria by spraying the plants at different times. The ascospore and bacterial solutions were prepared as previously described. Plants were inoculated with ascospores only, ascospores 1 d and 2 d prior to bacteria, ascopores and bacteria at the same time, and ascopores 1 d and 2d after bacteria. Plants were sprayed with the inoculum until run-off, after being kept in humidity chamber for 4.5 days (24/16 °C, 16 photoperiod and RH=87%), plants were then moved to the growthroom (24/16 °C, 16 photoperiod). DI was taken 1 week after pathogen inoculation, and DS was taken 2 weeks after pathogen inoculation. Data was analysed by LSD test using SAS software (Crow 2002). The DI and DS were recorded as previously described. The experiment had been conducted earlier by keeping plants in the humidity chamber for 2 days after inoculation, and then moving to 21/16 °C growthroom.

Ten petals per treatment were sampled from the treatments that ascospores were inoculated 1 d and 2 d after bacteria, as well as that ascospores and bacteria were

inoculated at the same day. After 10 seconds of sonication, a serial dilution was conducted using half NA mended with 100 ppm rifampicin and plates were incubated at 30 °C for 1 to 2 days. Bacterial colonies were counted from the plates.

3.3.9 Evaluation of biocontrol agents under field conditions

Biocontrol of *S. sclerotiorum* by bacterial strains #41, PA-23, E16 and BS6 was evaluated under field conditions at the University of Manitoba Carman Research Station in Carman, Manitoba in 2003. The experiment was conducted in two individual completely randomized design (CRD) (Savchuck 2002) trials each with eight treatments and four replications. In Trial One (southern plots), the pathogen was inoculated only by ascopores at 50% bloom stage. Breeder tents (courtesy, Advanta Seeds Inc. Winnipeg, MB) were set up over the plots before flowering in Trial One to help pathogen establish the infection. In Trial Two (northern plots), pathogen was inoculated by both *S. Sclerotiorum* sclerotia and ascospores. Sclerotia were inoculated on the surface of the soil in the previous fall (2002), and the ascospores were inoculated at the 50% bloom stage. Prior to seeding, a shallow tillage was conducted in northern plots. Treatments were as follows: (1) ascospore inoculation (control); (2) Fungicide spray (Rovral Flo® 240 g/L iprodione, Bayer CropScience, Cargary, Alberta, Canada) at the recommended rate of 0.85 L/acre; (3) #41 and (4) PA-23, at one spray application; (5) PA-23 at two spray applications; (6) E16 and (7) BS6, at one spray application; (8) BS6 at two spray applications. Bacteria were sprayed at a concentration of log 8 cfu/ml. Ascospores were sprayed at a concentration of log 4 cfu/ml. All plots received an ascospore application.

Each trial consisted of 32 4×4 m plots on a 60×35m block with 3.0 m or 4.0 m alleyways between plots and 3.5 m around the entire trial. A 6.0 m alleyway was set up between the two trials. The block was situated on a well-drained, clay loam soil (Denham clay loam). Plots were seeded with Roundup Ready canola (LG3235) on May 28 using a seeding rate of 10 kg/ha. Fertilizer (23-24-0) was applied with the seed at a rate of 80 kg/ ha. The northern plots were reseeded on June 13 with a seeding rate of 18kg/ha, due to the uneven earlier germination. Fall rye was seeded between the plots on June 20. The canola seeds were pre-treated with HELIX, which contains insecticides (thiamethoxam) and the fungicides (difenoconazole, metalaxyl-M and fludioxonil). The plots were treated with Roundup Transorb™ (480 g/L glyphosate, Monsanto, Missisauga, Ont., Canada) at a rate of 0.33 L/acre, and DECIS® (27.5 g/L deltamethrin, 811g/L liquid hydrocarbons, Bayer CropScience) at a rate of 0.3 L/acre.

Plots were sprayed at 30% bloom with the bacterial and fungicide treatments on July 7 (Trial One) and July 22 (Trial Two), with the second bacterial spray applied at 50% bloom on July 10 (Trial One) and July 25 (Trial Two). Bacteria were grown until early stationary phase of growth and diluted to log 8 cfu/ml in phosphate buffer (pH = 7.0) prior to spraying. Ascospores were washed off from aluminium foil and diluted to log 4 cfu/ml prior to spraying on July 8 (Trial One) and July 26 (Trial Two). Two drops per litre Tween 20 were added as a surfactant for all inoculums. Each plot was sprayed with a uniform 4.0 L volume using a backpack sprayer. A field misting system (Lamari and Smith, University of Manitoba, *unpublished*) was employed. It consisted of 64 nozzles in each trial with two in the opposite corners of each plot. The misting system was activated for 3 min. every 2 h for 10 days after ascospore inoculation in the Trial

One, and then ran for 6 min. every 1.5 h for two weeks in the Trial Two. Each nozzle (SP16-340 Micro-Bird spinner-type) delivered water at a rate of 1.526 L /min.

Petal infestation levels were assayed by sampling 20 petals from each of two replicates of each treatment in Trial One on July 12 (3.5 days after ascospore inoculation). Petals were taken randomly from the plot and put onto ice immediately. Petals were placed onto PDA media amended with 35 ppm streptomycin sulphate (Sigma®, St. Louis, MO, U.S.A.) and potential *S. sclerotiorum* colonies identified visually within five days and affirmed through sub-culturing procedures.

Bacterial survival ability under the field condition was investigated for strains PA-23 and BS6. Twenty petals were sampled from three out of four plots for PA-23 and BS 6 double application treatments in Trial Two. Bacterial concentrations on blossom were studied as previously described in greenhouse experiments.

DI on stem was assessed on August 2 (in half of the plots) and August 22 in Trial One, and DI and DS on stem were investigated on August 25 in Trial Two, in a 1×1 m quadrate in the centre of each plot. Forty (August 2) or sixty plants (August 22 and 25) per plot were visually rated. DI was recorded as 0 (no symptom) and 1 (visual symptom). DS was recorded as 0-3: 0, no stem lesion; 1, 1-10 cm stem lesion; 2, upper branch was infected (half of plant was infected); 3, whole plant was infected. Results of DI and DS in the field were analyzed separately for each date. The results of petal infestaion level, DI and DS were analyzed using LSD test between different treatments. All statistical analyses were conducted using ANOVA by SAS software (Crow 2002).

3.4 Results

3.4.1 Bacterial isolation and identification

Nineteen bacterial strains were isolated from canola leaves (Table 3.1). They were all white colour and wavy looking when growing on NA, PYDA and LBA media. Eight strains that have antifungal activity to *S. sclerotiorum* in the general plate assay were Gram stained and shown to be Gram-positive (Table 3.1). Strains BS6, BS8 and H were identified using Biolog® as *Bacillus* spp. The identifications and sources of bacteria which were used in greenhouse and field study are shown in Table 3.2.

3.4.2 Evaluation of bacterial antagonism through general plate inhibition assays, inhibitory volatile production and oxidase enzyme presence

Sixteen strains were found to have antifungal activity to *S. sclerotiorum* in at least one kind of media (PDA and/or LBA) in the initial general plate assay (eight strains were isolated from this study and the others were avalaible in the lab). Eight strains showed inhibition of *S. sclerotiorum* hyphal growth on both PDA and LBA plates. *S. sclerotiorum* hyphal growth was significantly inhibited by these strains, and a clear inhibition zone was observed between bacterial colonies and fungal hyphae (Figure 3.1).

In volatile test, all sixteen strains were observed to produce inhibitory volatile compounds against *S. sclerotiorum*. However, the variance was very high between replicates. Complete inhibition and no inhibition of fungal growth were sometimes observed using the same bacterial strain. Strains BS8, H, E12 and E16 may be the most

Table 3.1. Nineteen bacterial strains isolated from canola leaves.

Bacterial Strain	Sample Location	Selecting Media	Gram-Stain	Cell shape
B1	Carman, Manitoba	PYDA	+	rod
B2	Carman, Manitoba	PYDA		
B3	Carman, Manitoba	PYDA	+	rod
B4	Carman, Manitoba	PYDA		
B5	Carman, Manitoba	PYDA		
B6	Carman, Manitoba	PYDA	+	rod
B7	Carman, Manitoba	PYDA		
B8	Carman, Manitoba	PYDA	+	rod
B9	Carman, Manitoba	PYDA		
B10	Carman, Manitoba	PYDA		
B11	Carman, Manitoba	PYDA		
BS1	Saskatchewan	NA		
BS3	Saskatchewan	PYDA	+	rod
BS4	Saskatchewan	PYDA	+	rod
BS6	Saskatchewan	PYDA	+	rod
BS7	Saskatchewan	NA		
BS8	Saskatchewan	NA	+	rod
BS9	Saskatchewan	PYDA		
BS11	Saskatchewan	NA		

PYDA: 2 g peptone, 2 g yeast extract, 5 g dextrose, 15 g agar L^{-1};
NA: Nutrient agar

Table 3.2. Bacterial sources and identifications.

Strain	Source	Identification	Reference
PA-23	soybean rhizosphere	*Pseudomonas chlororaphis*	Savchuck and Fernando 2004
#41	canola root tips	*Pseudomonas* spp.	Savchuck 2002
BS6	canola leaf	*Bacillus amyloliquefaciens*	this study
BS8	canola leaf	*Bacillus thringensis/cereus*	this study
H	wheat	*Bacillus subtilis*	Fernanado et al. 2002
E16	canola leaf endophyte	*Staphylococcus* spp.	Ramarathnam and Fernando, unpublished

effective in inhibition of fungal growth according to the results from several times of repeating (Figure 3.2).

The sixteen strains were also tested for oxalate oxidase presence through three replications, and no clear zone on plates was observed during the following 7 days after inoculation.

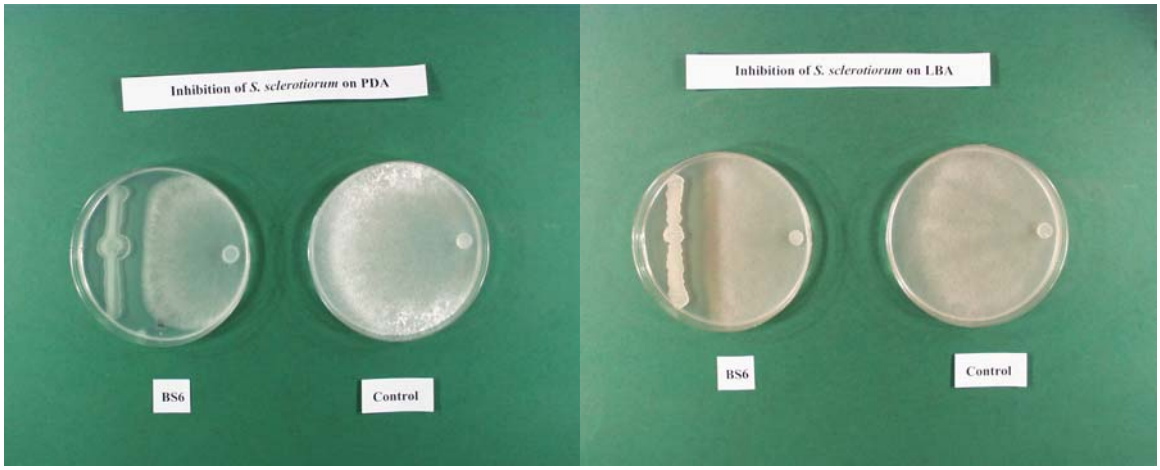

Figure 3.1. *S. sclerotiorum* growth inhibition by strain *Bacillus amyloliquefaciens* BS6 on PDA and LBA. Strain BS6 was inoculated on one side of the plate, and incubated at 30 ºC for 24 h before the inoculation of *S. sclerotiorum* mycelial plug on the other side. Mycelia growing without bacteria on the plates were used as controls. Inhibition was observed 72 h after mycelial inoculation.

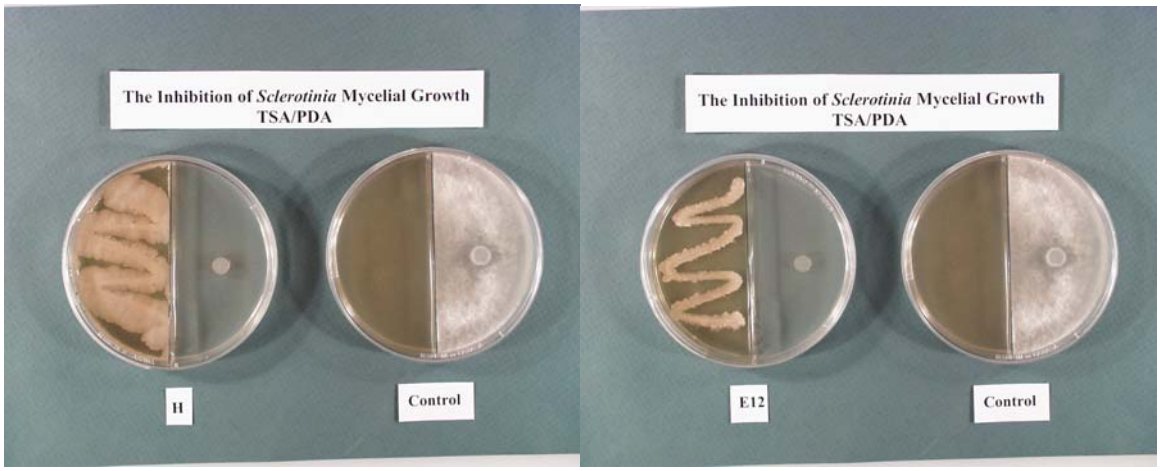

Figure 3.2. *S. sclerotiorum* growth inhibition by inhibitory volatiles produced by *Bacillus subtilis* H and *Staphylococcus* spp. E12. Bacteria were streaked on TSA, and incubated at 30 ºC for 5 days before the inoculation of *S. sclerotiorum* mycelial plug on PDA. Inhibition was observed 72 h after mycelial inoculation.

3.4.3 Production of rifampicin-resistant strains and establishing of growth curves

After three days incubation, spontaneous mutants growing on rifampicin containing media were observed. No visible difference of bacterial growth was found when single colony was restreaked on media with or without rifampicin (150 ppm). No difference was found in bacterial growth rate though serial dilution between strain BS6 mutant and mildtype after growing them at 30˚C in LB broth with/without rifampicin (100 ppm) (Data not shown). The relationship between bacterial cell density and OD value was established for rifampicin resistant strains of PA-23, #41, BS6, BS8, H and E16 (Fig.3.3) for the future greenhouse and field inoculation.

3.3.4 Evaluation of biocontol agents against sclerotinia on canola in the greenhouse

Seventeen days after inoculation, Disease incidence (DI) on leaves was 0.0-50% and 100% for bacteria and ascospores treated (BAT) plants and ascospores-treated (AT) control plants respectively (Figure 3.4). DI on stems was 0.0-12.5% for BAT plants and and 75% for AT plants, respectively (Figure 3.4). Strain BS6 achieved complete disease suppression. BAT plants had only 0.0-1.0 disease severity (DS) compared to 4.5 in AT control plants (Figure 3.5). Disease progressed much slower in plants treated with any one of the bacterial strains compared to the control plants (Figure 3.5).

The BAT plants also had healthy seeds and significantly higher yields than those of AT plants, and strain BS6 treated plants had the highest yield (Figures 3.6 and 3.7).

3.3.5 Effect of the time of application of strain BS6 against sclerotinia on canola in the greenhouse.

The experiment on the time of application of bacterial treatment showed all the bacterial treatments had significantly less DI and DS ($P<0.05$) than those of control plants (inoculated with *Sclerotinia* only). When strain BS6 was inoculated at the same time as pathogen, the highest disease suppression was achieved (Figures 3.8 and 3.9). An earlier study using less inoculation time and lower growth temperature also suggest that all the bacterial treatments had less DI on leaf than that of control plants, but symptoms are not as obvious as the later experiment.

The study of bacterial survival ability on petals showed that strain BS6 population was 10^5 cfu per petal, and it increased 10 fold after 24 hours regardless wheather plants were kept in humidity chamber or not. However, the bacterial population stared to decrease after 24 hours, and it dropped to less than 10^2 cfu per blossom (detection level) after 3 days. Similar results were observed in the earlier study, however, instead of increasing, strain BS6 population maintained its population after 24 hours.

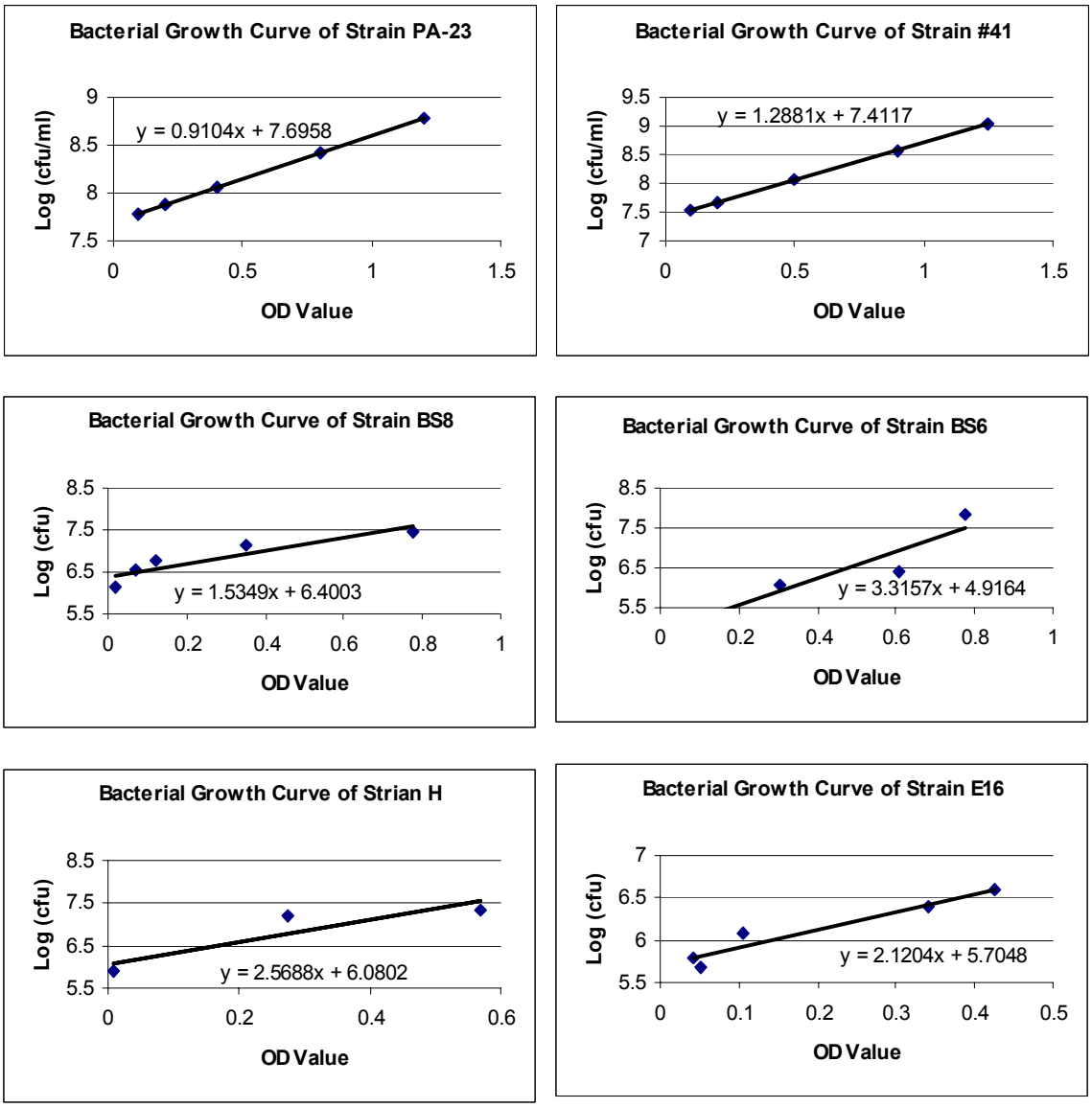

Figure 3.3. Bacterial growth curves for rifampicin resistant strains *Pseudomonas chlororaphis* PA-23, *Pseudomonas* spp. #41, *Bacillus thringensis/cereus* BS8, *B. amyloliquefaciens* BS6, *B. subtilis* H, and *Staphylococcus* spp. E16. Strains were grown in LBA at 28 ºC (PA-23 and #41) or 30 ºC (BS8, BS6, H and E16) at 180 rpm for 16 h before the measurement of OD values and the serial dilutions were conducted.

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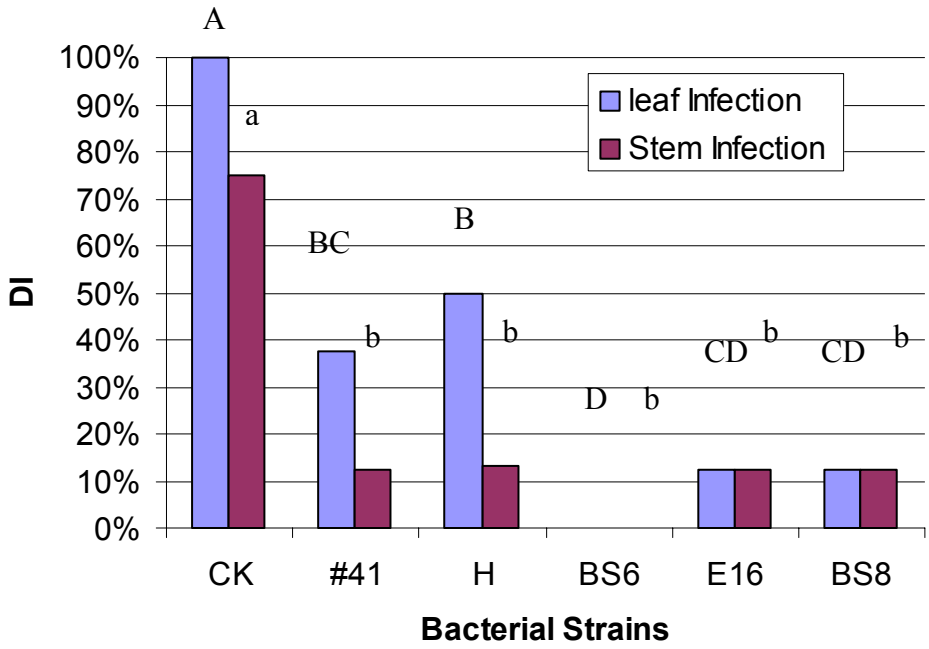


Figure 3.4. Sclerotinia disease incidence (DI) in the greenhouse test using five bacterial antagonists. Plants were inoculated with petals that were dipped into bacterial solutions (10^8 cfu/ml) and then *S. sclerotiorum* ascospore solutions (10^5 spores/ml). Symptoms were observed 5 and 8 days after inoculation. Treatments with same capital letters or lower case letters are not significantly ($P < 0.05$) different in leaf infection or stem infection, respectively. Leaf infection: LSD=0.3693; stem infection: LSD=34.82%.

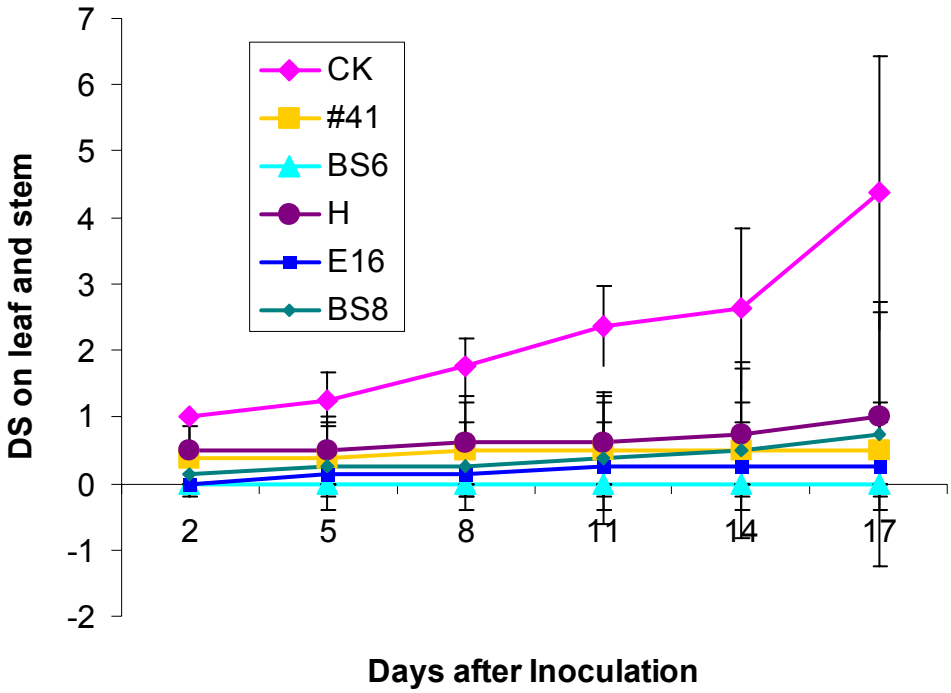


Figure 3.5. Inhibition of sclerotinia disease progression in the greenhouse by using five bacterial antagonists. Plants were inoculated with petals which were dipped into bacterial solutions (10^8 cfu/ml) and then *S. sclerotiorum* ascospore solutions (10^5 spores/ml). Symptoms were observed every 3 days after inoculation (Day 0). Bars represent standard deviation.

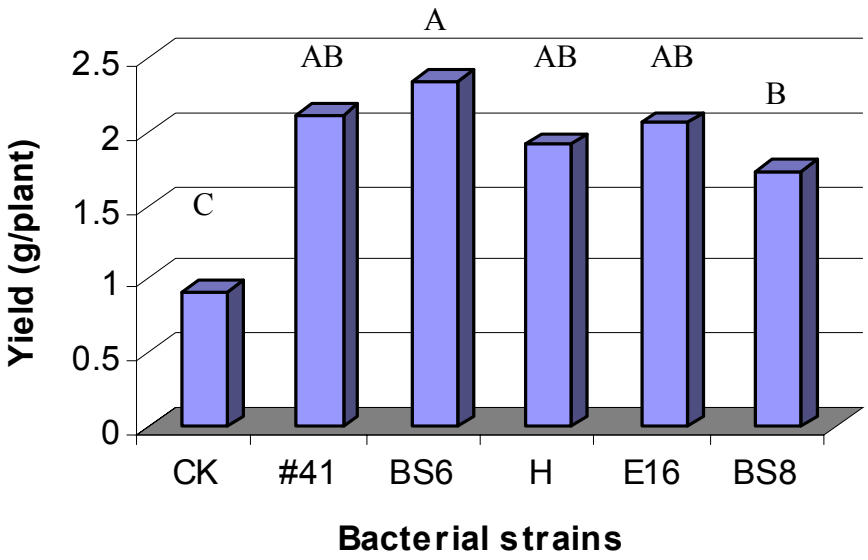


Figure 3.6. Canola yield harvested from sclerotinia diseased plants co-inoculated with bacterial antagonists in the greenhouse. Plants were inoculated with petals that were dipped into bacterial solutions (10^8 cfu/ml) and then *S. sclerotiorum* ascospore solutions (10^5 spores/ml). Canola seeds were harvested when plants reached full maturity. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 0.6078

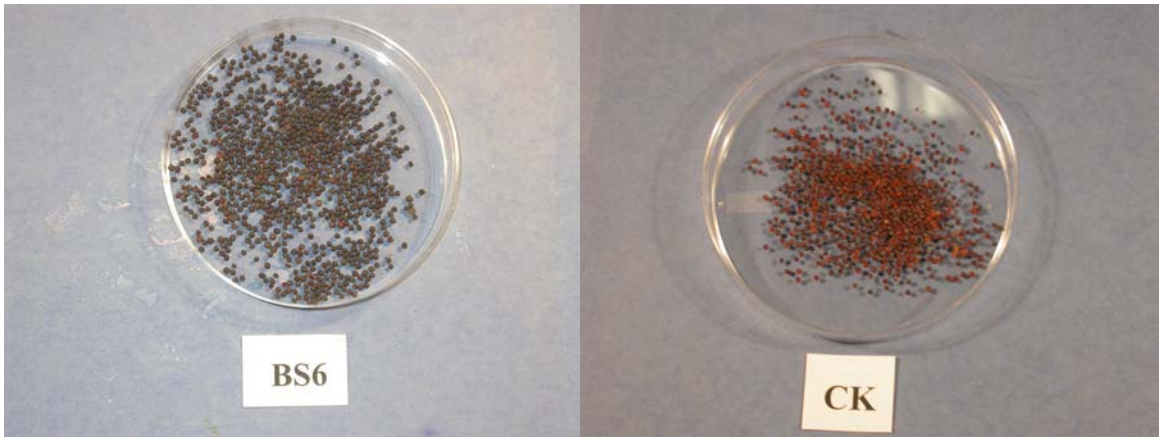


Figure 3.7. Canola seeds harvested from sclerotinia diseased plants inoculated with /without *B. amyloliquefaciens* BS6. Plants were inoculated with petals that were dipped into bacterial solutions (10^8 cfu/ml) and then *S. sclerotiorum* ascospore solutions (10^5 spores/ml). Canola seeds were harvested when plants reached full maturity.

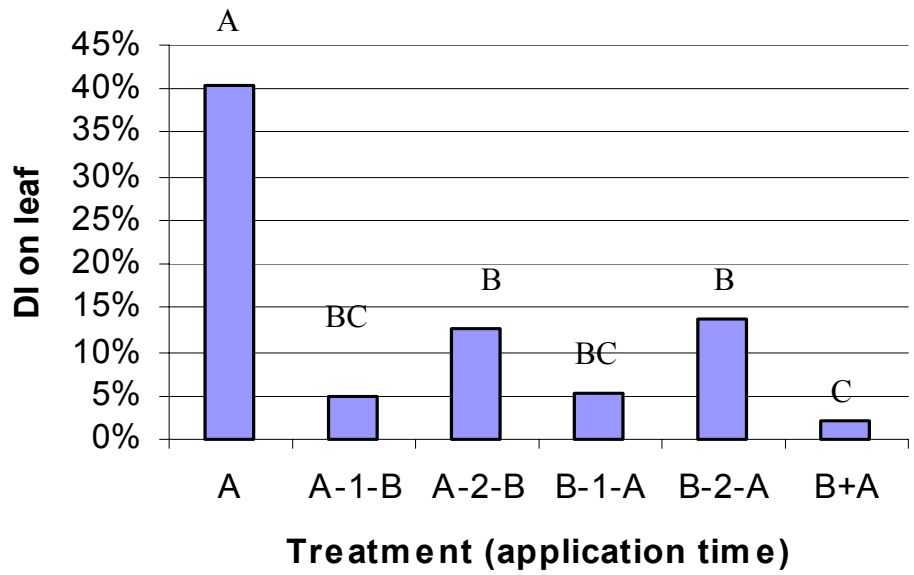


Figure 3.8. Sclerotinia disease incidence (DI) one week after ascospore inoculation. Plants were inoculated by BS6 at different time regions in the greenhouse. Treatments in order: A: ascospore only; A-1-B: ascospore 1 day prior to BS6; A-2-B: ascospore 2 days prior to BS6; B-1-A: BS6 1 day prior to ascospore; B-2-A: BS6 2 days prior to ascospore; B+A: BS6 and ascospores were inoculated on the same day. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 9.1 %

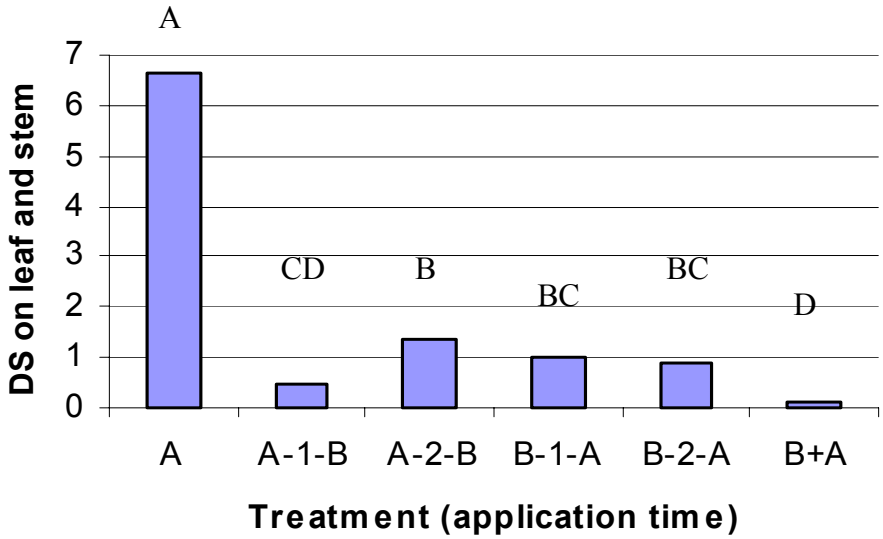


Figure 3.9. Sclerotinia disease severity (DS) two weeks after ascospore inoculation. Plants were inoculated by BS6 at different time regions in the greenhouse. Treatments in order: A: ascospore only; A-1-B: ascospore 1 day prior to BS6; A-2-B: ascospore 2 days prior to BS6; B-1-A: BS6 1 day prior to ascospore; B-2-A: BS6 2 days prior to ascospore; B+A: BS6 and ascospores were inoculated on the same day. Plants were rated based on a 0-7 scale. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 0.6875

3.4.6 Evaluation of biocontrol agents under field conditions

The field experiment of both Trial One and Trial Two showed significant disease suppression when using bacterial antagonists and achieved similar results as the fungicide Rovral Flo® (Figures 3.11-3.14). Petal infestation level during Trial One (within tents) is shown in Figure 3.10. All bacterial and fungicide treatments had less petal infestation levels, but only #41, E16 BS6 single application and PA-23 double application had significant ($P<0.05$) reduction of petals compared to that of the control plot.

In Trial One, DI on stem of plants pre-treated wit bacteria were 5-23.8% after flowering, compared to 10% in fungicide Rovral Flo® treatment and 33.8% in control plots (Figure 3.11). The DI reduction by #41, E16 and PA-23 single application were not significant ($P<0.05$) compared to that in control plots. Other bacterial treatments had significantly ($P<0.05$) less DI than that in control plots, with BS6 double application achieving the highest disease reduction. However, BS6 double application and #41 did not significantly ($P<0.05$) reduce the DI after plants reached full maturity, while all the other bacterial treatments achieved significant disease suppression ($P<0.05$) (Figure3.12). In Trial Two, when plants just reached maturity, 5.84-12.5% DI on stems was observed in bacteria-pre-treated plots, compared to 6.7% and 20.4% in the fungicide treatment and control plots, respectively (Figure 3.13). The DS on stems was 0.121-0.283 on bacteria-pre-treated plots and 0.167 in fungicide treatment compared to 0.426 in control plots (Figure 3.14). The results of DI in Trial One (Figure 3.12) and DS in Trial Two (Figure 3.14) showed that all bacterial treatments were not significantly ($P<0.05$) different from

each other, which indicated the four tested bacterial agents might have similar disease suppression abilities. The single application and double application did not have significantly effect on disease suppression by strains PA-23 and BS6 (Figures 3.11-3.14).

Both populations of strain PA-23 and BS6 on petals in the field Trial Two were $<10^2$ cfu per blossom 24 h after first inoculation; however, a significant population increase to $>10^3$ cfu per blossom of strain BS6 was detected 24 h after the second spray. No significant change in population levels of strain PA-23 was found after the second time spray.

3.5 Discussion

Results from the field study showed that all the bacterial treatments have almost similar disease suppression ability as the commercially used fungicide (Rovral Flo®). Since strains *P. chlororaphis* PA-23 had shown significant ($P < 0.05$) disease suppression in the field in 2001 (Savchuck 2002) and now in 2003, it has been consistently effective in controlling sclerotinia stem rot in two years and three station years (two identical experiments in 2003). Although the four bacterial strains tested in the field achieved generally similar disease suppression in the two field trials in this study, whether the disease suppression is through similar or different mechanisms is unknown.

The study on population dynamics is important in screening biocontrol bacteria applied on the phyllosphere. The population dynamics of *B. amyloliquefaciens* BS6 in the greenhouse is similar to those of strain #41 tested by Savchuck and Fernando (2004), despite that BS6 is a *Bacillus* spp. and #41 is a *Pseudomonas* spp.

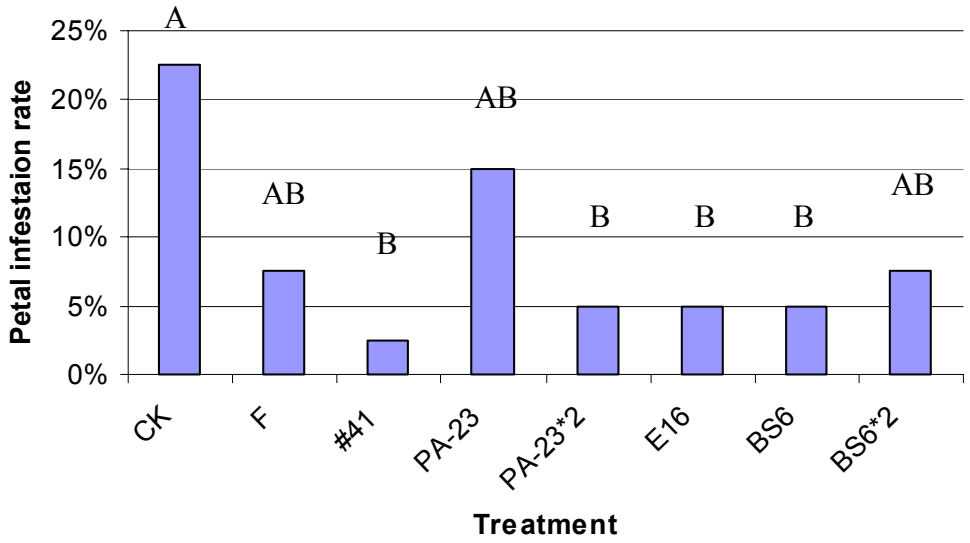


Figure 3.10. Petal infestation level in Trial One 4 days after *S. sclerotiorum* ascospore inoculation (on July 12). Bacteria and fungicide treatments were applied on July 7 and July 10 (only for PA-23*2 and BS6*2). Twenty petals per plot and two plots per treatment were sampled in Trial One. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 14.98%

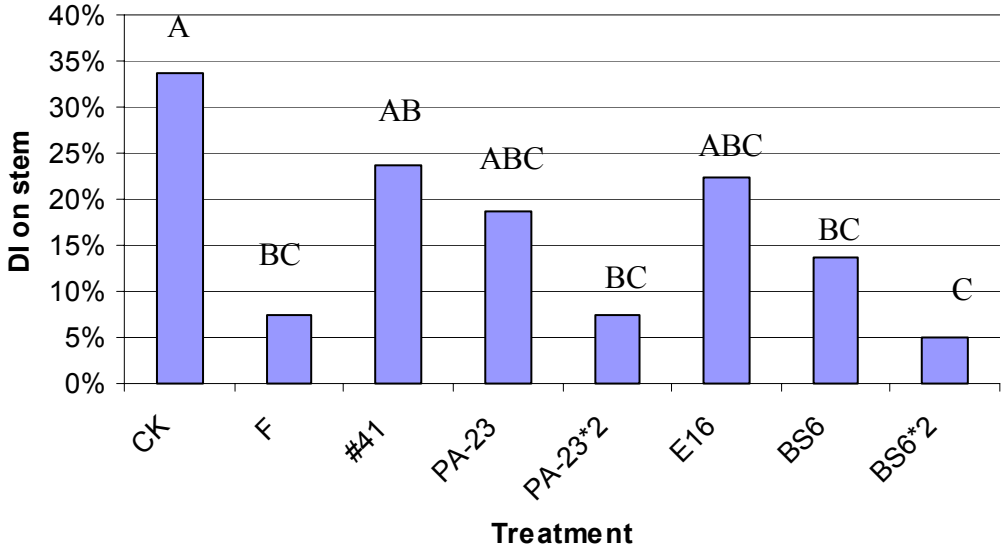


Figure 3.11. Sclerotinia disease incidence (DI) in Trial One observed right after canola flowering stage (August 2). Bacterial and fungicide treatments were applied on July 7 and July 10 (only for PA-23*2 and BS6*2). *S. sclerotiorum* ascospores were inoculated on July 12. Forty plants per plot in the 1x1 m central area were sampled from 2 plots per treatment in Trial One. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 18.68%

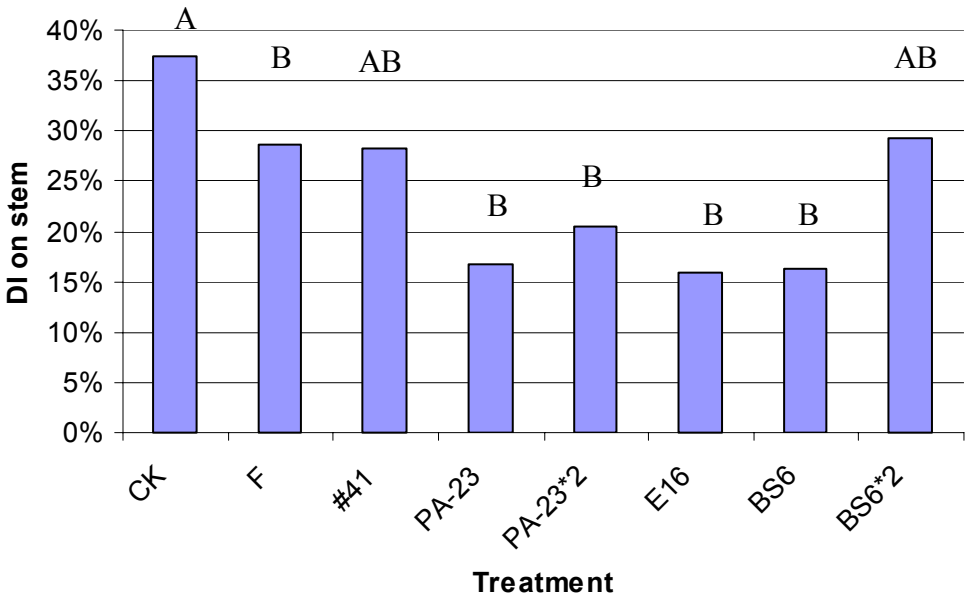


Figure 3.12. Sclerotinia disease incidence (DI) in Trial One observed when plants reached full maturity. Bacterial and fungicide treatments were applied on July 7 and July 10 (only for PA-23*2 and BS6*2). *S. sclerotiorum* ascospores were inoculated on July 12. Sixty plants per plot in the 1x1 m central area were sampled from each plot in Trial One. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 16.86%

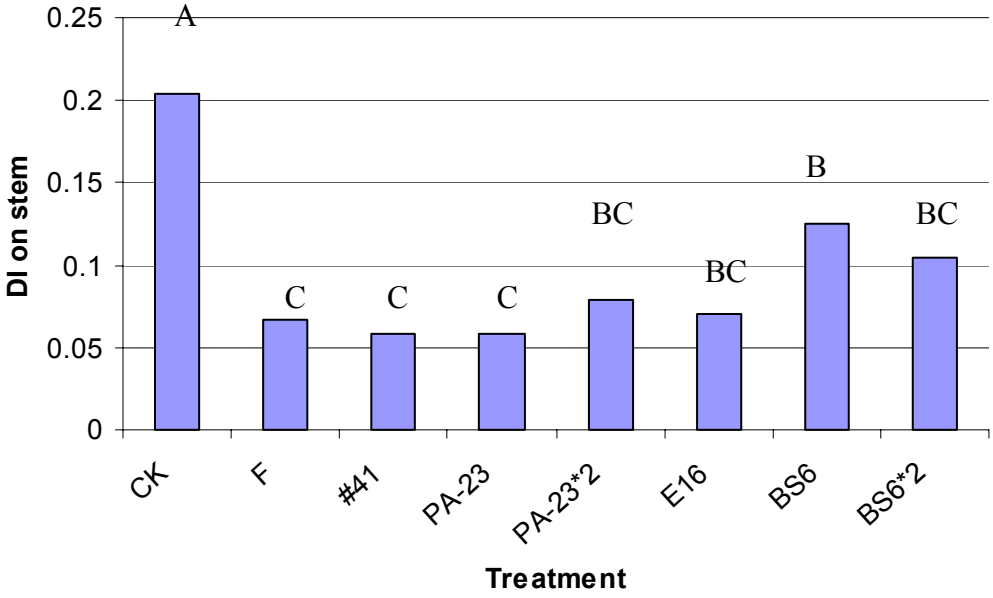


Figure 3.13. Sclerotinia disease incidence (DI) in Trial Two observed when plants just reached maturity. Bacterial and fungicide treatments were applied on July 22 and July 25 (only for PA-23*2 and BS6*2). *S. sclerotiorum* ascospores were inoculated on July 26. Sixty plants per plot in the 1x1 m central area were sampled from each plot in Trial One. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 5.48%

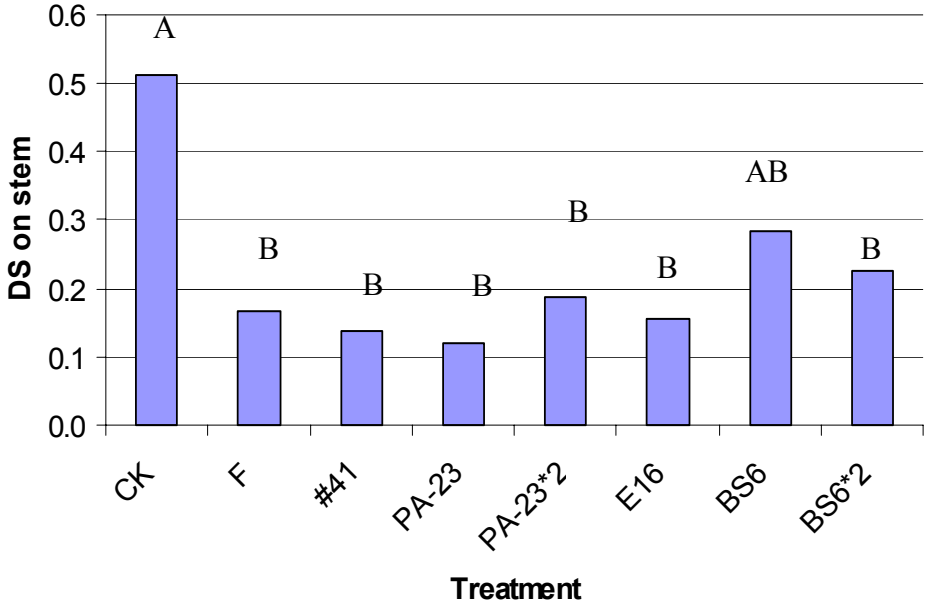


Figure 3.14. Sclerotinia disease severity (DS) in Trial Two observed when plants reached maturity. Bacterial and fungicide treatments were applied on July 22 and July 25 (only for PA-23*2 and BS6*2). *S. sclerotiorum* ascospores were inoculated on July 26. Sixty plants per plot in the 1x1 m central area were sampled from each plot in Trial One, and rated based on a 0-3 scale. Treatments with same letters are not significantly ($P < 0.05$) different. LSD = 0.1842

The multiplication of the bacterial population on blossom after inoculation was also observed by Yuen et al. (1994). They found that populations of three *Erwinia herbicola* strains, which were used to control white mold on dry bean caused by *S. sclerotiorum,* multiplied from $< 10^2$ cfu per blossom at initial level to $>10^7$ cfu per blossom within 16 h at 25 °C. However, it is unlikely that the *Pseudomonas* and *Bacillus* strains used in this study would reach such a high population level on blossom. They also found that the bacterial population level was lower in the field than in the greenhouse, which agrees with the result from this study. The results of population dynamics under field conditions suggested that *B. amyloliquefaciens* BS6 has longer survival potential than *P. chlororaphis* PA-23. This could be explained by the fact that *B. amyloliquefaciens* BS6 could probably form endospores when it encountered adverse environmental conditions. However, when comparing the DI and DS from the Trial Two, BS6 single application was not as effective as PA-23 single application in disease suppression; it achieved significantly ($P < 0.05$) less DI but not DS compared to that of control plots. However, both double application of strains BS6 and PA-23 have achieved similar disease suppression. This suggested that disease suppression ability of *B. amyloliquefaciens* BS6 might be more dependent on its population level compared to *P. chlororaphis* PA-23. *P. chlororaphis* PA-23 can achieve significant ($P < 0.05$) disease suppression even when the population on blossom is very low, and this may suggest that plant induced resistance might play a role in disease suppression. Since *P. chlororaphis* PA-23 may also produces antibiotic phenazine (Fernando, unpublished), further study on multiple mechanisms involved in disease suppression is needed. However, the DI in Trial One (Figure 3.12) and DS in Trial Two (Figure 3.14) of strains PA-23 and BS6 single applications

(population levels were both very low when encountering ascospore inoculation) are not significantly ($P < 0.05$) different from each other and it suggests that sclerotinia disease suppression by *B. amyloliquefaciens* BS6 may also involve plant induced resistance.

However, strain #41 single application and BS6 double application in Trial One did not significantly ($P < 0.05$) reduce DI compared to that of control plots when plants were mature. This might be due to plant logging within the tents. Several plots were observed with severe plant logging during and after canola flowering, and probably because of the high humidity within tents. Logging offered more plant-to-plant contact and allowed pathogen spread by mycelial infection. An intense misting system (at least run 6 min per 1.5 h after inoculation) without tents is recommended for future biocontrol tests in the field to control sclerotinia stem rot on canola.

Petal infestation level has been used for forecasting sclerotinia on canola (Turkington et al. 1991a). In this study, petal infestation level vigorously reflected the DI in Trial One in the field. Lack of accuracy could be because of disease epidemics consisting of several factors, including weather changes, relative humidity in plots and host variety etc. It could also be because of a relatively low sampling size in this study (2 sites per treatment, 20 petals per site). Sampling sizes were discussed by Turkington et al. (1988) for forecasting sclerotinia stem rot of canola using the petal test. They pointed out the minimum sample size required to estimate DI at specified levels of precision varied widely among the experiments. Increasing sampling sites or petals sampled per site might be needed in similar plot design in a future study. The study by Savchuck (2002) has shown un-uniform petal infestation by *S. sclerotiorum* according to the data collected

from 7 sampling days within 13 days. This might be explained by the random landing of ascospores in the natural system, for the plots were not inoculated with ascospores. No significant ($P < 0.05$) reduction of leaf infection was observed in 2001 and only *P. chlororaphis* PA-23 double application was able to significantly ($P < 0.05$) reduce the stem infection (Savchuck 2002). This might be explained by the low disease pressure, which indicated earlier by the petal infestation level. The application of ascospores in this study offered a more uniform and higher disease pressure compared to that in the study of Savchuk (2002). In 2002, a similar study was carried at Carman Research Centre, Manitoba, and the pathogen was inoculated by spreading over-wintered sclerotia on the surface of soil in the beginning of spring. Sclerotinia disease was rarely found after canola flowering. Even in the present study, no apothecia germination was found in Trial Two before or at the early flowering stage, based on the visual investigation of half of the plots. An ascospore inoculation (10^4 cfu/ml) is recommended for further *S. sclerotiorum* inoculation in field experiment, and misting system during the flowering stage is needed (could be activated two days before inoculation and run for at least 2 weeks after inoculation) to offer enough relative humidity for pathogen infection and disease development. Setting up tents for helping the inoculation in field is labour consuming, especially when large tents are used. The combination of tents and misting system are effective to help *Sclerotinia* ascospore inoculation in the field (Clinton Jurke, personal communication). But when both of them are used, crop lodging might happen easily.

Unfortunately the yield data from Trial Two was lost due to losing of the harvested canola samples in a big wind at the end of the season. However, the results from greenhouse experiments showed that all tested strains (BS6, BS8, H, E16 and #41)

have significantly reduced disease and increased the canola yield. Comparing yield from this study with non-treated healthy plants and plants treated only with *B. amyloliquefaciens* BS6 could help understand whether this strain has the plant growth promoting effects or not.

At the beginning of this study, several Gram-positive strains that were isolated from canola leaves were found to have antagonistic effects against *S. sclerotiorum in vitro*. The inhibition of fungal mycelial growth on LBA and PDA with zones of inhibition suggested that antibiosis might play a vital role in disease suppression *in vivo*. Bacterial antibiotics involved in fungal disease suppression have been reported in numerous studies, including phenazine-1-carboxylic acid (PCA) (Mavrodi et al. 1998; Delaney et al. 2001), 2,4-diacetylphloroglucinol (2,4-DAPG) (Nowak Thompson et al. 1994; Mavrodi et al. 2001), pyoluteorin (Plt) (de Souza and Raaijmakers 2003; Nowak Thompson et al. 1999), pyrrolnitrin (Prn) (Chernin et al. 1996; Hammer et al. 1999; Kirner et al. 1998), zwittermycin A (Raffel et al. 1996), hydrogen cyanide (Flaishman et al. 1996), kanosamine (Milner et al. 1996a), butyrolactones (Gamard et al. 1997), oligomycin A (Kim et al. 1999), Oomycin A (Howie and Suslow 1991), etc. Further studies focusing on antibiotics produced by these bacterial biocontrol agents are needed.

Since there is no inhibition zone observed in the oxalic acid degradation selective media, the bacteria tested in this study might not have the ability to degrade oxalic acid, which is the major pathogenesis factor of *S. sclerotiorum*. Savchuck (2002) also found strains PA-23 and #41 do not have the ability to produce oxalate oxidase.

Inhibitory organic volatiles seem to be commonly produced in gram-positive bacterial antagonists. All the strains tested in this study showed the inhibition of *S. sclerotiorum* hyphal growth by bacterial organic volatiles in the divided plates, but the results between different strains were hardly compared due to the high variation among replicates (data not shown). The theory of testing the inhibitory volatile is to spatially separate the bacteria and target pathogen, but allow them to share the same headspace. High variation among replicates in the results might be due to the limitations of the test method (Fernando and Linderman 1994) which would allow certain amounts of volatiles to be released out of the plate when the inoculation of fungal mycelia was being conducted a few days after bacterial growth. A recent study by Fernando et al. (2004) used several methods to test the bacterial inhibitory volatile production, including similar divided plate assay as previously described, a method with three-compartment divide plates with/ without charcoal, and sealed plated method which combined two bottom plates with bacteria and fungi on each of them. The latter two methods might give better results in the inhibitory volatile test, since no opening of the plate is needed after initial inoculation. Since this study is to look for biocontrol agents being sprayed at canola flowering stage, the screening of bacterial candidates was not based on inhibitory volatile production. However, these strains could be tested to control other soilborne diseases or reducing sclerotia germination by using as soil drainage.

Bacterial biocontrol agents have been studied in several host-pathogen systems. Bacterial biocontrol products have a great potential to be used in disease control, since their mass production is much easier than that of fungi (Shoda 2000). *Pseudomonas* spp. have been extensively studied as biocontrol agents. The use of *Bacillus* spp. as biocontrol

agents are relatively rare compared to Gram-negative bacteria, however, as *Bacillus* spp. have the characteristics of high thermal tolerance and ready formulation of endospores, their potential to be developed to commercial products is high (Shoda 2000). *Bacillus subtilis* strain GB03 has been successfully registered as a commercial product Kodiak® (Gustafson, Inc, Plano, TX, USA). It has shown a mixture of cotton growth promotion (increased root mass) and disease suppression of *Rhizoctonia* and *Fusarium* spp., when it was used as a cotton seed treatment (Brannen and Kenney 1997). Bacterial agents to control *S. sclerotiorum* have only been investigated in the recent decade. The four strains tested in the field have great potential to be developed to commercial products. Based on the present literature, this is the first extensive study undertaken to collect and screen *Bacillus* spp. (including other Gram-positive strains) for the control of *S. sclerotiorum* on the canola phyllosphere.

4.0 DETECTION OF ANTIBIOTIC-RELATED GENES FROM BACTERIAL BIOCONTROL AGENTS USING POLYMERASE CHAIN REACTION

4.1 Abstract

Eight bacterial isolates were screened against *Sclerotinia sclerotiorum in vitro* and/or *in vivo*. The presence of biosynthetic or self-resistant genes of bacterial antibiotics was investigated using polymerase chain reaction (PCR) method. Thirty-two specific primers were used to amplify the specific antibiotic target genes. *Pseudomonas fluorescens* 2-79, *P. fluorescens* Q2-87, *P. fluorescens* Pf-5 and *Bacillus cereus* UW85 were used as positive controls in some of the PCR reactions to detect phenazine-1-carboxylic acid (PCA), 2, 4-diacetylphloroglucinol (2,4-DAPG), pyoluterorin (Plt), pyrrolnitrin (Prn), and zwittermicin A, respectively. PCR products were sequenced and BLAST searched in the Genebank. Results showed the fungal antagonists *Pseudomonas chlororaphis* PA-23 to contain biosynthetic genes for PCA, Prn and probably 2,4-DAPG, and *Bacillus thuringiensis/cereus* BS8, *B. cereus* L, and *B. mycoides* S contain zwittermicin A self-resistant gene. The results also suggeted that *P. chlororaphis* PA-23 may not contain Plt biosynthetic gene and zwittermicin A self-resistant gene, and *Pseudomonas* spp. #41 and *B. amyloliquefaciens* BS6 may not contain PCA, Plt, Prn, 2,4-DAPG biosunthetic genes and zwittermicin A self-resistant gene. These results suggested that antibiosis might be involved in the bacterial antifungal activity and disease suppression.

4.2 Introduction

Antibiosis has been widely studied as one of the most important biocontrol mechanisms in controlling plant pathogens. Biocontrol bacteria *Pseudomonas fluoresens* 2-79 and *P. fluoresens* Q2-87 were reported to produce phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (2,4-DAPG), respectively (Thomashow and Weller 1988; Harrison et al. 1993). *P. fluorescens* Pf-5 was reported to produce 2,4-DAPG, pyoluterorin (Plt) and pyrrolnitrin (Prn) (Nowak Thompson et al. 1994; de Souza and Raaijmakers 2003). *B. cereus* UW 85 was reported to produce both zwittermin A and kanosamine (Silo Suh et al. 1994; Milner et al. 1996a). The production of antibiotics by these strains has been found relating to the suppression of several different plant pathogens (Thomashow et al. 1990; Chin-A-Woeng et al. 1998; Chernin et al. 1996; Cronin et al. 1997a; Howell and Stipanovic 1979; Homma et al. 1989; Keel et al. 1992). Extensive genetic studies have also been conducted in antibiotic-related genes in these biocontrol agents (Delaney et al. 2001; Pierson, III et al. 1995; Bangera and Thomashow 1996; Bangera and Thomashow 1999; Kraus and Loper 1995; Nowak Thompson et al. 1999; Hammer et al. 1997; Kirner et al. 1998; Stohl et al. 1999b; Stohl et al. 1999a; Milner et al. 1996b), and these strains have become model strains in bacterial antibiosis study.

The biosynthetic genes for PCA, 2,4-DAPG, Prn and Plt, and zwittermicin A self-resistant gene have been fully sequenced (Hammer et al. 1997; Nowak Thompson et al. 1999; Stohl et al. 1999b; Mavrodi et al. 1998; Bangera and Thomashow 1999). The availability of sequenced antibiotic-biosynthetic genes and self-resistant gene facilitated

the development of specific primers that can be used to detect naturally occurring antibiotic-producing bacteria (Raaijmakers et al. 1997). Several specific primers have been developed from the conserved regions of antibiotic biosynthetic genes or self-resistant gene (Raaijmakers et al. 1997; McSpadden Gardener et al. 2001; de Souza and Raaijmakers 2003; Delaney et al. 2001; Mavrodi et al. 2001; Milner et al. 1996b). For example, primers PHZ1and PHZ2 were used to detect phenazine biosynthetic genes, which were designed to amplify a 1.4 kb DNA fragment containing *phzF* and *phzA* genes from *P. aureofaciens* 30-84 or *phzC* and *phzD* in *Pseudomonas fluorescens* 2-79 (Delaney et al. 2001). Several primers were developed from a 745 bp DNA fragment amplified from 1,001 bp *phlD* gene sequence of *P. fluorescens* Q2-87 by primers Phl2a and Phl2b; *phlD* is responsible for the production of monoacetylphloroglucinol (MAPG), the precusor of 2,4-DAPG (McSpadden Gardener et al. 2001).

These specific primers for PCA, 2,4-DAPG, Prn, Plt, and zwittermicin A have been used to detect antibiotic-producing bacterial strains by polymerase chain reaction (PCR) method (Bangera and Thomashow 1999; de Souza and Raaijmakers 2003; Raaijmakers et al. 1997; McSpadden Gardener et al. 2001; Raffel et al. 1996; Picard et al. 2000). Though this method has mainly been used to detect biocontrol agents in soil, as PCR is a rapid method to screen antibiotic-producing bacteria among a large quantity of microorganisms, some studies also suggested that this method could be used to identify individual bacterial strains with specific antibiotic-producing ability (Raffel et al. 1996; Hammer et al. 1999).

In an earlier study (see Chapter 3), eight bacterial biocontrol agents were screened against *Sclerotinia sclerotiorum* de Bary *in vivo* and/or *in vitro*. The direct fungal growth inhibition on PDA/LBA plates suggests that antibiosis might be one of the most important mechanisms involved in the antifungal activity or disease suppression in the lab, the greenhouse and the field. The objective of this study is to identify antibiotic biosynthetic or self-resistant genes from these potential biocontrol bacterial agents in order to better understand the involvement of bacterial antibiotics in pathogen suppression by making Tn5 mutants in the future.

4.3 Materials and methods

Eight potential bacterial biocontrol strains were tested: *P. chlororaphis* PA-23, *Pseudomonas spp.* #41, *Bacillus amyloliquefaciens* BS6, *B. thuringiensis* BS8, *B. subtilis* H, *B. cereus* L, *B. mycoides* S and *Bacillus* spp. B70 (Savchuck and Fernando 2004; Fernando et al. 2002; Rob Duncan, personal communication). *Pseudomonas fluorescens* 2-79, *P. fluorescens* Q2-87, *P. fluorescens* Pf-5 and *Bacillus cereus* UW85 (provided by Dr. Linda Thomashow, Dr. Joyce Loper, Dr. Carolyn Press, Dr. Jo Handelsman) were used as positive controls in some of the PCR reactions to detect phenazine-1-carboxylic acid (PCA), 2, 4-diacetylphloroglucinol, pyoluteorin, prrolnitrin and zwittermicin A, respectively. Thirty primers (Invitrogen^{TM}, Carlsbad, CA, U.S.A.) were used in the PCR reactions to amplify the specific target genes (Table 4.1).

Table 4.1 Bacterial antibiotic-gene-specific primers used in this study**.**

Primer	Sequence	Antibiotics related	Reference
PHZ1	GGC GAC ATG GTC AAC GG	PCA*	Delaney et al., 2001
PHZ2	CGG CTG GCG GCG TAT AT	PCA	Delaney et al., 2001
PHZX	TTT TTT CAT ATG CCT GCT TCG CTT TC	PCA	Delaney et al., 2001
PHZY	TTT GGA TCC TTA AGT TGG AAT GCC TCC	PCA	Delaney et al., 2001
PCA2a	TTG CCA AGC CTC GCT CCA AC	PCA	Raaijmakers et al.,1997
PCA3b	CCG CGT TGT TCC TCG TTC AT	PCA	Raaijmakers et al., 1997
Phl2a	GAG GAC GTC GAA GAC CAC CA	2,4-DAPG**	Raaijmakers et al., 1997
Phl2b	ACC GCA GCA TCG TGT ATG AG	2,4-DAPG	Raaijmakers et al., 1997
BPF2	ACA TCG TGC ACC GGT TTC ATG ATG	2,4-DAPG	McSpadden Gardener et al.,2001
B2BF	ACC CAC CGC AGC ATC GTT TAT GAG C	2,4-DAPG	McSpadden Gardener et al.,2001
BPF3	ACT TGA TCA ATG ACC TGG GCC TGC	2,4-DAPG	McSpadden Gardener et al.,2001
BPR2	GAG CGC AAT GTT GAT TGA AGG TCT C	2,4-DAPG	McSpadden Gardener et al.,2001
BPR3	GGT GCG ACA TCT TTA ATG GAG TTC	2,4-DAPG	McSpadden Gardener et al.,2001
BPR4	CCG CCG GTA TGG AAG ATG AAA AAG TC	2,4-DAPG	McSpadden Gardener et al.,2001
PrnAF	GTG TTC TTC GAC TTC CTC GG	Pyrrolnitrin	Carolyn Press, personal communication
PrnAR	TGC CGG TTC GCG AGC CAG A	Pyrrolnitrin	Carolyn Press, personal communication
PRND1	GGG GCG GGC CGT GGT GAT GGA	Pyrrolnitrin	de Souza and Raaijmakers, 2003
PRND2	YCC CGC SGC CTG YCT GGT CTG	Pyrrolnitrin	de Souza and Raaijmakers, 2003
PrnCf	CCA CAA GCC CGG CCA GGA GC	Pyrrolnitrin	Mavrodi et al. 2001
PrnCr	GAG AAG AGC GGG TCG ATG AAG CC	Pyrrolnitrin	Mavrodi et al. 2001
PltCreg1F	AGG CAA TCA CTA CCA TCC GTG CGC	Pyoluteorin	de Souza and Raaijmakers, 2003
PltCreg2r	ATG AGG AGC AGG AGG TGT CGA GCA C	Pyoluteorin	de Souza and Raaijmakers, 2003
PLTC1	AAC AGA TCG CCC CGG TAC AGA ACG	Pyoluteorin	de Souza and Raaijmakers, 2003
PLTC2	AGG CCC GGA CAC TCA AGA AAC TCG	Pyoluteorin	de Souza and Raaijmakers, 2003
PltBf	CGG AGC ATG GAC CCC CAG C	Pyoluteorin	Mavrodi eta al. 2001
PltBr	GTG CCC GAT ATT GGT CTT GAC C	Pyoluteorin	Mavrodi eta al. 2001
Plt1	ACT AAA CAC CCA GTC GAA GG	Pyoluteorin	Mavrodi eta al. 2001
Plt2	AGG TAA TCC ATG CCC AGC	Pyoluteorin	Mavrodi eta al. 2001
678	ATG TGC ACT TGT ATG GGC AG	Zwittermicin A	Milner et al., 1996
667	TAA AGC TCG TCC CTC TTC AG	Zwittermicin A	Milner et al., 1996

* phenazine-1-carboxylic acid

** 2, 4- diacetylphoroglucinol

4.3.1 Genomic DNA extraction

A revised protocol (Ausubel et al. 1995) was used for DNA isolation. Bacteria were grown in 5 ml LB broth 18-20 h at 28 °C at a rotation speed of 200 rpm. 1.5 ml culture was centrifuged for 2 min in an eppendorf tube (Fisherbrand®, Fisher Scientific™, U.S.A.). After discarding the supernatant, the pellet was resuspended in 576 µl TE buffer (10 mM Tris.Cl, 1 mM EDTA, pH = 8). Ten percent SDS and 20 mg/ml Proteinase K (Sigma®, St. Louis, MO, U.S.A) were added to make final concentrations of 5% and 0.5%, respectively. Samples were mixed thothroughly, and incubated at 37 °C for 1 h. After adding 5 M NaCl to 714 mM and CTAB/NaCl (CTAB: hexadecyltrimethl-ammonium bromide, Sigma®, Sigma Chemical Co., St. Louis, MO., U.S.A) to 10%, the samples were incubated at 65 °C for 10 min. Samples were extracted with equal volume of 24:1 chloroform /isoamyl alcohol (chloroform: Fisher Scentific™, Fair Lawn, New Jersey, U.S.A.) and then 25:24:1 phenol /chloroform /isoamyl alcohol (Phenol: Sigma®, St. Louis, MO, U.S.A.; chloroform and isoamylalcohol: Fisher Scentific™, Fair Lawn, New Jersey, U.S.A.), and centrifuged at 13.000 rpm for 10 min. The supernatant was transferred to a fresh eppendorf tube after each centrifugation. DNA was precipitated by adding 0.6 vol. cold isopropanol (Sigma®, St. Louis, MO, U.S.A). After 5 min the samples were centrifuged again at 13,000 rpm for 10 min. The supernatant was discarded and the pellet was washed by 70% ethanol. The eppendorf tubes were placed upside down to let them air dry for about 30min. The pellet was resuspended in 100 µl of warm TE buffer (10 mM Tris.Cl, 1 mM EDTA, pH = 8). The DNA extracts were stored at 4 °C.

In order to catalyze the hydrolysis of RNA, Ribonuclease A (Sigma®, St. Louis, MO, U.S.A) were added to 0.02 µg, and then incubated at 37 °C for 30 min. DNA was precipitated again with isopropanol and resuspended in 100 µl of TE buffer as previously described. Samples were stored at –20 °C.

4.3.2 DNA quantification

DNA quantification was determined by gel electrophoresis and the spectrophotometer (GeneQuant®, Biochrom™, distributed by Fisher Scientific™, Nepean, Ontario, Canada). Agarose gel (Agrarose DNA Grade, Electrophoresis Grade, Fisher Scientific™, Fair Lawn, New Jersey, U.S.A.) of 1.2% with ethidium bromide (0.5µg/ml) and 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0) was used in the electrophoresis. The 1 kb DNA size marker was used to estimate the size of the extracted DNA. The gel was run at 150 volts for approximately 40min. A photograph was taken under the UV transluminator (Gel Doc 2000, Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.).

OD values of DNA were read at 260 nm and 280 nm using the spectrophotometer. The ratio of OD_{260} to OD_{280} was calculated to assess the DNA purity. The dilution of the DNA was calculated based on 1 OD_{260} = 50 µg/ml and the OD_{260}.

4.3.3 PCR reactions

Each 25µl PCR reaction mixture contained 25 pmol of each of the two primers, 1x PCR buffer, 800 µM dNTP's, 1.5 mM $MgCl_2$, 1.0-2 units Taq DNA polymerase

(Invitrogen™, Carlsbad, CA, U.S.A.). The PCR cycling conditions were employed according to the original reference publications (Raaijmakers et al. 1997; McSpadden Gardener et al. 2001; de Souza and Raaijmakers 2003; Raffel et al. 1996; Giacomodonato et al. 2001; Delaney et al. 2001; Mavrodi et al. 2001). PCR reactions were carried by (Delaney et al. 2001) using 0.2 ml PCR tubes (Rose Sclientific Ltd., Edmonton, Alberta, Canada) with TECHNE Genius® PCR machine (Techne Ltd. and Techne Inc., Cambridge, UK and New Jeysey, U.S.A.) or 0.6 ml PCR tubes (Rose Sclientific Ltd., Edmonton, Alberta, Canada) with Programmable Thermal Controller (PTC-100™, MJ Research, Inc., Watertown, Mass, U.S.A.). The amplification products were separated on 1.2-1.5 % agarose gel (Agrarose DNA Grade, Electrophoresis Grade, Fisher Scientific™, Fair Lawn, New Jersey, U.S.A.) with ethium bromide (0.5 µg/ml) and 1x TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). The 100 bp size marker was used to estimate the size of PCR products. The gel was run at 80 volts for approximately 1.5 h. A photograph was taken under UV transilluminator (Gel Doc 2000, Bio-Rad Laboratories Inc., Hercules, CA, U.S.A.). All reactions were conducted at least twice.

In order to confirm the positive results in the previous amplifications, the reactions were conducted again with positive control strains. Strain 2-79, Q2-87 and Pf-5 were used as positive controls and amplified with strain PA-23 using primers PHZ1/PHZ2, BPF3/BPR2 and PltBf/PltBr respectively. Strain UW85 was used as positive control using primers 677/678. PCR reactions amplified with water instead of genomic DNA were used as negative controls.

4.3.4 Sequencing and BLAST search

To further confirm the positive results from PCR reactions, the desired PCR products were sent for sequencing (University Core DNA & Protein Services, University of Calgary, Calgary, AB). Fifty or 100 µl PCR reaction was conducted and purified by High Pure PCR Product Purification Kit (Roche®, Roche Applied Science™, Mannheim, Germany). For the reaction with primers BPF3/BPR2 and strain PA-23, the desired band (470 bp) was cut from the agorase gel, and then the DNA was purified with 5M NaCl and 95% ethanol. After re-dissolution in water, the purified DNA was used as template to amplify the desired PCR product again with primers BPF3/BPR2. The PCR product was checked and purified as described previously. The DNA was quantified after gel electrophoresis, by comparing with 1650 bp band of 1 kb DNA ladder. Each 12 µl premixed sample for sequencing contained 0.01 µg of template DNA per 100 bp, and 3.2 pmol primer. The results were aligned on the BLAST search at National Centre for Biotechnology Information (NCBI) using nucleotide-nucleotide BLAST (blastn) and align two sequences (bl2seq).

4.4 Results

4.4.1 Genomic DNA

Genomic DNA was visualised as white pellets after isopropanol precipitation, and re-dissolved in TE buffer. The gel electrophoresis suggested the genomic DNA extracts were intact. No smear was found after adding the RNase A, which suggested that the RNA was cleaned out. The OD260/OD280 value (1.6 - 1.8) suggested that the purity

of genomic DNA should be enough for PCR reaction. The genomic DNA concentrations and the amount of genomic DNA needed for the PCR reactions were calculated based on the OD260.

Genomic DNA was stored at –20 ºC, and diluted to 20 ng/µl by water and stored at 4ºC for a short time prior to PCR reaction.

4.4.2 PCR reactions

P. chlororaphis PA-23 showed a 1400 bp (Figure 4.1), and an 850 bp (Figure 4.5) band in the gel electrophoresis when amplified with primers PHZ1/PHZ2 and PltBf/PltBr, respectively. The results were confirmed by amplification along with the positive control strains *P. fluorescens* 2-79 (Figure 2) and *P. fluorescens* Pf-5 (Figure 5), respectively. Identical bands were found from *P. chlororaphis* PA-23 and positive control *P. fluorescens* Pf-5 using primers PrnAF /PrnAR, PRND1/PRND2 and PrnCf/PrnCr (Figure 3), and the bands are approximately 1050 bp, 786 bp and 719 bp, respectively. *P. chlororaphis* PA-23 and positive control *P. fluorescens* Q2-87 also showed a 470 bp band and a few other weak bands when amplified with primers BPF3/BPR2 (Figure 4). *B. thuringiensis* BS8, *B. cereus* L and *B. mycoides* S amplified a 950 bp PCR product, respectively, using zwittermicin A self-resistant gene-specific primers 678/677, along with positive control *B. cereus* UW 85 (Fig.5). A very weak band was observed from *B. amyloliquefaciens* BS6 when amplified for the first time with primers 678/677, but the result was not repeatable. No PCR product was amplified from *Pseudomonas* spp. #41.

4.4.4 Sequencing and BLAST search

The BLAST search of the sequence of the 1400 bp PCR product showed >90% identity with phenazine biosynthetic genes of *P. chlororaphis* strains in the genebank. The PCR products (1050 bp band) amplified from *P. chlororaphis* PA-23 by primers PrnAF/PrnAR showed similarity to pyrrolnitrin biosynthetic genes (*prnABCD*) of *P. fluorescens*, *Burkholderia pyrrocinia* and *P. chlororaphis* strains. High identity (93%) was found between the sequences of *P. chlororaphis* PA-23 and *P. fluorescens* Pf-5 amplified with primers PrnAF/PrnAR. The sequence of 470bp PCR product amplified from *P. chlororaphis* PA-23 using primers BPF2/BPR3 (for 2,4-diacetylphloroglucinol biosynthetic gene) did not look clean and signals of each peak were weak, but it showed 100% identity with a 25 bp fragment of *phlD* gene sequence of several *P. fluorescens* strains, including *P. fluorescens* CM1'A2, Q65c-80, 42-36, 42-27, 39-8, 37-27, 22-27, 19-41, 19-30, 19-7, 18-33, 11-18, 7-37, 6-28, 3-1, etc. The sequence of 850bp PCR product amplified from *P. chlororaphis* PA-23 with primers PltBf/PltBr did not find similarity with that from *P. fluorescens* Pf-5 or any pyoluteorin biosynthetic gene from bacteria. The results indicate that *P. chlororaphis* PA-23 might produce phenazine-1-carboxylic acid, pyrrolnitrin and 2,4-diacetylphloroglucinol, but not pyoluteorin. The BLAST search of the three sequences of 950 bp PCR products amplified with primers 678/677 (*B. thuringiensis*/*cereus* BS8, *B. cereus* L and *B. mycoides* S) showed > 98% identity with *B. cereus* zwitermicin A-resistant gene (*zmaR*), which indicates that *B. thuringiensis* BS8/*cereus* BS8, *B. cereus* L and *B. mycoides* S might produce zwitermicin A.

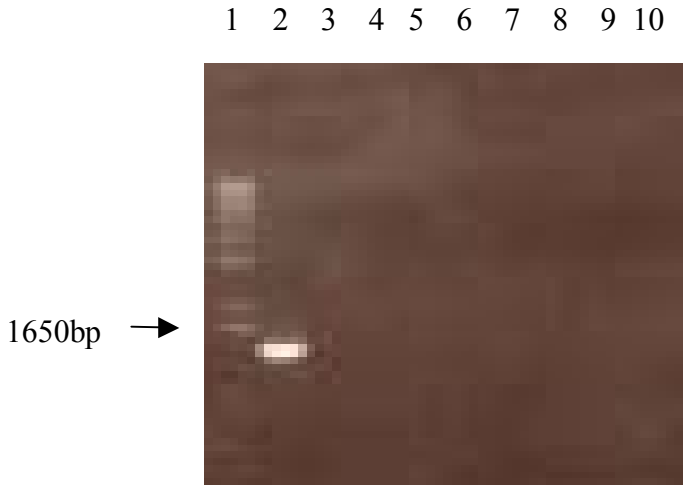


Figure 4.1. PCR products amplified with primers PHZ1/PHZ2 separated by agarose gel electrophoresis to identify phenazine biosynthetic gene. Lane1: 1 kb DNA ladder; Lane 2-9: *P. chlororaphis* PA-23, *Pseudomonas spp.* #41, *Bacillus amyloliquefaciens* BS6, *B. thuringiensis* BS8, *B. subtilis* H, *B. cereus* L, *B. mycoides* S and *Bacillus* spp. B70; Lane 10: negative control (water).

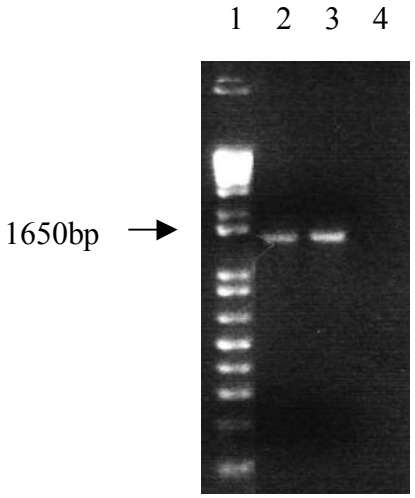


Figure 4.2. PCR products amplified from strain *P. chlororaphis* PA-23 and *P. fluorescens* 2-79 using primers PHZ1/PHZ2 separated by agarose gel electrophoresis to identify and confirm the presence of phenazine biosynthetic gene. Lane1: 1 kb DNA ladder; Lane 2: strain PA-23; Lane 3: strain 2-79 (positive control); Lane4: negative control (water).

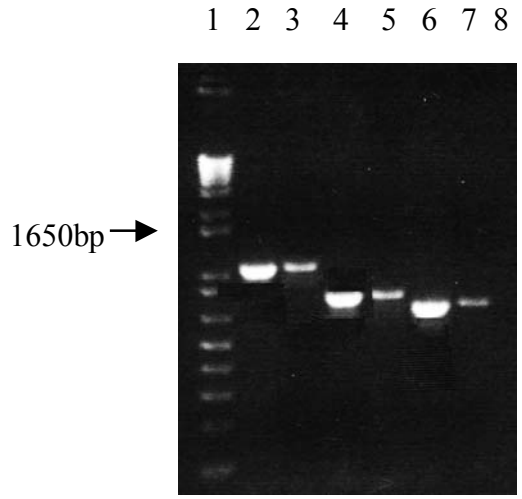


Figure 4.3. PCR products amplified from *P. chlororaphis* PA-23 and *P. fluorescens* Pf-5 with primers PrnAF/RF, PRND1/PRND2 and PrnCf/PrnCr separated by agarose gel electrophoresis to identify pyrrolnitrin biosynthetic gene. Lane1: 1 kb DNA ladder; Lane 2: strain PA-23, primers PrnAF/RF; Lane 3: strain Pf-5, primers PrnAF/RF (positive control); Lane 4: strain PA-23, PRND1/PRND2; Lane 5: strain Pf-5, PRND1/PRND2 (positive control); Lane 6: strain PA-23, PrnCf/PrnCr; Lane 7: strain Pf-5, PrnCf/PrnCr (positive control) ; Lane 8: negative control (water).

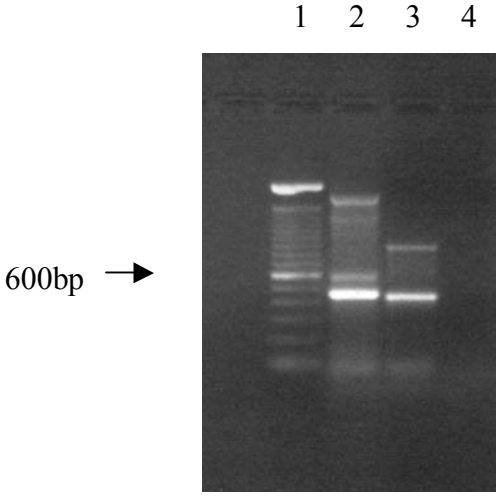


Figure 4.4. PCR products amplified from strain *P. chlororaphis* PA-23 and *P. fluorescens* Q2-97 using primers BPF3/BPR2 separated by agarose gel electrophoresis to identify 2,4-DAPG biosynthetic gene. Lane1: 100 bp DNA ladder; Lane 2: strain PA-23; Lane 3: strain Q2-87 (positive control); Lane4: negative control (water).

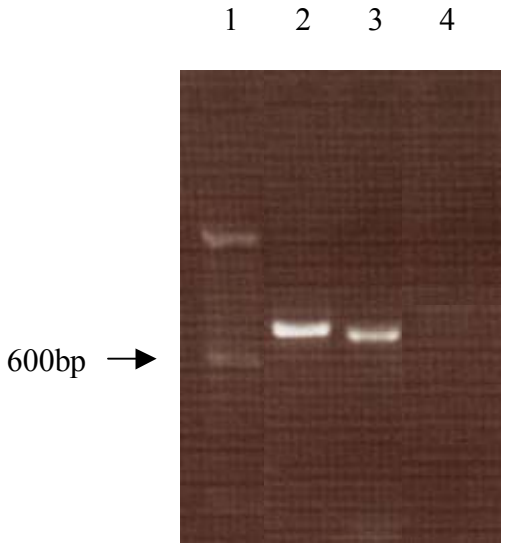


Figure 4.5. PCR products amplified from strain *P. chlororaphis* PA-23 and *P. fluorescens* Pf-5 using primers PLT BF/PLT BR separated by agarose gel electrophoresis to identify pyoluterorin biosynthetic gene. Lane1: 100 bp DNA ladder; Lane 2: strain PA-23; Lane 3: strain Pf-5 (positive control); Lane 4: negative control (water).

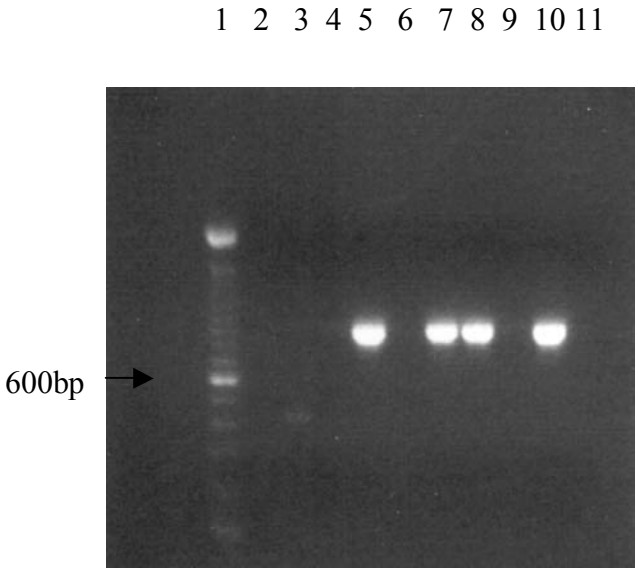


Figure 4.6. PCR products amplified with primers 678/677 separated by agarose gel electrophoresis to identify zwittermicin A self-resistant gene. Lane1: 100 bp DNA ladder; Lane 2-9: *P. chlororaphis* PA-23, *Pseudomonas spp.* #41, *Bacillus amyloliquefaciens* BS6, *B. thuringiensis* BS8, *B. subtilis* H, *B. cereus* L, *B. mycoides* S and *Bacillus* spp. B70; Lane 10: *B. cereus* UW85 (positive control); Lane 11: negative control (water).

4.5 Discussion

The PCR and sequencing results showed the high possibility of the presence of phenazine-1-carbocylic acid (PCA) and pyrrolnitrin, but not pyoluterorin biosynthetic genes in strain PA-23. Sequence analysis of PCR products showed similarity to PCA and pyrrolnitrin genes of several *P. fluorescens* and *Burkholderia* spp. strains deposited in the GenBank. The presence of pyrrolnitrin biosynthetic gene was also further confirmed by southern hybridization (Chrystal Berry, personal communication). The presence of 2,4-DAPG biosynthetic gene *phlD* was not very clear based on PCR and sequencing results. In addition, further confirmation using southern hybridization also failed to give a clear answer, but mainly due to technical difficulties (Chrystal Berry, personal communication). However, the 2,4-DAPG bacterial extraction from strain PA-23 by the method of Rosales et al. (1995) showed the same RF (retention factor) value as the 2,4-DAPG commercial product (2,4-diacetylphloroglucinol, Toronto Rereach Chemicals Inc., North York, ON) (Nakkeeran Sevugapperumal, personal communication). Further confirmation of 2,4-DAPG-producing ability of strain PA-23 is necessary, and analysis with high performance liquid chromatography followed by mass spectroscopy is probably a good choice (Raaijmakers et al. 1999; Picard et al. 2000; Bonsall et al. 1997; Mavrodi et al. 2001).

Pseudomonas spp. #41 has not shown any specific amplification using all thirty primers in this study, which was consistent with PCR results carried by Chrystal Berry (unpblished) when the primers PrnAF/PrnAR, PRND1/PRND2, PrnCf/PrnCr (for pyrrolnitrin biosynthetic gene *PrnA*) and Phl2a/Phl2b (for 2,4-DAPG biosynthetic gene

phlD) were used. However, southern hybridization showed that *Pseudomonas* spp. #41 genomic DNA was hydridazied at low strigency (50°C) with the probes of pyrrolnitrin biosynthetic gene *PrnA* of *P. fluorescens* Pf-5 and 2,4-DAPG biosynthetic gene *phlD* of *P. fluorescens* Q2-87, respectively (Chrystal Berry, personal communication). The presence of biosynthetic genes of pyrrolnitin and 2,4-DAPG in *Pseudomonas* spp. #41 is not clear.

The PCR results also suggested that *B. amyloliquefaciens* BS6 may not contain PCA, Plt, Prn, 2,4-DAPG biosunthetic genes and zwittermicin A self-resistant gene. Since *B. amyloliquefaciens* BS6 amplified a weak band of 950 bp using primers 678/677 and the result was unrepeatable, further confirmation was conducted by using two novel primers (Ramarathnam and Fernando, unpublished) designed for zwittermicin A biosynthetic gene. No product was amplified from strain BS6, while a bright band of 800 bp was observed from the positive strain UW85 and several other *Bacillus* strains (Rajesh Ramarathnam, personal communication).

All the tested strains in this study had showed antifungal activity in the plate assay, and those tested in the greenhouse and field as well, but whether these identified antibiotic-related genes were expressed and whether these antibiotics were responsible for sclerotinia disease suppression *in vivo* need to be investigated. Tn5 mutagenesis is a common method used to evaluate the biosynthetic gene expression and study the role of antibiosis involved in disease suppression in biocontrol (Thomashow and Weller 1988; Hill et al. 1994; Salcher and Lingens 1980; Bangera et al. 1994; Heungens and Parke 2001). A Tn5 mutant of *P. fluorescens* 2-79, which was deficient in PCA production, was

less suppressive to take-all on wheat caused by *Gaeumannomyces graminis* var. *tritici* than the parental strain (Thomashow and Weller 1988). A Tn5 mutant of *P. fluorescens* CHA0, which is defective in the production of 2,4-DAPG, showed reduced suppression of black root rot on tobacco caused by *Thielaviopsis basicola* (Keel et al. 1992). Results of Tn5 mutant of biocontrol agent *Burkholderia cepacia* AMMDR1 suggest no other mechanism other than antibiosis could be identified in suppression of oomycete pathogens of pea (Heungens and Parke 2001). Earlier studies by Fernando showed an orange colour compound produced by *P. chlororaphis* PA-23 on LBA and PDA (Fernando, unpublished), which indicates that this strain is probably a PCA producer; however, whether this antibiotic is involved in sclerotinia disease suppression has not been clear. Since *P. chlororaphis* PA-23 possibly produces three antibiotics (PCA, 2,4-DAPG and Prn), Tn5 mutagenesis and subsequent testing of mutants and the wild type strains on plants will help understand which antibiotic is responsible for sclerotinia disease suppression.

Inhibition of sclerotial and/or spore germination by 2,4-DAPG extracted from *P. chlororaphis* PA-23 has also been observed against several plant pathogens, including *S. sclerotiorum, Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii, Fusarium oxysporum, Alternaria solani, Botryodiplodia theobromae* (Nakkeeran, personal communication). Antibiotics PCA, pyrrolnitrin and 2,4-DAPG are major determinants of biological control of plant pathogens. The ability of producing multiple antibiotics by strain PA-23 might explain its wide-spectrum disease suppression of plant pathogens. Further work on Tn5 mutagenesis will help understand which antibiotic is responsible for disease suppression in different host-pathogen systems.

5.0 PLANT INDUCED RESISTANCE MEDIATED BY *SCLEROTINIA SCLEROTIORUM* AND BACTERIAL BIOCONTROL AGENT *BACILLUS AMYLOLIQUEFACIENS* BS6

5.1 Abstract

An earlier study (chapter 3) has shown that bacterial strain *Bacillus amyloliquefaciens* BS6 gave excellent protection to the canola plant against *Sclerotinia sclerotiorum* ascospore infection under lab, greenhouse and field conditions. The significant disease suppression and the low level of survival of strain BS6 on petals indicated that plant-induced resistance might be one of the most important mechanisms triggered by the bacterium. To test this hypothesis, plants were pre-treated with water or strain BS6 (10^8 cfu/ml) by spraying the inoculum on to petals at the 30% bloom stage, and then inoculated two days later with petals which were treated either with only sclerotinia ascospores (10^5 spores/ml) (S petals) or with both strain BS6 (10^8 cfu/ml) and sclerotinia ascospores (10^5 spores/ml) (B+S petals), by placing petals on three leaves per plant. Disease incidence on canola stems was reduced by 60% on plants pre-treated with strain BS6 and inoculated with S petals compared to plants pre-treated with water and also inoculated with S petals. Leaves of plants pre-treated with water and then inoculated with B+S petals had localized water-soaked lesions compared to plants pre-treated with BS6 and also inoculated with B+S petals, which had no disease symptom (0%) on either leaves or stems. Disease severity (DS) progressed rapidly in the absence of BS6 and reached a rate of > 5 (on a 0-7 scale) after 19 d; however, the BS6-treated plants had no disease (DS=0). Phenolic compounds of canola leaves were extracted using 80% methanol and fractioned using ethyl acetate. The HPLC results suggest that both *B.*

amyloliquefaciens BS6, and *S. sclerotiorum* result in the induction of phenolic compounds in canola leaves, but additional experiments are required to clearly demonstrate the concentration changes over time.

5.2 Introduction

Sclerotinia stem rot caused by *Sclerotinia sclerotiorum* is one of the most economically important diseases of canola in Canada. Usually cultural control methods do not work due to the extremely wide host range of this pathogen and the long survival time of sclerotia in soil. We have isolated a bacterial strain *Bacillus amyloliquefaciens* BS6 that gave excellent protection to the canola plant against ascospore infection under lab, greenhouse and field conditions (see Chapter 3). Although there was significant disease suppression in the field, the low level of survival of strain BS6 on petals indicated that plant-induced resistance might be one of the most important mechanisms triggered by the bacterium.

Plant induced resistance is a process which is analogous to immunization on the plant to trigger the natural defence machinery and results in the prevention of disease caused by a variety of plant pathogens (van Loon 1997; Boyetchko 1999). Plant induced resistance was often categorized as systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR refers to the plant resistance triggered by necrotizing pathogens (Bakker et al. 2003). SAR could express locally at the site of primary inoculation but also systemically in tissues remotely located from the initial treatment. ISR was described as the mode of action of disease suppression by non-pathogenic rhizosphere bacteria (van Peer et al. 1991; Wei et al. 1991).

Very little work has been done in induced resistance to *S. sclerotiorum* on canola/oilseed rape. Systemic resistance against *S. sclerotiorum* in oilseed rape was first reported by Toal and Jones (1999). Plants treated by oxalic acid were reported to have significantly smaller leaf lesions than the control plants after being challenged by the pathogen. **Phenolic compounds, which associate with induced plant resistance against *S. sclerotiorum*, have also rarely been studied in this system.** Studies by Li (1999) and Bodnaryk (1992) on changes of glucosinolates treated with *S. sclerotiorum* or by wounding suggest that glucosinolates' induction might be related to the induced resistance to *S. sclerotiorum* in oilseed rape. Brassinin, methoxybrassinin, and cyclobrassinin were the first phytoalexins reported from *Brassica* spp. induced by *Leptosphaeria maculans* (Soledade et al. 1992). However, no study has been reported on the relationship between phenolic compounds and induced resistance on canola/ oilseed rape against *S. sclerotiorum*.

The purpose of this study was (i) to investigate induced resistance mediated by *S. sclerotiorum* and bacterial strain BS6 in canola in the greenhouse, (ii) to isolate phenolic compounds from canola leaves, and investigate their role in induced resistance in the canola plant by monitoring their changes over time.

5.3 Materials and methods

5.3.1 Greenhouse experiment

Canola plants (var. Westar) were grown in the growthroom at 16/ 21 °C and 16 h photoperiod. Plants were pre-treated with water (CK) or strain BS6 (10^8 cfu/ml) by spraying the inoculum onto petals at the 30% bloom stage, and then inoculated two days

later with petals which were treated either with only *Sclerotinia* ascospores or with both stain BS6 and *Sclerotinia* ascospores by placing them on three leaves per plant (Figure 5.1). Eleven plants were treated in each of the five treatments. Five plants in each treatment were used to record the disease incidence (DI) and disease severity (DS), and the other six plants were used for leaf sampling.

One loop of fresh bacteria grown on Nutrient Agar (Difco Laboratories, Detroit, Mich., U.S.A.) was put into 150ml Luria Bertani broth [LB broth contains 10.0 g tryptone peptone, 5.0 g yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 5.0 g NaCl L^{-1}], and incubated at 30 ºC and rotated at 180 rpm for 16-18 h. Ascospores of *S. sclerotiorum* (University of Nebraska) were washed with a brush and distilled water to release them from the aluminium foil. The concentrations of bacterial (10^8 cfu/ml) and ascospore (10^5 spores/ml) solution were checked using a spectrophotometer and a hemacytometer (Bright-Line®, Reichert, Buffalo, N.Y., U.S.A.), respectively. Petals were sampled from other untreated plants and dipped into bacterial and/or ascospore solution, and placed on water agar for 1-2 h before placing them onto the leaves. Tween 20 (0.02%) was used in all inoculum. After inoculated with petals, plants were kept in the humidity chamber for 3.5 days (24/16 °C, 16 photoperiod and RH = 87%) before being moved to a growth cabinet (25/19 °C, 16 photoperiod). DI and DS were recorded every 3 days in the following two weeks in the growth cabinet. DI of each plant was calculated as percentage of infected leaves, and data of each treatment were averaged from 5 plants. DS was rated the same as previous greenhouse experiments, except that the water-soaked lesion was recorded as 0.5 (Figure 5.2 D) to distinguish with more obviously necrotizing leaf lesion.

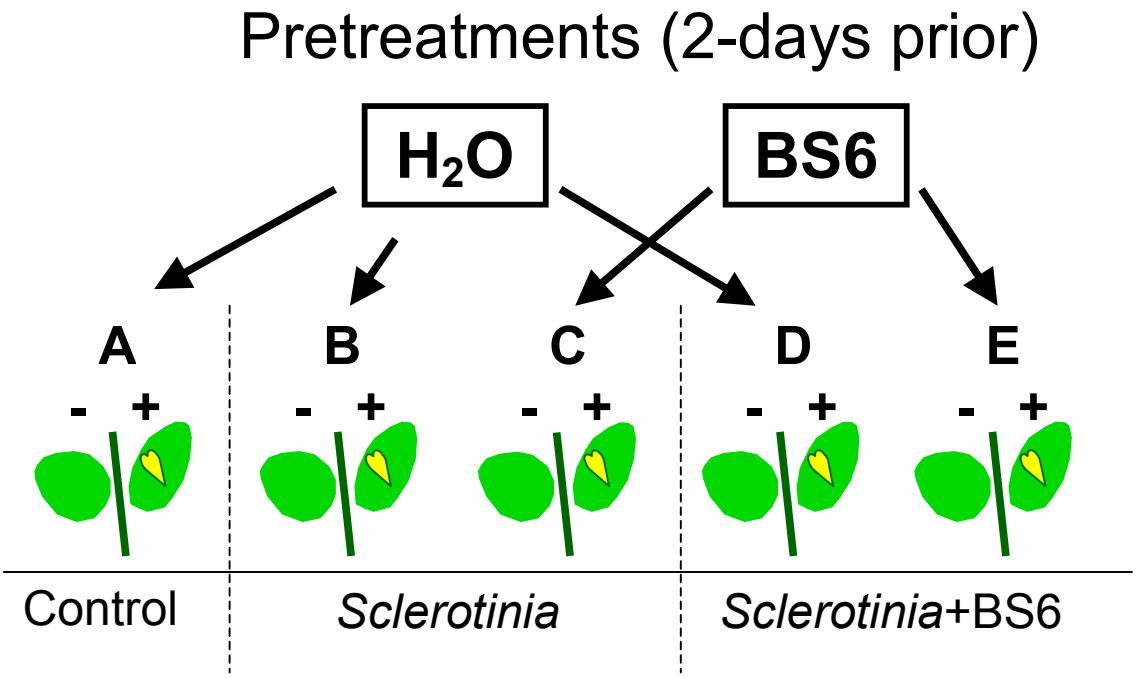


Figure 5.1 Diagram depicting inoculations on canola plants and leaves. Plants were pre-treated with water or *B. amyloliquefaciens* BS6 (10^8 cfu/ml) by spraying the inoculum on to petals at the 30% bloom stage, and then inoculated two days later with petals which were treated either with only *Sclerotinia* ascospores (10^5 spores/ml) or with both *B. amyloliquefaciens* BS6 (10^8 cfu/ml) and *Sclerotinia* ascospores (10^5 spores/ml) (B+S petals) by placing petals on three leaves per plant. +: presence of petal inoculum; -: absence of petal inoculum.

DI and DS were compared between different treatments using LSD test in ANOVA by SAS software (Crow 2002).

Canola leaves with petal inoculation (+) and without petal inoculation (-) (Figure 5.1) in each treatment were sampled separately –2, 0, 3, 5, and 7 days after pathogen inoculation (DAI), and recorded as A, B-, B+, C-, C+, D-, D+, E- and E+ for each treatment. Leaves were put onto ice immediately after sampling to bring back to the lab, weighed and then stored at –80 °C.

5.3.2 Phenolics extraction and fractionation

Free phenolics were extracted using a modified method of Daayf et al. (1997). Leaves stored at –80 °C were grounded in liquid N_2, and immediately put into 80% methanol (Fisher Scientific^{TM}, Fair Lawn, New Jersey, U.S.A) in 50 ml or 150 ml flasks with the ratio of 10ml 80% methanol /1 g fresh leaf weight. The flasks were wrapped with aluminium foil and incubated at room temperature for 24 h at 200 rpm. Each sample was then vacuum filtrated using Waterman No. 1 filter paper, and was collected to a 50 ml centrifuge tube (Fisherbrand®, Ficher Scientific, Pittsburgh, PA, U.S.A.). Methanol (100%) was used to wash each flask. Samples were evaporated using N_2 at 40 ºC in the water bath, until the volume left in each tube was approximately 20% of original volume. Petroleum ether (Fisher Scientific^{TM}, Fair Lawn, New Jersey, U.S.A) (vol. 1:1) was used twice to exclude the chlorophyll, carotenoids, lipids, and waxes. After petroleum ether was added, the tube was thoroughly mixed and then let stand for 10 min. The acquous fraction (lower part), which contains the phenolic compounds, was then partitioned with ethyl acetate (Fisher Scientific^{TM}, Fair Lawn, New Jersey, U.S.A) (vol. 1:1). The ethyl acetate fraction was evaporated using N_2 as previously described, and was then re-

dissolved in 100% methanol and the concentrations were adjusted to 8 g of fresh material per millilitre of methanol. Samples were stored at –20ºC for further analysis. The samples from 5DAI were extracted with petroleum ether after the ethyl acetate extraction, and then extracted again with H_2O and ethyl acetate.

5.3.3 HPLC analysis

Samples were analysed by high performance liquid chromatography (HPLC) with two separate runs: one injection the first time, and another two injections the second time. Fifteen micro litres of each sample were used in each injection (25 µl and 35 µl were prepared in the test tube, respectively). The HPLC was equipped with a Waters 996 photodiode array detector, a Waters 2690 separations module, and fitted with a C18 reverse-phase column (Lichrosorb® RP-18 (7 µm), Kartuschenhalter Cartridge holder, E. Merck, Darmstadt, F.R. Germany). Results were analyzed with the Millennium[32] Software. The column was eluted with a gradient of acetonitrile - 0.1% phosphoric acid (acetonitrile, Fisher Scientific™, Fair Lawn, New Jersey, U.S.A; 1% phosphoric acid, Fisher Scientific™, Fair Lawn, New Jersey, U.S.A) as follows: [time (minutes) /acetonitrile (percent) /1% phosphoric acid (percent)] c [5 /5 /95, 10 /5 /95, 14 /10 /90, 20 /20 /80, 23 /20 /80, 30 /35 /65, 35 /35 /65, 43 /50 /50, 48 /75 /25, 55 /100 /0, 60 /100 /0, 62 /0 /100, 65 /0 /100]. Samples were monitored at the wavelength between 220.0 nm to 370.0 nm.

5.4 Results

5.4.1 Greenhouse experiment

The difference in disease symptoms between each treatment was visualised 5-6 days after pathogen inoculation (DAI) (Figure 5.2). No infection was visualized on either leaf or stem in treatments A (water-treated control) and E (pre-treated by strain BS6 and inoculated with B+S petals), which means that plants pre-treated by *B. amyloliquefaciens* BS6 and then co-inoculated with both *B. amyloliquefaciens* BS6 and *S. sclerotiorum* ascospores have achieved complete disease suppression (Figure 5.3 and 5.4). DI on leaf of treatments B (pre-treated by water and inoculated with S petals) and C (pre-treated by strain BS6 and inoculated with S petals) was observed as 73.3% and 66.7% 4DAI, respectively, which are not significantly different. However, DI on stem was reduced by 60% in treatment C comparing to treatment B 7DAI (Figure 5.4). In treatment C, disease lesions are mainly restricted on the inoculated leaves instead of developing onto the stems (Figure 5. 2 C). A significant ($P<0.05$) reduction of DI on canola leaf 4DAI was also observed in treatment E (pre-treated with strain BS6 and inolulated with B+S petals), compared with the treatment D (pre-treated with water and inoculated with B+S petals) (Figure 5.3), but no such significant reduction was found on canola stem 7DAI (Figure 5.4).

Disease severity (DS) progressed rapidly in the absence of BS6 and reached a rate of >5 (on a 0-7 scale) in treatment B after 19 d; however, the BS6-treated plants had DS<2 (treatment C, DS<2; treatment D, DS<1; treatment E, DS=0) (Figure 5.5). Only water-soaked lesions were found in plants pre-treated by water and inoculated with B+S

petals (treatment D), with a DI of 33.3%, and no system was found on stems (Figure 5.3 and 5.4). Significant diseased plants were found in treatment B 25 DAI (Figure 5.2 F).

5.4.2 HPLC analysis

Three majar peaks were found to be related to the pre-treatment by strain BS6 and/or *S. sclerotiorum* inoculation, and they were designated as compounds 1-3. Their retention time and max absorbance by UV light are shown in Figure 5.6 A and Figure 5.7. Compounds 1, 2, and 3 generally showed higher concentrations in samples from 5 DAI in all *Sclerotinia* inoculated plants than those in the control (treatment A), except for treatment E-, in which plants were pre-treated with strain BS6 and B+S petals were deposited on the adjacent leaves (Figure 5.6). Plants in treatment E did not have visible symptoms, even on the inoculated leaves (E+) in the greenhouse experiments (Figure 5.2 E). The compound 2 was found to be greatly induced in treatment B-, in which leaves were without inoculum of *Sclerotinia* (*Scelrotinia* was inoculated on adjacent leaves) and plants were pre-treated with water, as comparedto that in the control (treatment A).

The concentrations of compounds 2 and 3 of samples from treatment C- and C+ were found to be much higher than those in the treatment E- and E+ (Figure 5.6) (Samples of C- and C+ were 15 times diluted comparing to other samples in the HPLC analysis); plants in both treatments C and E received a pre-treatment of strain BS6, but were inoculated with S petals and B+S petals, respectively.

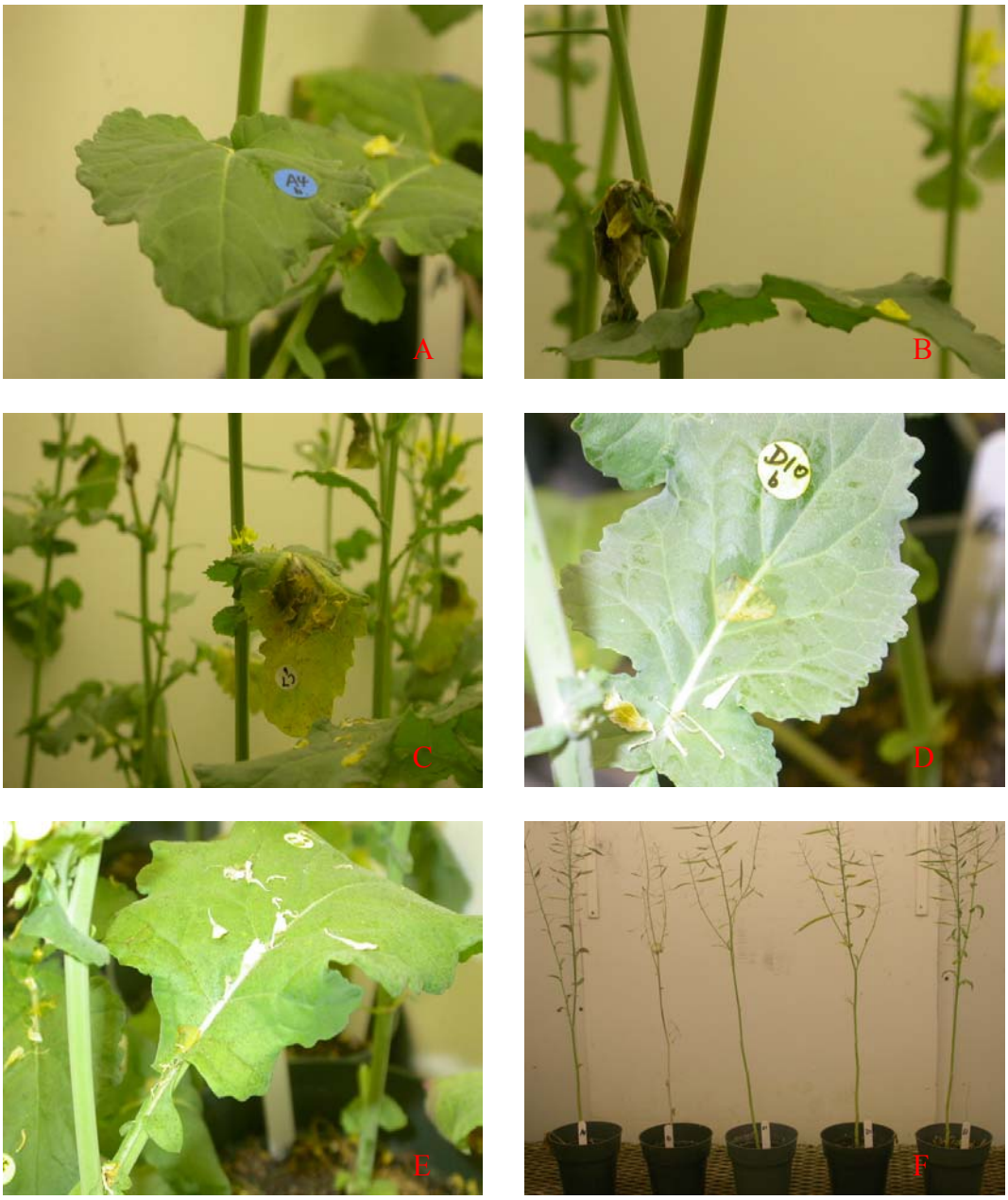


Figure 5.2 A-E: Typical disease symptoms on canola of Treatment A, B, C, D, and E, respectively, observed 6 Days after disease inoculation (DAI); F: Disease symptoms of treatment A-E observed 25 DAI. Treatments C and E were pre-treated with *B. amyloliquefaciens* BS6 (10^8 cfu/ml) by spraying; Treatments A (control), B, D were pre-treated with water. Treatment B and C were inoculated with *Sclerotinia* ascospores (10^5 spores/ml); Treatments D and E were inoculated with both *B. amyloliquefaciens* BS6 (10^8 cfu/ml) and *Sclerotinia* ascospores (10^5 spores/ml). Three leaves per plant were inoculated with infested petals.

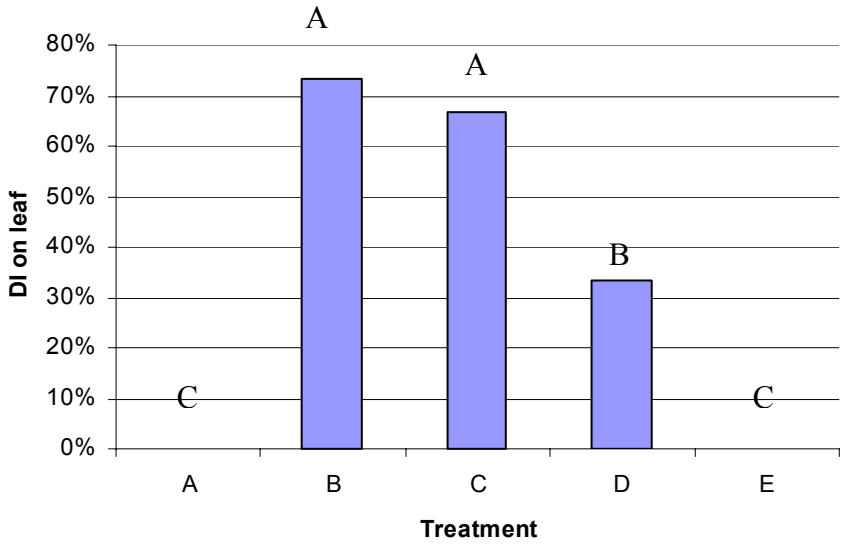


Figure 5.3 Disease incidence (DI) observed on canola leaves 4 days after pathogen inoculation (4DAI). Treatments C and E were pre-treated with *B. amyloliquefaciens* BS6 (10^8 cfu/ml) by spraying; Treatments A (control), B, D were pre-treated with water. Treatment B and C were inoculated with *Sclerotinia* ascospores (10^5 spores/ml); Treatments D and E were inoculated with both *B. amyloliquefaciens* BS6 (10^8 cfu/ml) and *Sclerotinia* ascospores (10^5 spores/ml). Three leaves per plant were inoculated with infestated petals, and DI was calculated as percentage of infected leaves. Treatments with same letters are not significantly ($P < 0.05$) different. LSD=0.1866

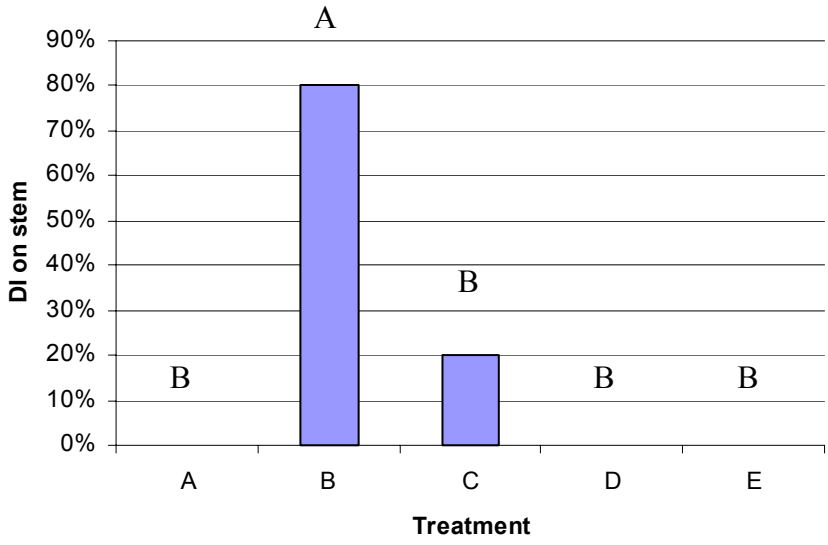


Figure 5.4 Disease incidence (DI) observed on canola stems 7 days after pathogen inoculation (DAI). Treatments C and E were pre-treated with *B. amyloliquefaciens* BS6 (10^8 cfu/ml) by spraying; Treatments A (control), B, D were pre-treated with water. Treatment B and C were inoculated with *Sclerotinia* ascospores (10^5 spores/ml); Treatments D and E were inoculated with both *B. amyloliquefaciens* BS6 (10^8 cfu/ml) and *Sclerotinia* ascospores (10^5 spores/ml). Three leaves per plant were inoculated with infested petals. Treatments with same letters are not significantly ($P < 0.05$) different. LSD=0.3731

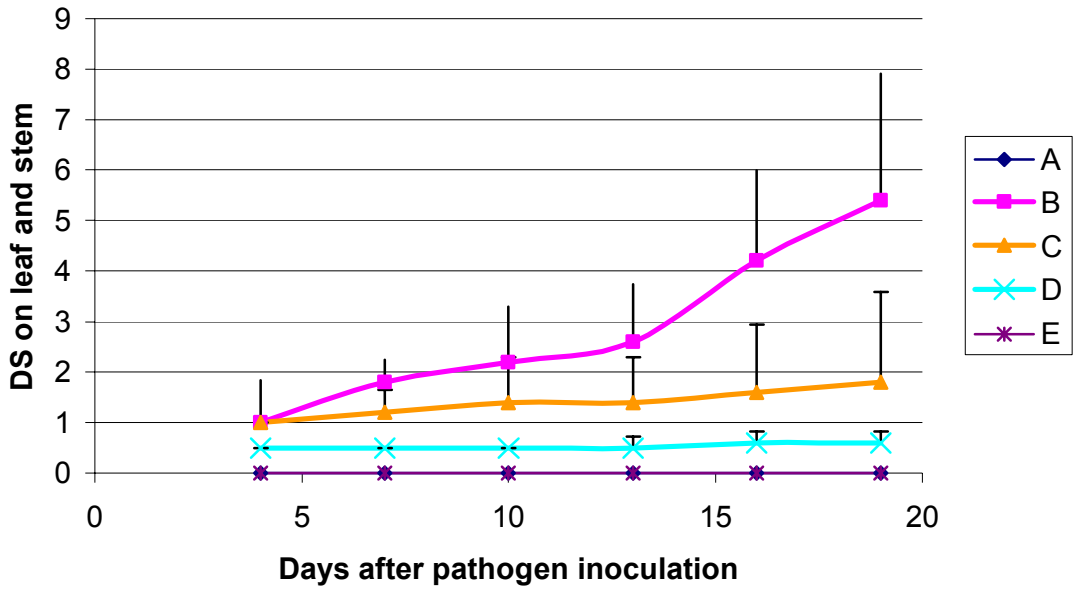
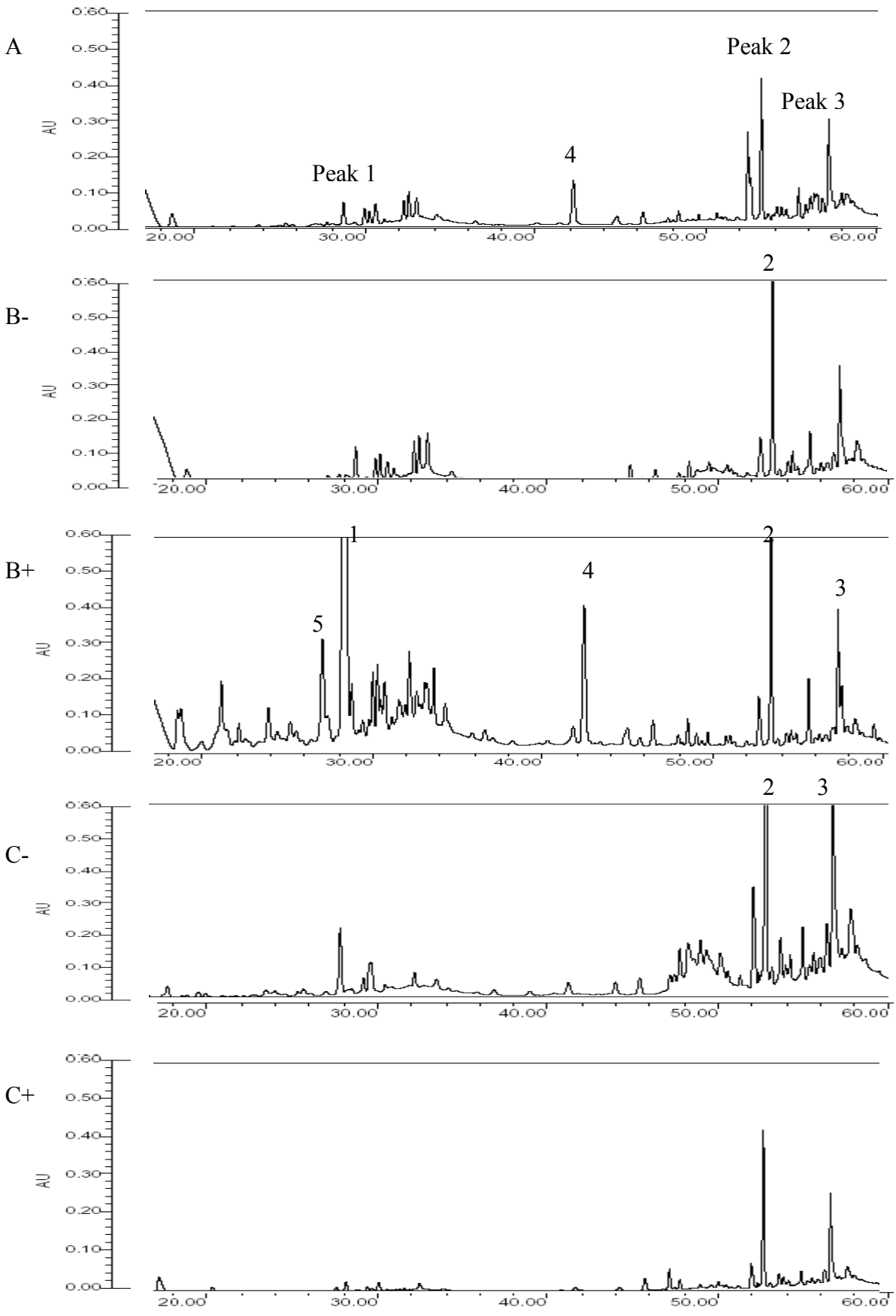


Figure 5.5 Disease progress over time on canola plants. Treatments C and E were pre-treated with *B. amyloliquefaciens* BS6 (10^8 cfu/ml) by spraying; Treatments A (control), B, D were pre-treated with water. Treatment B and C were inoculated with *Sclerotinia* ascospores (10^5 spores/ml); Treatments D and E were inoculated with both *B. amyloliquefaciens* BS6 (10^8 cfu/ml) and *Sclerotinia* ascospores (10^5 spores/ml). Three leaves per plant were inoculated with infested petals, and Disease severity (DS) was rated every 3 days 19 DAI. Bars represent standard deviation.


A
Peak 2
Peak 3
4
Peak 1
B-
2
B+
1
2
4
3
5
C-
2
3
C+
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0.60
0.50
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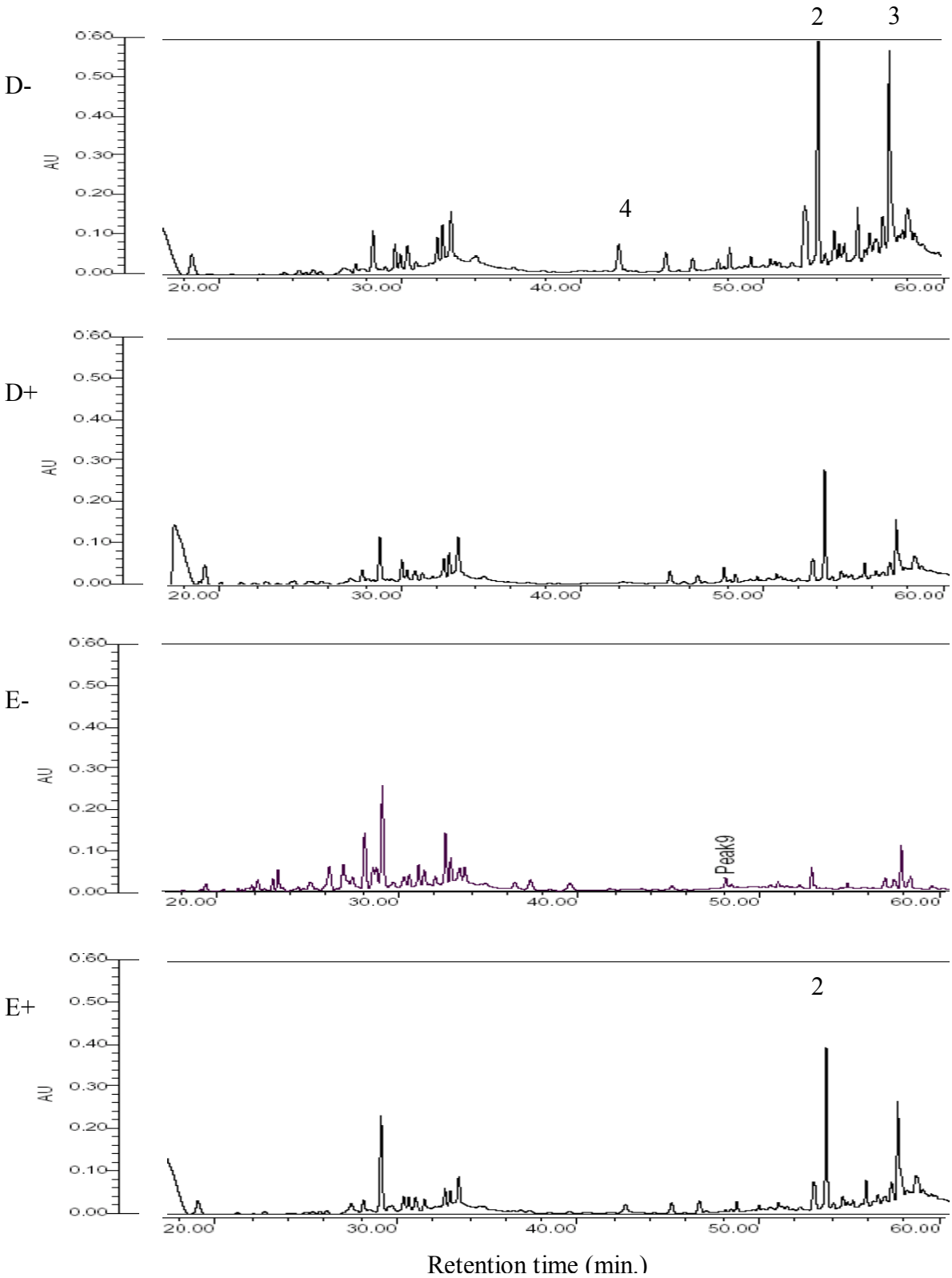

Figure 5.6 HPLC chromatograms from leaf samples 5 days after pathogen inoculation (DAI). The absorbance was monitored from 220 nm - 370 nm. The samples of treatements C- and C+ were diluted 15 times compared to the other samples.

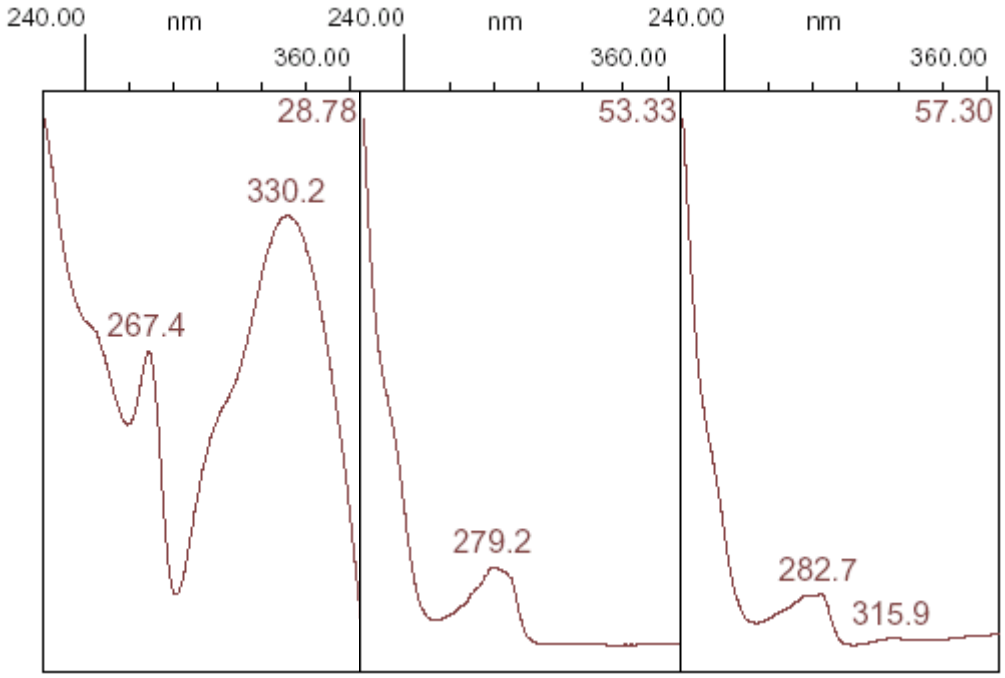

Figure 5.7 Spectrum index plots of 3 compounds from canola leaf sampled 5 days after pathogen inoculation (DAI). Samples were analysed by Waters HPLC, and the UV absorbance was monitored between 220 nm and 370 nm. Three major peaks (Figure 5.5) were found to be related to the difference between treatments, and designated as compounds 1-3. Numbers above peaks in the upper graph indicate the max absorbance. The retention time of the 3 compounds are 28.776, 53.327, 57.29 min, respectively.

The samples from 3 DAI and 7 DAI (data not shown) showed similar results but are not as clear as those from 5 DPI. The compound 1 seems to reach the highest concentration 3 DAI, and the compound 2 and 3 seems to reach the highest concentration 5 DAI.

5.5 Discussion

Disease incidence (DI) on canola stems was reduced by 60% on plants pre-treated with *B. amyloliquefaciens* BS6 and inoculated with *Sclerotinia* ascospores (treatment C) compared to plants pre-treated with water and also inoculated with *Sclerotinia* ascospores (treatment B). Plants pre-treated with *B. amyloliquefaciens* BS6 and inoculated with both *B. amyloliquefaciens* BS6 and *Sclerotinia* ascospores (treatment E), had no disease symptom (0%) on either leaf or stem, compared to plants pre-treated with water and then inoculated with both *B. amyloliquefaciens* BS6 and *Sclerotinia* ascospores (treatment D) with localized water-soaked lesions. The two cases of comparison suggest that pre-treatment with *B. amyloliquefaciens* BS6 did reduce the sclerotinia disease. However, the pretreatment of *B. amyloliquefaciens* BS6 only achieved a significant ($P < 0.05$) reduction of DI on canola leaf 4DAI, but not on canola stem 7DAI, by comparing the treatments D and E (Figure 5.3 and 5.4). Therefore, even though induced resistance by *B. amyloliquefaciens* BS6 on canola plants may involve be in the disease suppression, the latter seems to be mainly due to a direct bacteria-fungi interaction.

In this experiment, treatment E where plants were pre-treated with *B. amyloliquefaciens* BS6 and then inoculated with both *B. amyloliquefaciens* BS6 and

Sclerotinia ascospores achieved complete disease suppression. In an earlier study about the effect of time of application of strain BS6 (see chapter 3), treatment B+A (strain BS6 and ascospores inoculated at the same day) had the best disease control among all the treatments, including treatment B-2-A (BS6 inoculated 2 days prior to ascospore) (Figure 3.9) that was equivalent to treatment C (pre-treated with water and inoculated with B-S+ petals) in this experiment. The results from the two experiments are not contradictory; however, the combination of the results demonstrates that when *B. amyloliquefaciens* BS6 inoculated with *Sclerotinia* ascospores at the same day (Figure 3.9) or when plants were pre-treated with strain BS6 2 days prior to ascospore inoculation, strain BS6 had significant disease suppression but did not achieve complete disease suppression; However, only when *B. amyloliquefaciens* BS6 was used as both pre-treatment and co-inoculum with *Sclerotinia*, the complete disease suppression was achieved.

The question here might be the disease suppression by *B. amyloliquefaciens* BS6 pre-treatment is due to the bacterial residue on the plant leaf (even bacterial population level might be low), which might be toxic to ascospores, or to the induced resistance by the plant itself. The HPLC results of treatments C (inoculated with ascospores) and E (inoculated with B+S petals), where plants were both pre-treated with strain BS6, demonstrate that the induction of compounds 2 and 3 are from plants other than from bacteria, since treatment C has much higher concentration of the two compounds than treatment E. Interestingly, the elicitation of compounds 2 and 3 by *B. amyloliquefaciens* BS6 seems to happen only when the plants are under high disease pressure, such as *Sclerotinia* ascospores are active or in high concentration in infecting plants. This might be the reason why even treatments C and E are both pre-treated with

strain BS6, but the two compounds are found at high concentration only in treatment C, but not in E, in which *Sclerotinia* ascosproes might be greatly inhibited by the bacteria.

The study by Collins et al. (2003) showed that the bacterial cell density of *B. subtilis* BacB isolated from sugarbeet phyllosphere was not strongly correlated to the disease suppression of sugarbeet cercospora leaf spot, caused by *Cercospora beticola* under field conditions. Their greenhouse study also showed that plants that were pre-treated by *B. subtilis* BacB 3 days prior to *C. beticola* inoculation have significant better control than those treated with bacteria right before the pathogen, which may indicate that the foliar-applied *B. subtilis* BacB had induced the sugarbeet to build up its resistance to *C. beticola*. Similar results were found by Liu et al. (1995), Hoffland et al. (1995) and Meena et al. (1999) using foliar-applied plant growth-promoting rhizobacteria (PGPR). Therefore, the ISR might not be only a common phenomenon of PGPR when bacteria are applied on rhizosphere, but also common in foliar-applied biocontrol bacteria agents. Since the foliar-applied bacterial agents and the *S. sclerotinia* both target plant phyllosphere (or blossoms), the inoculation method of studying induced resistance needs to be especially concerned. The inoculation could either be done by spatially separating the inoculations of bacteria and *S. sclerotinia*, or by adding a bacterial treatment (without *S. sclerotinia*) to the experimental design of this study. A better design will help clarify the role of the pathogen and the bacteria in the process of induced resistance in canola.

The induction of phenolic compounds in treatment B+ and B- (plants were pre-treated with *B. amyloliquefaciens* BS6, and then *Sclerotinia* was inoculated on B+ leaves) compared to control (treatment A) is an indication of systemic acquired resistance

mediated by the pathogen *S. sclerotiorum.* However, whether this form of induced resistance will deviate the further pathogen attacking is unknown. Systemic resistance to *S. sclerotiorum* in oilseed rape was first reported by Toal and Jones (1999) by pre-treated oilseed rape with oxalic acid. Oxalic acid is the major pathogenesis factor of *S. sclerotiorum.* Plants treated with oxalic acid were found to have significantly smaller leaf lesions than the control plants after being challenged by the pathogen. Whether the phenolic induction in this experiment was due to the pathogenesis factor oxalic acid or other determinants of *S. sclerotiorum* is unkonwn.

Since induced resistance is generally expressed against a broad spectrum of organisms, it usually results in preventing a variety of plant diseases (Kuc 2001). The study by Bonnet et al. (1996) showed that elicitins, which are a family of proteins excreted by *Phytophthora* spp., not only induced resistance to the *Phytophthora parasitica* var. *nicotianae* on tobacco, but also to *S. sclerotiorum* on oilseed rape. Therefore it is also valuable to investigate the induced resistance elicited by *B. amyloliquefaciens* BS6 against other canola pathogens, such as *Leptosphaeria maculans.* Integrating plant induced resistance as one of the strategies or mechanisms in disease control under field conditions will be promising, since there are different pathogens existing in the natural farm system.

6.0 GENERAL DISCUSSION AND CONCLUSIONS

Antibiosis and plant induced resistance appear to be two separate mechanisms of disease suppression, and the their combination may lead to improved efficacy of biological control. Bakker et al. (2003) stated that antibiosis would first weaken or even partly kill the population of the pathogen and, subsequently, this weakened population of the pathogen would be confronted on a plant that is in a state of enhanced defensive capacity, thus resulting in much less disease development. Therefore, integrating the two mechanisms in biocontrol of *Sclerotinia* on canola should be a promising prospect.

Study of bacterial population dynamics is important in biocontrol of plant pathogens. The understanding of bacterial population and the time of application is essential to help understand biocontrol mechanisms. In this study, the bacterial population dynamics were investigated with *B. amyloliquefaciens* BS6 and *Pseudomonas chlororaphis* PA-23, and the effect of time of application and plant induced resistance were tested with *B. amyloliquefaciens* BS6; however, whether plant induced resistance is involved in the disease suppression by other bacteria that were used in the field study is unknown.

Most studies on induced systemic resistance (ISR) are on plant growth promoting rhizobacteria (PGPR) (van Loon et al. 1998), and studies on ISR mediated by foliar applied bacteria are comparatively rare. Leaf application of PGPR strains which induce resistance on plants were reported by Liu et al. (1995), Hoffland et al. (1995) and Meena et al. (1999). *Bacillus subtilis* BacB was isolated from phyllosphere and has been found to induce resistance on sugar beet against Cercospora leaf spot when used as a

foliar application (Collins and Jacobsen 2003). The preliminary study of induced resistance mediated by *B. amyloliquefaciens* BS6 (chapter 5) has already strongly suggested that induced resistance by foliar applied bacteria may be involved in sclerotinia disease control on canola. Therefore, the induced resistance mediated by *P. chlororaphis* PA-23 and other bacteria should be evaluated. Investigating plant induced resistance mediated by foliar applied bacteria will help us to establish a better system to control *Sclerotinia sclerotiorum* by combining different mechanisms in a single biocontrol agent.

P. chlororaphis PA-23 has shown successful disease control in three field trials in two years (one in 2001 and then in 2003), even though the population density was quite low in some of the cases. The high disease suppression of sclerotinia stem rot by *P. chlororaphis* PA-23 seems to involve multiple mechanisms. It was found by Kavitha et al. (2003) that *P. chlororaphis* PA-23 had significant increase in plant growth as well as disease control of damping-off in chilli. It was also found that plants pre-treated with *P. chlororaphis* PA-23 and challenged with *Pythium aphanidermatum* in tomato had higher levels of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and β-(1,3)-glucanase, which indicated it may have triggered induced systemic resistance in plants. This study has shown that *P. chlororaphis* PA-23 could probably produce two or three antibiotics (chapter 4). The future work on Tn5 mutagenesis of the wild-type strain, and testing antibiotic deficient mutants and wild type strians against *S. sclerotiorum* on canola, will help understand the role of phenazine, pyrrolnitrin and perhaps 2,4-diacetylphloroglucinol in controlling *Sclerotinia* on canola.

Bacillus spp. are well known to have good survival under field conditions due to the production of endospores (Boyetchko 1999; Collins and Jacobsen 2003). *Bacillus amyloliquefaciens* BS6 was observed to survive longer than *P. chlororaphis* PA-23 in the field study (chapter 3), but single application of *P. chlororaphis* PA-23 had better disease control than that of *B. amyloliquefaciens* BS6 in Trial Two (without tents). Therefore, the disease suppression under field conditions does not necessarily correlate with bacterial survival ability and this should be taken into account when biocontrol agents are screened and developed. It was suggested that effective screening of biocontrol bacteria could be conducted by screening on media plates first and following it with a field experiment that includes as much genotypically-diverse bacterial strains as possible (Roberts and Lohrke 2003). *Bacillus* spp. have an advantage to commercialization due to their endospore forming ability (Collins and Jacobsen 2003). The commercialization of *Pseudomonas* spp. as biocontrol agents seems to be much more difficult than that of *Bacillus* spp. (Scherm et al. 2004). Slininger et al. (1996) stated, "Barriers to the commercial use of phenazine-producing pseudomonads, such as strain 2-79 include the lack of liquid-culture and formulation technologies needed to optimize cost-effective mass production and application." However, the study by Janisiewics and Jeffers (1997) have shown that the formulation of *P. syringae* (ESC-11) as a biofungicide (Bio-Save 11), did not have adverse effect on disease control of blue mold and gray mold of apples, compared with fresh bacterial cells. Therefore, the study on formulation of *P. chlororaphis* PA-23 towards commercialization is needed, since it might have greater disease suppression than *Bacillus* spp. when appropriately applied on canola plants. In addition, as different bacterial strains may be involved in different mechanisms, it is useful to develop

formulations to combine the bacterial strains, which are capable of producing antibiotics, inducing plant resistance and promoting plant growth.

S. sclerotiorum is comparatively easy to handle *in vitro*, but its inoculation on canola plants is difficult, especially under field conditions. In the greenhouse studies (chapter 3 and chapter 5), different inoculation conditions were employed, and this was mainly due to lack of standard protocol. In the study by Jurke (2003), several *Sclerotinia* inoculation methods were compared for selecting lines of sclerotinia stem rot resistance in *B. napu*s , and mycelium-infested petal technique (Mypetal) appeared to be the most accurate, reliable and efficient disease inoculation method. In his study, ascospore-infested petal technique (Ascpetal), which is similar to the dipping petals method in this study, was also regarded as the most accurate measure of physiological resistance since it mimics the natural infection process; however, it was found that Ascpetal technique had low efficiency and high variability in growthroom trials, so it was not recommended for disease inoculation in growthroom for screening resistance against *Sclerotinia*. However, *S. sclerotiorum* infects canola plants mainly through ascospore-infected petals, and petals offer a critical nutrient factor for ascospores to germinate. So many of the other methods, such as Mypetal technique are more artificial than the inoculation described in this study. Mypetal technique is not recommended for screening biocontrol agents in the greenhouse, for it might mask the real biocontrol ability of potential biocontrol agents. Ascospore inoculation was used in both greenhouse and field in this study, and they both appeared effective and reliable if proper conditions such as a misting system were provided. The dipping petal method used in this study was modified from the work of Savchuck (2002), by placing petals on water agar for 1 h instead of 24 h before placing

onto plants. The dipping petal method described in chapter 3 and following with an incubation period of 3-4 days with daytime temperatures of 23-24 °C in humidity chamber is recommended for *Sclerotinia* ascospore inoculation in the greenhouse. After the incubation period, plants should be kept no less than 23 °C (25 ° C is probably the best) to allow the disease to progress.

There is also no generally used standard for *Sclerotinia* disease evaluation on canola so far. The disease evaluation used in both greenhouse study (chapter 3 and chapter 5) and field study (chapter 5) is recommended for future disease rating of *Sclerotinia* stem rot on canola, accompanied by the recommended inoculation methods.

In summary, several potential biocontrol agents against *S. sclerotiorum* were screened in the lab and greenhouse in this study. The two field trials showed that *P. chlororaphis* PA-23, *Pseudomonas* spp. #41, *B. amyloliquefaciens* BS6, and *Staphylococcus* spp. E16 achieved significant disease suppression, and their disease suppression ability may not depend on bacterial survival. The presence of antibiotic biosynthetic or self-resistance genes in several potential biocontrol bacteria and the evidence of induced resistance mediated by *B. amyloliquefaciens* BS6 have strongly suggested that antibiosis and plant induced resistance might be two important mechanisms responsible for the effective sclerotinia disease control on canola.

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