Chemical and Biological Control of *Sclerotinia* Diseases in Sunflower and Study of Biocontrol Mechanisms Involved

by

Sarangi Nirosha Priyajeevani Athukorala

A Thesis

Submitted to the Faculty of Graduate Studies of

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GENERAL ABSTRACT

Athukorala, Sarangi Nirosha Priyajeevani. M.Sc., The University of Manitoba, March, 2008. Chemical and Biological Control of Sclerotinia Diseases in Sunflower and Study of Biocontrol Mechanisms Involved. Major Professors; W.G. Dilantha Fernando and Khalid Y. Rashid.

Sclerotinia sclerotiorum (Lib.) de Bary is a devastating pathogen affecting over 400 different crop species and causes head rot, root rot and wilt, and mid-stalk rot, in sunflower (*Helianthus annuus* L.) worldwide. The disease management strategies in Canada include mainly cultural practices like crop rotation, tillage practices since there are no highly resistant cultivars or registered fungicides available. Biological control of wilt using fungal antagonists has been mostly experimental. The use of bacterial agents against this pathogen in sunflower is uncommon in Canada. Therefore, two bacterial strains *Pseudomonas chlororaphis* PA23 and *Bacillus amyloliquefaciens* BS6 which exhibited *in vitro* and *in vivo* inhibition of *S. sclerotiorum* in canola were used with the fungicide Ronilan (vinclozolin) in a field study at Morden, Manitoba against head rot and wilt diseases in 2006 and 2007. In 2006 none of the strains or the fungicide was successful against head rot due to the high disease pressure that prevailed. However, with some modifications in the application procedure a reduction in head rot by PA23, BS6 and Ronilan could be observed in the 2007 head rot trial although the differences were not significant. Only Ronilan showed significant reduction of wilt in both years. In addition, growth promotion by these two strains was monitored in two field trials located at Morden and Winnipeg in 2007. None of the strains in both locations promoted growth of sunflower. The laboratory experiment carried out to determine the involvement of different genes in the production of organic antimicrobial volatiles by PA23 showed that

none of the tested genes affect the antibiotic production but they seem to affect the amount of volatiles produced by PA23 to various extents. Further research is suggested to test this hypothesis. The greenhouse study to determine the effect of two different antibiotics, phenazine and pyrrolnitrin, produced by PA23 on its colonization ability showed that antibiotics play a role when nutrients in the rhizosphere become limited. In addition, various other factors including siderophores seem to affect its colonization efficiency, their antibiotic production and biocontrol ability. Antibiotic deficient mutants showed relatively reduced colonization ability compared to the wild type PA23. Furthermore, combining PA23 with mutant strains of PA23 also reduced its efficiency but the relative effect of one antibiotic-deficiency over the other was not clear. In the investigation of antibiotics produced by *Bacillus* sp. isolated from different habitats using PCR, the presence of biosynthetic genes for surfactin and iturin A were identified in 20/21 of strains. Only a few strains showed the presence of other antibiotic genes tested. Sequence analysis showed that BS6 was positive for all genes tested. The MALDI-TOF MS analysis further confirmed the production of particular antibiotics by these strains. This indicates that antibiosis may be one of the mechanisms involved in the biocontrol exhibited by these *Bacillus* strains. In summary, this study demonstrated the potential use of the fungicide Ronilan to control wilt of sunflower and the importance of studying the effect of environmental factors influencing the efficacy of a biocontrol agent when using it in the rhizosphere and the phyllosphere.

FORWARD

This thesis is written in manuscript style. Each manuscript has its own abstract, introduction, materials and methods, results and discussion sections. There is a general abstract, general introduction and literature review prior to manuscripts. The literature cited section follows after a general discussion and conclusions.

1.0 INTRODUCTION

Cultivation of sunflower (*Helianthus annuus* L.) began in temperate North America and has become the world's fourth largest oilseed crop (Kleingartner, 1997). Manitoba is one of the major producers of sunflower in Canada. Approximately 80% of this production is confection type (Manitoba Agriculture, Food and Rural Initiatives, 2004). Canada is a competitive partner of the growing international market and the demand for Canadian grown sunflower seeds is on the rise. For example, the birdseed market for Canadian-grown sunflower seeds is growing at an annual rate of 10% in North America (Agriculture and Agri-Food Canada, 2007).

Diseases are one of the main factors that limit the sunflower production worldwide. *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the common and widespread pathogens (Bisby, 1921) responsible for three main diseases, namely, head rot, mid-stalk rot and wilt in sunflower (sclerotinia wilt). Since 1921, sclerotinia diseases have been recorded to bedestructive to sunflower in Canada and United States. In terms of yield loss, sclerotinia wilt is considered the most challenging sunflower disease in North America (Gulya, 1985, 1996). Head rot and mid-stalk diseases are caused by air borne ascospores produced by apothecia. This is a result of carpogenic germination of over-wintering sclerotia of *S. sclerotiorum*. Wilt and root rot are caused by hyphae which are produced by myceliogenic germination of buried sclerotia (Willetts & Wong, 1980).

Compared to other crops, management of *S. sclerotiorum* in sunflower is limited. Chemical fungicides are not currently registered for this pathogen in Canada. (Mestries et al., 1998; Rashid & Dedio, 1992). Breeding for resistance is economical; however, this is

not always possible due to the lack of resistant germplasm among sunflower varieties to this pathogen. In addition, the resistance is not consistent from year to year (Rashid & Dedio, 1992). The hybrids with high resistance to wilt have not always had satisfactory resistance to head rot due to the involvement of different genetic factors (Rashid & Dedio, 1992). Cultural practices including crop rotation are not always successful and are highly dependant on the prevailing environmental conditions. Biocontrol is another applicable method which is an eco-friendly way of combating plant diseases. This method also provides safe and long-term protection to the crop (Fernando et al., 2005). Fungal antagonists such as *Coniothyrium minitans* Campbell, *Gliocladium catenulatum* Gilman and Abbott and *Trichoderma viride* Pers. ex Fr. have been successful against sclerotinia wilt of sunflower in Canadian Prairie conditions (Huang, 1980a).

However, use of bio-control agents (BCA) against airborne ascospores (head rot and mid-stalk rot) and the use of antagonistic bacteria against sclerotinia diseases in sunflower are rare or at experimental level in Canada. There are a few situations where bacterial species (such as *Bacillus cereus* Frankland and Frankland, strain alf-87A, *Bacillus subtilis* (Ehrenberg) *Pseudomonas chlororaphis* Guignard and Sauvageau strain PA23 and *Bacillus amyloliquefaciens* n. sp. Fukumoto strain BS6) have been tested against basal pod rot of pea (Huang et al., 1993), white mold of bean (Boland & Hall, 1987) and stem rot of canola (Savchuk & Fernando, 2004; Zhang, 2004), respectively.

Therefore, the first main objective of this research is to assess the effectiveness of the two bacterial species (*P. chlororaphis* PA23 and *B. amyloliquefaciens* BS6) against sclerotinia wilt and head rot diseases in sunflower compared to an experimental fungicide (Ronilan, active ingredient vinclozolin). This study will reveal the efficacy of these

antagonists in sunflower-sclerotinia host pathogen system. Data collected from field will be used to determine the commercial applicability of these bacteria. Furthermore, it will compare the efficacy of a *Pseudomonas* sp. against *S. sclerotiorum* over a *Bacillus* sp. and a fungicide. In addition, the growth promotion ability of the antagonists is also investigated under field conditions.

In the biocontrol of wilt disease, the root colonization ability of a biocontrol agent (BCA) is an important factor that facilitates the even distribution and superior performance of the BCA. Successful colonization of rhizosphere requires the adaptation and some selective advantage over the indigenous microorganisms (Mazzola et al., 1992). The ability of a BCA to produce antibiotics is considered an important trait that affects its rhizosphere colonization ability (Bruehl et al., 1969; Atlas & Bartha, 1987; Mazzola et al., 1992). This concept has been tested on several occasions and the results were inconsistent. In some studies the importance of antibiotics has been proven (Mazzola et al., 1992) while in other studies a significant effect has not been observed (Thomashow & Weller, 1988; Paulitz & Linderman, 1989; Caroll et al., 1995; Chin-A-Woeng et al., 2000). Furthermore, the root colonization and the antibiotic production are also affected by several factors (Duffy & Défago, 1997; Howie & Suslow, 1991; Ownley et al., 1992; Shanahan et al., 1992; Slininger & Jackson, 1992) including plant species and cultivars (Georgakopoulos et al., 1994; Maurhofer et al., 1995).

The effect of different antibiotics produced by a BCA on its root colonization ability and the effect of different plant species on the antibiotic production of BCAs have been studied (Howie & Suslow, 1991; Thomashow et al., 1990; Mazzola et al., 1992). However, particular interactions have not been studied for the sunflower host system.

Therefore, the second objective of this research is to investigate the effect of two main antibiotics; phenazine and pyrrolnitrin, produced by PA23 on its root colonization ability on a sunflower host system and the relative importance of the above mentioned antibiotics in wilt disease control. It is very important to understand particular interactions, especially when PA23 is used against wilt disease in sunflower.

S. sclerotiorum over-winters as sclerotia (Adams & Ayers, 1979). Myceliogenically germinated sclerotia are the primary source of inoculum for the wilt disease. Carpogenically germinated sclerotia are the source of ascospores, which act as the primary inoculum for head rot and middle-stalk rot diseases in sunflower. Therefore, the management of sclerotia in soil is important in order to minimize the disease incidence. The volatile antimicrobial compounds are more important over non-volatile antimicrobial compounds. They can diffuse through the soil and kill the pathogen without contact between the antagonist and sclerotia (Fernando et al., 2005). Previous studies identified that *P. chlororaphis* PA23 produces three organic volatiles; nonanal, 2-ethyl-1-hexanol, and benzothiazole, together with HCN. These are inhibitory to mycelial, sclerotial and ascospore germination of *S. sclerotiorum* under *in vitro* conditions (Fernando et al., 2005; Poritsanos et al, 2006). The production of volatile antibiotics is considered to be one mechanism of biocontrol of PA23. Therefore the third objective of the current study was to understand the involvement of different genes in the production of the above three organic volatiles of PA23. This objective helps to understand how volatile organic antimicrobial compounds are regulated. Production of a genetically enhanced BCA that contains all desirable characteristics will also be supported by this type of information.

Similar to *Pseudomonas* spp., *Bacillus* sp. have been studied as a BCA against plant pathogens (McKeen et al., 1986; Silo-suh et al., 1994) and the production of antibiotics such as surfactins, iturins, zwittermicin A, bacillomycins, fengycin, etc. has been found to be one major mechanism of its biocontrol (Stabb et al., 1994; Moyne et al., 2001; Asaka & Shoda, 1996; Leclère et al., 2005). Previous studies carried out in this lab isolated and identified *Bacillus* sp. which showed significant *in vitro*, green house and field control of *S. sclerotiorum* and *Fusarium graminearum* (Fernando et al, 2002; Zhang, 2004; Duncan et al., 2006). The production of antibiotics was thought to be one of the mechanisms of bio-control involved since a significant mycelial inhibition has been observed on agar diffusible plate assay (Zhang, 2004). Therefore, the fourth objective of this study was to detect the presence of antibiotic biosynthetic genes of 21 potential *Bacillus* BCAs using specific primers for the antibiotics, surfactin, iturin A, bacillomycin D, fengycin, mycosubtilin and zwittermicin-A through polymerase chain reaction (PCR). Furthermore, instead of the biochemical detection of the production of antibiotics, matrix-assisted laser desorption ionization-time of flight-mass spectroscopy (MALDI-TOF-MS) analysis (Vater et al., 2002a) will be done to further confirm their production.

Determining the efficacy of certain BCAs under field conditions is required before it can be released as a commercial product. Understanding the different traits that contribute to their biocontrol ability and understanding the mechanisms of biocontrol at molecular and biochemical level are important when developing strategies for application of antagonists and gathering desirable characteristics through genetic engineering. The outcome of this study will address these areas to some extent.

2.0 LITERATURE REVIEW

2.1 Sunflower: *Helianthus annuus* L.

Sunflower (*H. annuus* L.) is considered as one of the few crop plants that have been domesticated in temperate North America and whose wild progenitor still exists (Heiser, 1951). It is also considered as the only major agronomic crop which arose from the native North American species (van Wyk, 2005; Flora of North America, 2006). It is one of the most important oil-seed crops in the world (Putt, 1997; van Wyk, 2005).

The cultivated sunflower is a tall, unbranched annual plant belonging to the family Asteraceae with sturdy stems and large, coarsely hairy leaves and a single large flowering head (inflorescence, up to 0.5 m in diameter) borne at the stem tip. The fruits are one seeded achenes, which can be black, brown, grey or striped (van Wyk, 2005; Wikipedia Contributors - sunflower, 2007).

2.1.1. History

The origin of domesticated sunflower was initially thought to be in the east-central United States where the use of wild sunflowers by Native Americans as a food source eventually resulted in a camp-following weed that spread eastward and became domesticated (Heiser 1954, 1978). This has been supported by archaeological evidence (Brewer, 1973; Ford 1985; Crites, 1993; Smith, 2006) with the discovery of the carbonized achenes from the Hayes site in Middle Tennesee, which belongs to the middle

of the fifth millennium B.P. This single-origin hypothesis has also been substantially strengthened by recent genetic studies (Harter et. al., 2004; Lentz et. al., 2006). The discovery of 4,000 year old domesticated sunflower remains from San Andrés, Tabasco in Mexico questioned the single-origin hypothesis but this argument is lacking well documented description and diagnostic morphology (Smith, 2006). However it is suggested that the archaeological record of Mexico represents either the southward dispersal of domesticated sunflower from eastern North America or a second independent domestication of sunflower in Mexico (Smith, 2006).

The sunflowers are known to be widely used by North American Indians as food, medicine and for ceremonial use (Heiser, 1951). According to Dodge (1870) "the seeds are eaten raw, or pounded up with other substances, made into flat cakes and dried in the sun." Some tribes had roasted or ground seeds into a flour, and then made into cakes or mush (Heiser, 1951). Hidatsa Indians used pounded parched sunflower with beans, dried squash and corn to prepare a mixed meal called "four-vegetables-mixed" (Wilson, 1917). Furthermore, the seeds were parched, ground and made into palatable and nutritious bread. It is also said that the common sunflower was a staple food source which extended from the Arctic Circle to the tropics and from the Missouri river to the Pacific and it was commonly cultivated by the Indians, from Canada to Mexico, west and east of the Mississippi (Havard, 1895). In addition, 20 per cent of excellent table oil had been used for anointing their hair and skin by many tribes (Havard, 1895; Parker, 1910; Waugh, 1916; Jenness, 1934). Furthermore, an important use among the Hopi was the purple dye obtained from the achene coats, which was used for textiles and basketry. The dried stalk was used as ventilation structures (Whiting, 1939). According to Gilmore

(1913), Dakotas used an infusion made from the heads of sunflowers for chest pains. Furthermore, sunflower was used among the Zuni as a medicine for rattlesnake bite (Stevenson, 1915). Pounded and cooked seeds, mixed with roasted white corn and maple sugar, was used for bad cough and also for False Face ceremonies similar to modern day lard (Heiser 1951). The early use of the plant as a food might be the first step toward the eventual development of a cultivated sunflower (Heiser, 1949). However there are records from Hidatsa Indians that they collected seeds from big heads as much as eleven inches across, which is an indication of the use of cultivated sunflower by early Indians of North America.

The introduction of this exotic North American plant into Europe probably came from Mexico with Spanish explorers around the sixteenth century and subsequently from "Virginia" and Canada by the French and English (Heiser, 1951). The earliest record of transportation reveals that this movement initially occurred from New Mexico to Madrid in 1510 (Zukovsky, 1950) and later distributed eastward and northward over the continent of Europe (Putt, 1997). Initially the plant was popular throughout present-day Western Europe as an ornamental but some medicinal uses were also developed (Semelczi-Kovacs, 1975). For example, Putt (1997) states that sunflowers were produced in gardens in Belgium, the Netherlands, Switzerland, Germany and England by the late 16^{th} century. In 1664 it moved to countries like Hungary from Germany also as a garden plant (Semelczi-Kovacs, 1975). However, in 1716 an English patent was granted to Arthur Bunyan, for describing the extraction of oil from sunflower seeds (Putt, 1997) which extended its use in Europe beyond the garden.

Distribution of the sunflower in Russia took place in the 18th century through the seeds produced in the Netherlands (Semelczi-Kovacs, 1975). This introduction is very important in the history since today Russia is the world's top sunflower producer. Although it was initially as an ornamental there are records that sunflower became very popular as a cultivated plant in the 18th century mostly for oil production (Semelczi-Kovacs, 1975), where by 1830 the manufacture of sunflower oil was a commercial scale industry in Russia (Quesenberry et al., 1921). This might have been supported by the incidents that happened in Russia during that period where the Russian Orthodox Church forbade most oil foods from being consumed excluding sunflower (Heiser, 1955). With rapid expansion of cultivation after the introduction the breeding programs were commenced in 1890 with the search for resistance to the European sunflower moth (*Homoesoma nebulella* Hb.) (Gundaev, 1971) and later to increase the oil content. As a result Pustovoit was able to increase the oil content of the main cultivar to 550g/kg by 1965 from 330 g/kg in1940 in USSR (Putt, 1997). By 1915 two distinct types of seeds were deceloped, small ones for edible oil and large ones for direct human consumption, which today are referred to as oil-seed and confectionary (Putt, 1997).

Reintroduction of sunflower into North America occurred in the latter part of the 19th century (Putt, 1997) either through the ordering of seeds from Russia by American farmers (Semelczi-Kovacs, 1975) or via immigrants mostly as a silage crop (Putt, 1997). However the competition from other U.S. oilseed crops as well as the imported oils prevented the sunflower from evolving as a significant agricultural crop (Putt, 1997).

2.1.1.1 Sunflower in Canada

Sunflowers were grown in the prairie provinces of Canada during the early 1900's as a silage crop (Geise, 1974). The evidence from the descendants of Mennonite immigrants to Canada reveals that sunflower seeds were brought by their parents from Russia (Putt, 1997). However on the way of seeking for potential oilseed production in Canada, breeding research began at the Saskatoon Research Station, Agriculture Agri-Food Canada concerning the desirable agronomic characters of sunflower such as strength of stem, early maturity, vigour and high seed yield per plant as well as high oil concentration in the seed (Putt, 1997). Furthermore due to the restrictions in edible oil supply during the World War II the Canadian government promoted sunflower production (Putt, 1997). After 1943 sunflower cultivation moved almost entirely to the Red River Valley of south central Manitoba because of the availability of germplasm resources imported by Mennonites, and the extended growing season in the area (Putt, 1997). The mean annual gross return per hectare from sunflower during the periods of 1943 to 1948 and 1947 and 1948 was far greater than what was obtained from wheat, oat, barley and grain corn. With the sunflower rust epidemic in 1951 the cultivation and production in Manitoba dropped to the lowest level ever recorded since the reintroduction of the crop for oilseed. However, the discovery of rust resistant varieties was able to bring the mean crop growing area to almost 11,000 ha in Manitoba for the years from 1953 to 1959 (Putt, 1997). Discovery of the phenomenon of heterosis in sunflower around this time led hybrids to out yield the parental mean by almost 250% and the best open-pollinated cultivar by over 60%. The introduction of cultivars from the USSR with

much higher oil content and discovery of cytoplasmic male sterility and fertility-restoring genes around 1960 had an immense effect on the sunflower industry in North America.

The cultivation and production of sunflower in other continents in the world like Asia (China, India), Australia, Africa, and South America have been reported. However, when comparing the huge contribution done by North America and Russia to the evolution of sunflower, the role played by other countries seems not that prominent in the literature except that Argentina has been considered as second important area in the literature (Putt, 1997).

2.1.2 Agronomy and Production

The development and yield of sunflower is affected by two main factors; genetic background and environmental conditions (Blamey et al., 1997). Sunflowers are best grown on medium textured soils such as loams, silty loams and silty clay loams with good drainage. Sunflower is fairly drought tolerant (Robinson, 1978) because of the very deep root system and it provides the greater ability for roots to extract water under low water potential (Unger, 1990) they can produce satisfactory yield under water stress conditions compared to other crops. Unger (1990) states that by maintaining plant-available water (PAW) above 700 to 800 g/kg, near maximum yield could be obtained. Soil salinity and sodicity are major problems affecting sunflower production. Normally primary and secondary tillage operations are used to overcome the soil structural problems and also to control weeds and to prepare the seedbeds (Blamey et al., 1997). Furthermore planting date and soil and air temperatures above or below the optimal

temperature range are two main factors affecting germination and emergence. Below optimum temperatures germination and emergence decrease (Blamey et al., 1997). Nitrogen and phosphorus are the nutrients that are required by sunflower with occasional requirements for potassium on light textured soils. Normally the fertilizers are applied on the basis of a soil test (Manitoba Agriculture, Food and Rural Initiatives, 2004).

Sunflower seeding starts usually after May 1 and is completed by June 1 since the maturation will become an issue with late seeding specially for confection hybrids (Manitoba Agriculture, Food and Rural Initiatives, 2004). The optimum planting depth is from 3 to 7 cm (Shanthamallaiah et al., 1975). Recommended plant densities differ from oil seed cultivars to confectionary cultivars. For oil seed cultivars it is 40,000-75,000 plants/ha while for confectionary cultivars, it is 37,000-44,000 plants/ha since the seed size is more important in confectionary cultivars. Row spacing of 30-90 cm is recommended (Dedio et al., 1980). Sunflower is harvested in late September or October with a growing season of approximately 120 days (Hofman & Hellevang, 1997). Frost helps to dry the foliage and reduce moisture content (Hofman & Hellevang, 1997). Although the length of the growing season is dependent on various environmental factors (Hofman & Hellevang, 1997) plants are physiologically mature when the back of the head has turned from green to yellow (Allen et al., 1979). Moisture content of 20 to 25 % is optimum for harvesting with minimal damages by birds, seed shattering and plant breakdown. Seeds are further dried to about 10 to 12 % to avoid spoilage in the bin (Hofman & Hellevang, 1997).

Crop rotation is very important in sunflower agronomy specially to reduce the disease pressure. Normally the 4-5 year gap between two consecutive sunflower planting

seasons is recommended, where it is rotated with cereals like wheat (Young & Morris, 1927; McLaren et al., 1994). Cereals precede sunflower (Dedio et al., 1980) providing a sequence of hosts that are susceptible to different pathogens. Since sunflower has a high water use efficiency and can extract water from deeper soils, a crop with distinct nutrient and moisture requirements should be considered for a rotation system.

The plant growth stages (Table 2.1) developed by Schneiter and Miller in 1981 is well adapted for comparing differences in stages of development required by field scientists and producers to identify the perfect stage for applications (Blamey et al., 1997). This system is applicable to any kind of sunflower variety. Heliotropism is an interesting phenomenon shown by sunflower from emergence to anthesis and when anthesis is initiated this response ceases (Blamey et al., 1997).

About 95% of world sunflower production is the oilseed type while only 5% is the confectionary type. World sunflower oil-seed production has been relatively stable over the last few decades. For example, from 1987/1988 to 1990/1991 the mean world production was 21.5 million tonnes while from 1991/1992 to 1995/1996 it was 22.74 million tonnes (Mielke, 1996) with a low of 21.4 million tonnes (Mt) in 2001-2002 to a high of 27.3 million tonnes in 1999-2000 (Manitoba Agriculture, Food and Rural Initiatives, 2003). Over the ten years from 1981-1992 Russia, Argentina, Eastern Europe, USA, China, France, and Spain were among the top producers, which a ltogether

Table 2.1. Sunflower growth stages (Schneiter and Miller, 1981)

Stage	Description
VE Vegetative Emergence	Seedling has emerged and the first leaf beyond the cotyledons is less than 4 cm long.
V (number) Vegetative Stages ie:V1 V2 V3 etc.	These are determined by counting the number of true leaves at least 4 cm in length beginning as V1, V2, V3, V4, etc. If senescence of the lower leaves has occurred, count leaf scars (excluding those where he cotyledons were attached) to determine the proper stage.
R1 Reproductive Stages	The terminal bud forms a miniature floral head rather than a cluster of leaves. When viewed from directly above the immature bracts for a many-pointed star-like appearance.
R2	The immature bud elongates 0.5 to 2.0 cm above the nearest leaf attached to the stem. Disregard leaves attached directly to the back of the bud.
R3	The immature bud elongates more than 2.0 cm above the nearest leaf.
R4	The inflorescence begins to open. When viewed from directly above immature ray flowers are visible
R5 (decimal) ie:R5.1 R5.2 R5.3 etc.	This stage is beginning of lowering. The stage can be divided into sub-stages dependent upon the % of the head area (disk flowers) that has completed or is in flowering. ex: R5.3 (30%), r5.8 (80%) etc.
R6	Anthesis is complete and the ray flowers are wilting.
R7	The back of the head has started to turn a pale yellow colour.
R8	The back of the head is yellow but the bracts remain green.
R9	The bracts become yellow and brown. This stage is regarded as physiological maturity.

accounted for 84 per cent of the world's production of both oilseed and non-oil seed sunflower (North Dakota State University Agriculture and University Extension, 1995). In 1990-91 Russia produced about 27 per cent of the world production. In the 1970s the United States was the second largest producer in the world; however, in the 1980s Argentina quickly emerged in the second place (North Dakota State University Agriculture and University Extension, 1995). According to the 2005 statistics of UN Agriculture Organization (FAO) the first six top sunflower seed producing countries are Russia, Ukrain, Argentina, China, India and USA. In 2005 Russian seed production was 6.3 million metric tonnes while that of Argentina was 4.7 million metric tonnes (Wikipedia Contributors – sunflower seed, 2007).

After the shifting of cultivation of sunflower to the red river valley of south central Manitoba, the area planted increased rapidly to over 24,000 hectares in 1949. However, with the sunflower rust epidemic in 1951 the yield reduced to only 365 kg/ha (Putt, 1997). The planted area reached its peak in 1979 to approximately 170 thousand hectares. By 1986 the area reduced to about 25 thousand hectares due to problems of low prices as well as from weather, diseases and pests (Manitoba Agriculture, Food and Rural Initiatives, 2003). However according to statistics the sunflower area of production, yield and the volume of production keep fluctuating over the years. For example, in 1996 the lowest harvested area was recorded followed by 115 thousand hectares in 2003. In 2005, the area of production fell to 75 thousand hectares (Agriculture and Agri-Food Canada, 2007). However, according to Statistics Canada (seeded area), 2007 records sunflower seeded area is continuously declining from 118.5 thousand hectares in 2003 to 76.9 thousand hectares in 2007. Farm cash receipt of sunflower in 2006 was $ 32 million CDN

where it was a slight increase than in 2005 (Statistics Canada (farm cash receipts), 2007). Peak farm cash receipt in the recent past was reported in 2003 at 50.9 million CDN (Statistics Canada (farm cash receipts), 2007). Currently approximately 80 % of sunflower production is confectionary type and over 80 % is consumed domestically (Agriculture and Agri-Food Canada, 2007). Exports are significantly growing since 1991 and in 2005 the United State was the major importer of Canadian sunflower seeds (Agriculture and Agri-Food Canada, 2007).

In spite of the fluctuating area of production in different areas in the world, sunflower has become the world's fourth main oil-crop. The future of the world production over coming decades will be determined by the competitive ability, internal production policies of individual countries and trade policies (Kleingartner, 1997). Disease resistance and the improved yield capacities through genetic engineering will be other two factors determining the upcoming world sunflower production (Kleingartner, 1997).

2.1.3 Uses

There are two main types of cultivars in which the seed morphology differ from each other as do the end uses. Oil-type seed cultivars are characterized by black hulls and small seed size. Confectionary type seed cultivars have striped hulls and are relatively larger seed size than oil-types.

2.1.3.1 Oil-type sunflower cultivars

The industry utilizes every component of the seed for various purposes from which oil extraction is the major one. Eighty per cent of the value of the sunflower crop is accounted for by oil (Putnam et al., 1990). Commercial crushing of sunflower for oil began in Manitoba, Canada in 1944 (Dorrell & Vick, 1997). Production increased by mid 1970s with the introduction of hybrid cultivars which had higher yield with disease resistance (Dorrell & Vick, 1997). By today only in USA there are several plants that crush about 0.6 to 1 million tons of seed annually (Dorrell & Vick, 1997). Because of its light colour, high level of unsaturated fatty acids and lack of linolenic acid, bland flavour and high smoke points, sunflower oil is considered as one of the best oils (Putnam et al., 1990). In North America half of the oil sold as cooking oil is marketed as pure oil while the remainder is mixed with canola and soybean oils to enhance the flavour and odour of those vegetable oils (Dorrell & Vick, 1997). In addition sunflower oil is primarily used in salads and for processing margarine. In countries where the sunflower oil is the major oilseed, lecithin obtain from sunflower is used as an additive to poultry, swine and cattle feeds, and as food additive in products such as chocolate (Holló et al., 1993) and also in shrimp diet (Dorrell & Vick, 1997). Although the industrial uses of sunflower are limited because of higher prices compared to other sources, it has a considerable market for oil-based white and pastel paints where yellowing is a problem (Dorrell & Vick, 1997) and in other oil-based industries like soaps and detergents (Putnam et al., 1990). Furthermore bio fuel production from sunflower oil has been explored. Meal left behind after

extracting oil is an isonitrogenous diet for ruminant animals, swine and poultry feeding and is high in fiber (Putnam et al., 1990).

2.1.3.2 Confectionary type seeds

Confectionary sunflower seeds are used mainly for human and bird consumption. In Canada approximately 80 % of sunflower grown is confectionary type in the provinces of Manitoba, Saskatchewan, and Alberta (Manitoba Agriculture, Food and Rural Initiatives, 2004). In other parts of North America field production of confectionary type sunflower is concentrated in North Dakota and to some extent in South Dakota, Minnesota, Kansas, Colorado, California and Texas (Lofgren, 1997). Confectionary seeds are primarily marketed as roasted snack food with the shell. Dehulled seeds are widely used in salads (kernels or sprouted seeds), baking industry (Manitoba Agriculture, Food and Rural Initiatives, 2004) and to make butter called "Sunbutter" especially in China, Russia, the United States, the Middle East and Europe (Wikipedia contributors - sunflower, 2007). Relatively small seeds are used for bird food, either pure or mixed with wheat, oat, corn, proso millet and/or grain sorghum (Lofgren, 1997). In addition sunflower crops damaged by biotic and abiotic stresses are used as silage (Park et al., 1997).

Sunflower products in foods have proven health and nutritional benefits. Because of its high concentration of unsaturated fatty acids like linoleic acid, vitamin E, a moderate level of oleic acid and fiber, and a very low level of linolenic acid they are considered an excellent source of nutrients for humans and many livestock. For example

sunflower seeds contain 280 to 470 g/kg fat, 170 to 270 g/kg crude protein and 320 to 360 g/kg acid-detergent fiber (McGuffey & Schingoethe, 1982; Rafalowski & Park, 1982; Bath et al., 1993). The oil contains 850 g/kg of unsaturated fatty acids (McGuffey & Schingoethe, 1982; Finn et al., 1985; Drackley & Schingoethe, 1986). The crude fiber content of 160 to 310 g/kg present in seeds is found to avoid milk fat depression in high-producing cows fed high-energy rations (Park et al., 1997). The concentrated oil in sunflower seed is very useful for cows in early lactation (Park et al., 1997). Sunflower meal fed to animals contains 260 to 500 g/kg crude protein, 120 to 350 g/kg crude fiber and 10 to 90 g/kg crude fat (National Research Council, 1989). Sunflower silage has been found to have considerably more fiber and slightly more protein than that of corn (Park et al., 1997).

High content of phytosterols in sunflower seeds is found to reduce cholesterol levels and improve heart health (National Sunflower Association, 2007). Vitamin E in raw sunflower seeds is powerful antioxidant that avoids risk for heart disease.

2.1.4 Diseases of sunflower

Although sunflower is susceptible to over three dozen pathogenic organisms, only less than a dozen are successful in causing serious economic losses around the world (Gulya et al., 1997). Most of these pathogens have spread in every country. This is thought to happen with the introduction of sunflower into each continent except for North America (Gulya et al., 1997). In North America since the genus *Helianthus* is native; almost all the pathogens affecting it already exist. Sunflower is susceptible for pathogens

throughout its life cycle from seedling to flowering stage. Furthermore pathogens can attack every plant part from root to head. Downey mildew (*Plasmopara halstedii* (Farl.) Berl., and de Toni) is a major seedling disease (Gulya et al., 1997) which now has been reported in every continent with the exception of Australia (Leppik, 1962). The disease can be destructive when appropriate biological and environmental conditions are met. The important foliar diseases include sunflower rust (*Puccinia helianthis* Schwein), Alternaria leaf spots (*Alternaria helianthi* (Hansf.) Tubaki and Nishihara), septoria leaf spot (*Septoria helianthi* Ell and Kell.) and powdery mildew (*Erysiphe cichoracearum* DC. Ex Meret) along with different bacerial and viral diseases (Gulya et al, 1997). Verticillium wilt (*Verticiliium dahliae* Klebahnis), Phomopsis stem canker (*Phomopsis helianthi* Munt.-Cvet. et al.), phoma black stem (*Phoma macdonaldii* Boerma), charcoal rot (*Macrophomina phaseolina* (Tassi) Goid) and southern blight (*Sclerotinia rolfsii* Sacc.) are the main stem diseases (Gulya et al., 1997). *Sclerotinia sclerotiorum* (Lib.) de Bary is one of the most economically important pathogens of sunflower since it causes three main diseases; sclerotinia root rot or wilt, midstalk rot and head rot causing significant economic losses.

2.2 *Sclerotinia sclerotiorum* (Lib.) de Bary

2.2.1 Taxonomy and Nomenclature

In the very beginning of the history of the naming of *Scelrotinia sclerotiorum*, it had been named as *Peziza sclerotiorum* (Libert, 1837) followed by renaming it as *Sclerotinia libertiana* by Fuckel in 1870 (Purdy, 1979). However by considering the

International Rules of Botanical Nomenclature and earliest use of the particular name by de Bary in his 1884 contribution, the proper name and the authority for the fungus was given as *Sclerotinia sclerotiorum* (Lib.) de Bary (Purdy, 1979).

Macroscopic characteristics such as colour, size and shape of the apothecium, stipe and sclerotium; cultural characteristics such as size and distribution of sclerotial on agar plates; biological characteristics such as host, season, and part of substrate invaded and microscopic characteristics such as colour and dimensions of asci, paraphyses and ascospores were taken into consideration when designating a species (Kohn, 1979). However distinguishing between *Sclerotinia* species (mainly *S. minor*, *S. trifoliorum* and *S. sclerotiorum*) has been under question since different taxonomists have put emphasis on different characteristics. Valleau et al. (1933) used associated host and size of sclerotia as the basis for speciation while Keay (1939) stated that pathogenicity and the associated host are not important. Wolf and Cromwell (1919) identified the range of sclerotial sizes (0.3 to 10 mm) is a reliable measurements for the *S. trifloliorum.* Purdy (1955) could not distinguish between species on the basis of sclerotial size and included both *S. minor* and *S. trifoliorum* under *S. sclerotiorum.* However, with the development of new techniques current studies successfully differentiate between the *Sclerotinia* species (Hubbard et al., 1997; Halmimi et al., 1998) using host isolation, physical and physiological characteristics as well as genetic analysis (Errampalli & Kohn 1995; Kohli & Kohn 1996). Currently *Sclerotinia sclerotiorum* comes under Phylum Ascomycota in the Class Discomycete, the Order Helotiales and the Family Sclerotiniaceae (Ulloa & Hanlin, 2000).

2.2.2 Host Range

Sclerotinia sclerotiorum is one of the most nonspecific, omnivorous and successful plant pathogens (Purdy, 1979). As Purdy reports in 1979 plants from 64 families, 225 genera, 361 species and 22 other (cultivars, etc.) for a total of 383 species were susceptible to *S. sclerotiorum* including four Gymnosperms in the family Pinaceae. However, by 1994 host range has increased to 75 plant families, 278 genera, 408 species, and 42 subspecies from which over 100 (104 by 1976 as recorded by Morrall et al.) are present in Canada (Boland & Hall, 1994). These include economically important crops such as oil seed rape (canola), sunflower, tobacco, soybean, peanut and a range of vegetables such as lettuce, bean, cabbage, cauliflower, carrot and potato including a number of flower crops (Grau, 1988; Farr et al., 1989; Clarkson et al., 2003). In addition it is a pathogen of some herbaceous weeds (Hoes, 1969). Therefore, using *S. sclerotiorum* as mycoherbicide against weeds such as *Cirsium arvense* (L.) is under investigation in USA (Brosten & Sands, 1986) and In New Zealand (Bourdôt et al., 1993; Hurrell & Bourdôt, 1996; Mitchell & Davis 1996). Solanaceae, Cruciferae, Unbelliferae, Compositae, chenopodiaceae and Leguminosae are among the most affected families (Willetts & Wong, 1980; Mederick & Piening, 1982). Although *S. sclerotiorum* was not reported on flax (*Linum usitatissimum* L.) in Canada (Medrick & Peining, 1982), the fungus was found on flax in 1981 in Alberta (Medrick & Peining, 1982) as well as in Manitoba and Saskachewan recently (Rashid, 2000). In addition the first report of *S. sclerotiorum* on grass pea (*Lathyrus sativus* (L.) appears in 1990 in southern Manitoba, Saskatchewan and Alberta (Zimmer & Campbell, 1990). The aggressive nature of the

pathogen is well explained by the existence of the susceptible hosts from 26% of the plant families (Bailey & Bailey, 1976).

2.2.3 Geographical Distribution and Prevalence

S. sclerotiorum has a broad geographical and ecological distribution. It has been reported in many countries in all continents (Purdy, 1979). In China, which is the fourth largest sunflower-producing country in the world, *S. sclerotiorum* is considered as one of the most important pathogens of sunflower causing 10-50 % yield reduction and even crop failure in some areas (Liu & Li, 1988). Furthermore, *S. sclerotiorum* is one of the two *Sclerotinia* spp. reported in Canada (Bardin & Huang, 2001) and the population is genetically heterogenous with numerous clones (Kohn, 1995). In addition the presence of aberrant strains, which produce tan sclerotia and albino apothecia, is evident in association with sunflower in Canada (Huang, 1981). It is common in temperate regions where relatively cool and moist conditions prevail (Purdy, 1979; Boland & Hall, 1988). Now it has been found to occur in hot and dry areas as well (Purdy, 1979). In temperatures below the freezing point and higher than 32 ^{0}C the fungus is less active. High prevalence of disease is supported by the growing susceptible cultivars, agronomic practices such as tillage (Workneh & Yang, 2000) and row width (Steadman et al., 1973; Grau & Radke, 1984; Buzzell et al., 1993) in addition to cool and wet weather conditions. Workneh & Yang (2000) observed high disease pressure when yearly temperatures are below normal. *S. sclerotiorum* was a production problem on grass pea in south central Manitoba, Saskatchewan and Alberta only during times with high moisture (Zimmer &

Campbell, 1990). High yielding, short-season varieties adapted to cool conditions was found to be a main cause of increase in sclerotinia stem rot in canola in central and eastern Canada (Wrather et al., 2001). Most of the isolates from Canadian canola (*Brassica napus* L. & *Brassica rapa* L.) belong to two subpopulations of *S. sclerotiorum* which are widely dispersed in temperate and subtropical regions of United States, Europe and New Zealand and isolated from a range of crops such as canola, cabbage (*Brassica oleracea* L.), groundnut (*Apios americana* Medik.), Kiwi (*Actinidia chinensis* Planch.), tobacco (*Nicotiana tabacum* L.) and sunflower (Carbone & Kohn, 2001). Furthermore a study carried out by Hambleton et al, (2002) to examine the distribution of the *S. sclerotiorum* genotypes from the soybean production belt of Canada further strengthen the fact put forward by Carbone & Kohn (2001) that soybean in Ontario and Quebec as well as other crops of North America are infected with one relatively recently evolved, highly dispersed temporal and subtropical population and one older strictly temperate population.

With the high frequency of some genotypes and the potential of new genotypes to become widespread, management practices that aim at genotypes that are locally important as well as those known to occur across diverse regions on different hosts should be required to control this highly aggressive pathogen.

2.2.4 Life Cycle, Diseases and Epidemiology

S. sclerotiorum is a necrotrophic pathogen (Boland & Hall, 1994), in which the vegetative state is a white, cottony mycelial mass living saphrophytically in soils. It is

considered as homothallic fungus (Atallah et al., 2004). The life cycle of *S. sclerotiorum* is monocyclic, where the pathogen completes only one, or even part of one disease cycle in one year (Agrios, 2005).

The fungus overwinters or survives in the soil in the absence of a suitable host in the form of sclerotia (Rimmer, 1983). *Sclerotinia* spp spend approximately 90 % of their life cycle as sclerotia in soil (Adams & Ayers, 1979). Sclerotia are thick, black, hard structures that are capable of withstanding harsh environments. The formation of sclerotia starts as a thick mycelial mat which then turns into white mounds of mycelium covered with small liquid droplets (LeTourneau, 1979). These droplets disappear when the surface darkens and sclerotia increase in size. The sclerotial development in *S. sclerotiorum* is terminal type, where initials arise from anastomoses of long primary hyphae and several initials fused to form a large sclerotium. The mature sclerotium consists of a black thick walled rind about three cellwide and a thin-walled cortex and white medulla of prosenchymatous tissues embedded in a fibrillar matrix (Colotelo, 1974; Saito, 1974; Huang, 1982). Mature rind cells contain melanin (Jones, 1970). Organic acids (Humpherson-Jones & Cooke, 1977; Wang & LeTourneau, 1971) and enzymes like phenol oxidases including tyrosinase (Wong & Willets, 1974) may be involved in sclerotial formation. Furthermore, nutritional factors such as macronutirents P, K, Mg, S (Purdy & Grogan, 1954) especially Zn (Vega & LeTourneau, 1974), environmental factors such as a temperature range from 0 – 30 ^{0}C (LeTourneau, 1979) have been found to optimize the sclerotial production. In natural soils sclerotial densities may very from zero to less than 10 sclerotia per kilogram of soil in a field ready for planting (Adams & Ayers, 1979).

Depending on the nature of the fungus, various environmental factors and crop canopies (Bardin & Huang, 2001), sclerotia show two different types of germination; myceliogenic germination and carpogenic germination (Gulya et al., 1997). Sclerotia t buried from 2 cm to 10 cm (Adams & Tate, 1975; Marcum et al., 1977) can germinate myceliogenically and can infect hosts. Mycelial growth is enhanced between the temperature range 20 to 25 °C (Abawi & Grogan, 1975). Though mycelia can extend from 5 to 30 mm from scletotium without a close contact with the host, infection would not occur (Williams & Westerm, 1965; Newton & Sequeira, 1972). Although high humidity is required for myceliogenic germination of *S. sclerotiorum* (Huang et al., 1998), several researchers (Smith, 1900; Stone & Smith, 1900; Beach, 1921; Adams & Tate, 1975) observed that the soil dryness has induced lettuce drop caused by mycelial infection of *S. sclerotiorum*. Furthermore, the high degree of black melanin pigment in cell walls of the rind prevents the myceliogenic germination (Huang, 1985), where only tan sclerotia germinated in the absence of exogenous nutrients (Huang, 1981, 1983a). Mechanical damage (Huang, 1985), desiccant-dried treatments (Huang et al., 1998) or freezing (Huang, 1991) have been found to trigger myceliogenic germination of black sclerotia. Exogenous nutrient source requirements for the germination seem to vary slightly with the type of the host. Beach (1921) and Adams & Tate (1975) observed infection of lettuce via mycelia in the absence of exogenous food source, while Gulya et al. (1997) report that infection of sunflower roots by sclerotia is stimulated by root exudates. In spite of favourable factors inducing myceliogenic germination in soils the probability of sclerotia that do not overcome dormancy is very high (Abawi & Grogan, 1979).

Mycelia of germinated sclerotia can randomly infect actively growing roots primarily when they come in contact (Young & Morris, 1927; Hancock, 1972; Huang & Dueck, 1980). Root infections are favoured by wounds caused by mechanical damage, ruptured epidermis and cortex with emergence of root branches (Young & Morris, 1927). Relatively low temperatures of about 10 – 25 °C favour the infections (Abawi & Grogan, 1979). Secondary spread of myceliogenic infections are through plant-to-plant root contacts (Van der Plank, 1963; Jarvis & Hawthorne, 1972). Young & Morris (1927) and Hoes & Huang (1976) observed that spread of sclerotinia wilt in sunflower occur in the upper 20 cm of the soil where fibrous roots are distributed. High wilt incidence of sunflower observed with high plant populations (Kruger, 1975; Young & Morris, 1927) and low incidence observed with low crop densities (Hoes & Huang, 1985; Huang & Hoes, 1980) further support this hypothesis.

Carpogenic germination occurs through the production of several stipes and apothecia from the sclerotia close to the soil surface (Schwartz & Steadman, 1978). Usually sclerotia present between 2 to 5 cm soil layers can produce apothecia (Abawi & Grogan, 1979). Temperature and soil moisture are major factors governing apothecia formation by *S. sclerotiorum* (Phillips, 1987; Clarkson et al., 2001). High soil moisture favours carpogenic germination (Gulya et al., 1997). Carpogenic germination is always associated with long periods of rainfall (Gulya et al., 1997). For example, severe head rot of sunflower was observed with abonormally high rainfall during the flowering season in Manitoba (Hoes, 1969). Light is needed for stipes to differentiate into disks and stipes are positively phototropic (LeTourneau, 1979). Only light below 390 nm is effective in stimulating apothecium formation (Honda & Yunoki, 1977). In addition Radke and Grau

(1986) state that certain dinitroaniline and triazine herbicides such as trifluralin, pendimethalin, metribuzin, simazine, and atrazine stimulate carpogenic germination while Teo et al. (1992) and Huang and Blackshaw (1995) report reduction and prevention of carpogenic germination and apothecia production induced by trifluralin, metribuzin and simazine.

Although the mechanism of spore discharge is poorly understood, "forcible ejection by puffing" is supposed to be involved (Clarkson, et al., 2003). Ingold (1971) found that sudden changes in environmental conditions can trigger the puffing. Although spore release has been observed both in light and dark (Clarkson, et al., 2003) with a diurnal distribution pattern the peak level has been observed in the middle of the day under field conditions (Harthill, 1980, Raynal, 1990, McCartney & Lacey, 1991) which supports the observation of maximum discharge at 20 – 25 ^{o}C (Newton & Sequeria, 1972). Ascospore release both under saturated air (Newton & Sequeira, 1972; Raynal, 1990) and non-saturated air (65 – 75% rh) (Clarkson, et al., 2003) has been reported. However, Abawi & Grogan (1979) report an ascospore release up to 3 x 10^{7} while Clarkson, et al. (2003) report 7.6 x 10^{5} spores over 20 d.

Discharged ascospores can be dispersed from 1 km (McCartney & Lacey, 1991) to several kilometers (Brown & Butler, 1936) by air currents, splashing rain or by insects such as honey bees (Stelfox et al., 1978; McCartney & Lacey, 1991). Ascospores are short-lived (McCartney & Lacey, 1991) but can survive for a considerable time period depending on the environmental conditions (Abawi & Grogan, 1979). Ascospores survived for 12 days on bean leaves under field conditions have been reported (Abawi & Grogan, 1979). Stevens and Hall (1911) and Moore (1949) found that the ascospores

remained for 5 months and 5-6 weeks respectively under dry conditions. Low humidity and temperatures between 19 and 24 °C can cause ascospores to be viable for more than 45 days. Temperatures such as 5 °C can result in longer viability up to several months. Generally temperatures and humidity more than 25 °C and 35%, respectively, have adverse effects on ascospore survival (Caesar & Pearson, 1983). In addition, germinated ascospores and appressoria on or in plant tissue survive longer than ungerminated spores (Grogan & Abawi, 1975). Prevalent free water and exogenous energy source supplied by wounded or senescing plant tissues are needed for ascospores to germinate (Grogan & Abawi, 1975). On bean plants about 48 – 72 hr prevalence of leaf wetness was found to be needed for ascospore germination (Abawi & Grogan, 1979). In addition other nutrient sources such as exudates and sucrose from nectar glands in leaves, stems (Sedun & Brown, 1987) and wounds can support the germination and infection (Gulya et al., 1997).

The initial stage of infection depends on the type of inoculum, the nutrient status of the fungus, host characteristics and the surrounding environment (Lumsden, 1979). Germinated ascospores and mycelium from sclerotia or ascospores can initiate the infection. A mucilaginous material containing polysaccharides, glycoproteins and fibrillar materials discharged with the ascospores help them to attach to the host surface (Abawi & Grogan, 1979; Agrios, 2005). After the host-pathogen recognition, infection starts with host penetration. Host penetration is mainly through the cuticle in most hosts (Boyle, 1921; Purdy, 1958; Lumsden & Dow, 1973; Abawi et al., 1975) although entry via stomata can occur occasionally (Jones, 1976). Free water (Abawi & Grogen, 1979) and external organic matter (Lumsden, 1979) are prerequisites for penetration, though optimum temperature for infection can vary between 20 – 25 °C (Natti, 1971; Abawi &

Grogan, 1975). Initial penetration can occur as early as 9h postinoculation (Guimarães & Stotz, 2004). During the infection through ascospores, appressorium produced at the tip of the germ tube is important. Penetration of the cuticle is mainly through mechanical force given by the appressorium and there is no evidence of pre-penetration dissolution of the cuticle (Purdy, 1979). Numerous infection hyphae emerge laterally from the subcuticular vesicle. After complete colonization of the subcuticular zone hyphae enter into subepidermal tissue. Necrosis occurs in the epidermal cells when subcuticular region is fully covered by the pathogen. The penetration of the cell wall mainly occurs through cell wall degrading enzymes produced by *S. scletrotiorum*. Endopolygalacturonase is one of the major enzymes involved (Tariq & Jeffries, 1985). In addition cellulase (Lumsden, 1972), hemicellulases (Bauer et al., 1977), proteolytic enzymes also enhance cell wall degradation and at the same time provide nutrients for the pathogen (Lumsden, 1979). Oxalic acid is an important compound that is produced by *S. sclerotiorum* that is directly involved in pathogenesis (DeBary, 1887; Maxwell & Lumsden, 1970; Godoy et al., 1990; Jamaux et al., 1995). It lowers the pH so that the activity of cell wall degrading enzymes is optimized (Bateman & Beer, 1965; Lumsden, 1979). Sudden pH change caused by oxalic itself alone can affect cell viability and responsive ability against invasion (Maxwell & Lumsden, 1970). Furthermore oxalate crystals are responsible for symptom development specially wilt diseases either through vascular plugging (Pawlowski & Hawn, 1964) or a direct influence on water relationships in lamellar tissue (Lumsden, 1979). Recent studies (Guimarães & Stotz, 2004) report that oxalate induces stomatal opening and inhibits ABA induced stomatal closure, which is another possible reason for the wilting symptom. With the help of these compounds the fungus colonizes the tissue

interior reaching the lower cuticle in a leaf approximately 15 – 20 h postinoculation (Tariq & Jeffries, 1985). Simultaneously, symptoms such as watersoaked spots in most hosts or dry lesions with a sharp demarcation from the healthy tissue appear approximately 24 – 28 h from penetration followed by chlorosis and necrosis. Further attack totally disrupts parenchymatous tissues and remaining vascular tissues show shredded appearance (Purdy, 1979). When the optimum environmental conditions are met, the fungus produces white cottony mycelium on the host surface followed by sclerotia (Purdy, 1979). Sclerotia are added into soil upon death of the host completing the life cycle of the fungus. Enisz (1986) reports 50 – 100 sclerotia produced by wilted sunflower plants and the number is twice and five times higher produced by head and midstem rot. Wilt incidence of 17 to 79% resulted in 0.11 to 2 sclerotia per kg of soil (Holley & Nelson, 1986). Hoes and Huang (1975) found around 24 sclerotia per kilogram of soil in the rhizosphere of diseased sunflower plants.

Survival of sclerotia added into soil is affected by different factors. Although Adams & Ayers (1979) considered temperature and pH are not important, Cook et al. (1975) state that temperatures above 27 °C is injurious and 5 °C is favourable for sclerotial viability and survival. Wet conditions are detrimental to sclerotia. In soil flooded with water for 26-31 days, sclerotia were killed nearly 100% (Moore, 1949). Sclerotia buried in depths ranging from 5 to 30 cm were observed to survive longer than those were close to the surface (Williams & Western, 1965; Cook et al., 1975). However, Adams and Ayers (1979) stated that the most significant factor affecting survival of sclerotia in soil is biological, which are antagonistic or mycoparasitic microorganisms. More than 30 fungal species are found to colonize sclerotia (Adams & Ayers, 1979),

including *Trichoderma* spp. (Jones & Watson, 1969) and *Coniothyrium minitans* (Campbell, 1947). Depending on the prevailing soil conditions survival varies from soil to soil. For instance, In Nebraska, sclerotia survived for 3 yr (Cook et al., 1975), while in New York a few survived only 2 yr (Partyka & Mai, 1962). Under favourable conditions sclerotia of *S. sclerotiorum* can survive for at least 10 yr (Brown & Butler, 1936). Residues of hosts such as sunflower can provide nutrients needed for secondary sclerotia to be formed (Kruger, 1975), however, there are reports of sclerotia of *S. sclerotiorum* that produced secondary sclerotia in soil without a host (Williams & Western, 1965). In addition to buried sclerotia, mycelia from infected crops can survive on the soil surface, but do not survive appreciably to be primary inoculum (Huang & Kozub, 1993; Gulya et al., 1997).

Dissemination of *S. sclerotiorum* from field to field is mainly through windblown ascospores and contaminated seeds, farm equipment, animals or man and irrigation (Dillon-Weston et al, 1946; Starr et al., 1953; Steadman et al., 1975).

Ascospores cause stem blight, stalk rot, head rot, pod rot, white mold and blossom blight of plants (Bardin & Huang, 2001) resulting in diseases in many crops worldwide. In Canada, leaf blight of canola (Gugel & Morrall, 1986), head rot of safflower (Mündel et al., 1985a), pod rot of dry bean (Huang & Kemp, 1989; Boland & Hall, 1987), stem and pod rot of soybean (Sutton & Deverall, 1983; Boland & Hall, 1988), pod rot of dry pea (Huang & Kokko, 1992), Blossom blight of alfalfa (Holley et al., 1995; Huang et al., 2000a) and lettuce drop being one of the earliest disease recorded (Purdy, 1979; Mercier & Reeleder, 1987). Germinated sclerota infect root parts causing crown rot and root rot of carrots (Finlayson et al., 1989), wilt of greenhouse tomatoes (Dickson, 1920) and

Jerusalem artichoke (Huang & Stauffer, 1979). *S. sclerotiorum* is the causal organism of most common diseases; head rot (Huang, 1983), and basal stalk rot and wilt (Huang & Dueck, 1980) affecting sunflower worldwide. Sunflower as the world's fourth largest oil seed crop, *S. sclerotiorum* is one of the limiting factors in sunflower production.

2.2.5 Economic Importance

The vast range of economically important crops affected by *S. sclerotiorum* worldwide including canola, soybean, sunflower, explains the importance of this pathogen in the economy in most countries. For instance, in the province of Manitoba in Canada sclerotinia wilt is a limiting factor in sunflower production (Putt, 1958; Hoes & Huang, 1976). The damage and losses caused by this pathogen are both quantitative and qualitative (Putt, 1997) and can be at pre-harvest, harvest and/or post-harvest levels. Pre-harvest losses are resulted through the expenditure for fungicides, where by 1983 it was estimated to be about 50-55/ha ($20–22/acre) including application cost by air (Rimmer, 1983). At harvest level the fungus significantly reduces yield and the quality of the produce. Yield loss is through the reduction in seed number and or seed size and weight. Since the infected plants ripen prematurely, shattering occurs before the crop is harvested and the shrunken seeds may be lost with the chaff during combining in Canola (Morrall, et al., 1976). In 1986, an average of 34% reduction of sunflower seed weight caused by head rot was observed (Gulya et al., 1989). Significant reduction in oil and protein content (Dorrell & Huang, 1978) as well as mixing sclerotia with seedlots reduces the quality and subsequently market value. Gulya et al. (1989) report 2% reduction in oil

content in sunflower seeds associated with 34% reduction in seed weight. Seeds mixed with sclerotia are unacceptable for human or animal consumption (Cole & Cox, 1981) and small animals consumed relatively lesser amount when sclerotial bodies comprised of a portion of 2 to 8% (Ruddick & Harwig, 1975). Large losses during storage and/or transportation can occur at post-harvest level. Any temperature would not prevent the pathogen invasion when even a trace of infection is present in crops such as celery, carrots or beans being stored or transported (Rimmer, 1983).

The pressure put on the world economy by *S. sclerotiorum* is increasing. In 1982 overall loss in Canola resulted by stem rot disease in Manitoba was $10-15 million (Rimmer, 1983) while $24.5 million loss was resulted in 2000 in North Dakota and Minnesota (Sayler, 2003). Presently annual production losses done by *S. sclerotiorum* to dry bean, snap bean and sunflower production in United States is $26 million, $13 million and $15 million respectively. However the crop losses are strongly correlated with the disease incidence, which is determined by the prevailing weather condition in a particular year. Under the optimal environmental conditions the loss can be 100% (Purdy, 1979). The significant impacts exerted by *S. sclerotiorum* on world's economy have increased the need of sclerotinia research and control. Sclerotinia Initiative implemented by United States congress, which provided approximately $1 million in 2001 and $496,750 in 2003 for *S. sclerotiorum* research, is an outcome of such a need (Anonymous, 2003; Sayler, 2003).

2.3 Management of *Sclerotinia sclerotiorum*

The management of *S. sclerotiorum* is difficult, inconsistent and not economical due to the wide host range and sclerotia that survive for years (Steadman, 1979; Gulya et al., 1997). However the current strategies of control of *S. sclerotiorum* include cultural practices, development of "moderate levels of genetic resistance" in crops, chemical methods such as fungicide application and use of biological control agents (Gulya et al., 1997; Ferraz et al., 1999).

2.3.1 Cultural Management

Crop rotation, residue management (Ender & Kelly, 2005) including tillage practices and crop management practices are among the major cultural management practices that are currently used. All these methods aim either at reducing the survival of sclerotia or the production of apothecia and asocospores through microclimatic variations.

Cultivation of susceptible crops in consecutive years will increase the sclerotial density in a particular field (Fernando et al., 2004). Rotation of host crop species with non-host species will considerably reduce the inoculum density. Susceptible crops like rapeseed, yellow mustard (*Brassica alba*), field pea (*Pisum sativum* L. var. *arvense* (L) Poir.) induce the disease incidence (Gulya et al., 1997). The rotation time period varies slightly from crop to crop. For sunflower, at least 4-yr rotation is recommended in a field with a history of sclerotinia wilt (Young & Morris, 1927) whereas for bean white mold, it

was suggested as 3- to 5-yr period (Starr et al., 1953). Generally a minimum of 5 year rotation with two non host crops of *S. sclerotiorum* has been suggested to handle the infection level of the pathogen (Gulya et al., 1997). In southern Manitoba disease incidence was reduced by the use of non-host crops wheat and barley (*Hordeum vulgare* L.) used in the previous 5-yr crop rotation system (Hoes & Huang, 1976). However, the control is not always consistent with crop rotation due to the influence of other environmental factors. There are reports that under dry conditions sclerotia of *S. sclerotiorum* remained visible for at least 10 yr (Brown & Butler, 1936). Cook (1973) observed that in bean fields in Nebraska rotated with corn and sugarbeets every 3rd yr was not effective. Furthermore rotation should be accompanied with the effective control of weeds and volunteers and will not be effective for ascospore borne diseases as sunflower head rot since airborne ascospores can come from adjacent infected fields (Gulya et al., 1997). For such crops at least 1 km of spatial isolation is recommended from last year's heavily infested field (Masirevic & Gulya, 1992).

The effect of residue management and tillage practices is highly influenced by the environment factors and the type of inoculum acting as the primary inoculum for a particular disease. Therefore, different studies show controversial results of tillage. Cook et al. (1975) observed that shallow cultivation or zero-tillage resulted in fast deterioration of sclerotia on the soil surface reducing the amount of buried sclerotia in North America under cold temperatures. This will reduce the myceliogenic germination thereby reducing the root rot diseases but the probability of carpogenic germination will be remained unchanged (Gulya et al., 1997). In contrast, deep tillage buries the sclerotia increasing the chance of survival (Abawi & Grogan, 1975) and cause heavy root infections but on the

other hand reduce the ascospore release under high soil moisture content (Gulya et al., 1997). Duncan et al. (2006) found a significant negative relationship between sclerotial viability and burial depth, and between sclerotial viability and colonizing bacterial populations under zero tillage conditions. In theory, shallow or no-tillage in dry areas and deep tillage in wet areas would be effective on two epidemiological systems of *S. sclerotiorum* under different environmental conditions (Gulya et al., 1997).

Plant population density, irrigation and field sanitation issues have considerable impact on both epidemiological systems (Gulya et al., 1997) of *S. sclerotiorum*. Considering the plant population density, row spacing, growth habit and canopy structure of the crop is important. Dense canopies increase the disease severity by creating a favourable microclimate for the carpogenic germination and ascospore release (Weiss et al., 1980; Marcum et al., 1977). Vigorous viny cultivars of two dry edible bean types that produced dense canopy had the highest disease severity (Blad et al., 1978) and apothecia production compared to upright indeterminate and open bush types of bean cultivars (Schwartz Steadman, 1978). High N fertilization favoured the carpogenic germination and head infections of sunflower due to thick and dense canopy (Heber et al., 1985; Peres et al., 1989). Narrow row spacing and plant spacing within rows will increase the plant density thereby enhancing the root infections and plant-to-plant spread (Huang & Hoes, 1980; Hoes & Huang, 1985). In contrast, wide row spacing allows air circulation and drying of soil reducing the apthothecial formation and ascospore release and infections (Gulya et al., 1997). In addition, high irrigation causes rotting of sclerotia (Moore, 1949; Teo et al., 1989). Use of clean seeds without mixed sclerotia or dormant mycelia will also prevent the reintroduction of *S. sclerotiorum* in to clean fields.

2.3.2 Genetic Resistance

Anton de Bary in 1887 was the first to observe genetic resistance to *S. sclerotiorum* in *Phaseolus multiflorus* (*P. coccineus*) (Steadman, 1979). Contemporary studies to increase the genetic resistance involve conventional breeding and genetic transformations. Breeding for genetic resistance to *S. sclerotiorum* is held back due to the lack of germplasm with resistance or immunity to this pathogen (Rashid & Dedio, 1992). However, some progress has been achieved in developing resistant cultivars of some crops such as bean, safflower, sunflower and soybean (Bardin & Huang, 2001). Although breeding programs trying to increase resistance physiologically have limitations since the resistance to *S. sclerotiorum* is primarily controlled by additive gene action (Fuller et al., 1984), tolerance to white mold was discovered in white bean cultivar ExRico23, which is caused by lower rate of diffusion of oxalic acid in the infected leaf tissue (Tu, 1985, 1989). Canadian short season soybean 80, which has transformed wheat germin gene coding for oxalate oxidase was resistant to *S. sclerotiorum* both in greenhouse and field (Simmonds et al., 2001). Other desirable characteristics such as upright growth habit, narrow canopy and indeterminate growth habit have been looked at and were found to be very successful to avoid white mold (Saindon et al., 1993; Huang & Kemp, 1989). In addition, height, early maturity and greater lodging resistance have been successful in reducing *S. sclerotiorum* in safflower (Mündel et al., 1985a, 1985b) and soybean (Boland & Hall, 1987; Buzzell et al., 1993) by reducing favourable microclimate conditions for the disease development. In sunflower, wilt resistance has been observed among inbred lines (Huang, 1980b) and hybrids (Huang, 1980b; Rashid & Dedio, 1992). Furthermore,

selective breeding for the increased production of two terpenoids that inhibit hyphal growth of *S. sclerotiorum* was under investigation (Picman et al., 1990). In canola, complete resistance to *S. sclerotiorum* has not been achieved but varieties with partial field resistance such as Zhongyou 821exists (Li et al., 1999; Buchwaldt et al., 2003). "Combining quantitative trait loci (QTL) for physiological resistance with plant avoidance traits" is recommended for common bean as a better approach to improve resistance to *S. sclerotiorum* (Ender & Kelly, 2005), which would be applicable to other types of crops as well.

2.3.3 Chemical Treatments

Use of fungicide is one management option for *S. sclerotiorum*. Relative high cost of fungicide applications needs perfect timing for maximum benefit. Reliable forecast systems are important in this regard (Sun & Yang, 2000). Both foliar application and seed treatment of fungicides are in use. When the pathogen is prevalent two aerial applications may be required to combat the prevailing ascospores for a longer period (Patterson & Grogan, 1985). Combined application of two or more fungicides seems to be more effective compared to single application (Mueller et al., 1999).

Effect of different fungicides may differ from crop to crop. Benomyl, vinclozolin and iprodione have been successful against *S. sclerotiorum* in certain crops including bean (Natti, 1971; Hunter et al., 1978; Vulsteke & Meeus, 1982), cabbage (Gabrielson et al., 1973) and canola (Dueck, et al., 1983). For sunflower, benomyl has been effective (Auger & Nome, 1970). Effectiveness of a fungicide is strongly influenced by the growth

habit or canopy density of the crop (Steadman, 1979). For the maximum benefit the fungicide should adequately cover the target area (Letham et al, 1976). Gabrielson et al. (1973) report that poor coverage of bean plants by benomyl was due to the vigorous viny growth. In the case of sunflower complete coverage of stem, petioles and leaves along with the head is relatively difficult because of the thick canopy (Duncan, 2003). However, in most cases aerial application would give more satisfactory coverage than ground sprays (Letham et al., 1976).

Seed treatment with benomyl, vinclozolin, iprodione or procymidone eliminated seed-borne *S. sclerotiorum* (Herd & Phillips, 1988) and reduced early wilt in sunflower (Rashid & Swanson, 2002). Captan, thiophanate-mathyl (Tu, 1989), benomyl, thiabendazole and thiram (Homechin & Henning, 1983; Yorinori & Homechin, 1985) as seed treatments have been 100% effective against *Sclerotinia* infected seeds.

In addition certain herbicides such as metribuzin and diuron are reported to inhibit myelial growth while atrazine and simazine inhibited the apothecial formation or caused non-functional apothecia (Casale & Hart, 1986; Huang & Blackshaw, 1995). Urea (Huang & Janzen, 1991), calcium cyanamide (Huang & Sun, 1991) and formulated compounds such as S-H mixture (Huang and Sun, 1991) and CF-5 (Huang et al., 1997) reduced the carpogenic germination of sclerotia.

When it comes to registered fungicides, chemicals such as quadris, ronilan, topsin M, endura, T-methyl, etc. are registered in Northe Dakota and Minnesota against sclerotinia stem rot in canola (North Dakota Field Crop Fungicide Guide, 2007) while quadris, ronilan EG, rovral flo, lance, proline 480 SC are the ones that are commercially available in Canada to use *Sclerotinia* diseases in field beans (white mold) and canola

(stem rot) by 2007 (Guide to Field Crop Protection 2007, Maniotiba Agriculture, Food and Rural Initiatives). Thiophanate-methyl is a fungicide currently registered in the USA for seed-borne *Sclerotinia* (Gulya, et al., 1997). However, certain fungicidal treatments are at experimental levels and still no fungicide is registered against *S. sclerotiorum* in sunflower in Canada (K.Y. Rashid, personal communication). Even a registered fungicide would not be very economical because of the difficulty of the successful application of the fungicide with the morphology of the sunflower plant (Mestries, et al., 1998; Gentzbittel et al., 1998).

2.3.4 Biological Control

With the rising concern about the environment pollution, human health issues (Lugtenberg et al., 2001) through chemical residues and development of resistance among the plant pathogens due to repeated application of fungicides (Steadman, 1979), biological control became known as an attractive and sustainable (Sutton & Peng, 1993) alternative for the management of plant diseases. Biological control is defined as "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 & Baker, 1983). The first attempt to control a pathogen biologically was made between 1920 and 1940 by Hartley, who tried to control damping-off of pine seedlings by inoculating antagonistic fungi into forest nursery soils. The concept of suppressive soils that had been observed in the early stages of development of biocontrol explains the fact that it is a

naturally occurring phenomenon, which has been developed for the purpose of disease management.

Failure or success of biocontrol is dependent on the suitability of the biocontrol agent (BCA), method of introduction and maintenance, which determine the efficiency of colonization and survival of the BCA in a particular ecosystem or a host-pathogen system (Stack et al., 1988). So far aqueous suspensions, wettable powders or dusts have been tested as different types of delivery methods, from which spraying an aqueous suspension at a high concentration (Sutton & Peng, 1993) has been common due to the efficient coverage of the plant surface by the BCA, which facilitate colonization and protection from the pathogen. The microclimatic changes governed by the physical, chemical and biological factors in the environment play a vital role in this regard. For a soil-borne BCA, rhizosphere is a much more stable environment than a phylloplane surface, where the temperature and relative humidity fluctuations and both UV and visible light irradiation are common (Blakeman & Fokkema, 1982; Wilson et al., 1999). Viability and longevity of a BCA will subsequently affect frequency of applications thus production cost.

Successful biocontrol agent should act at the appropriate moment, on the target pathogen as well as on the target crop and the possibility of alternative hosts and detrimental effects on beneficial indigenous microflora should be minimal. To develop a thriving BCA and a successful delivery system, better understanding of the biology of the mycoparasite, its interaction with soil microflora and population dynamics, disease epidemiology and biology of the pathogen, economic aspects as well as the interactions

among these variables should be taken into consideration (Adams, 1990; Deacon, 1991; Sutton & Peng, 1993).

Biocontrol agents of plant diseases include bacteria, filamentous fungi as well as insects and other organisms (Steadman, 1979). The most studied fungal antagonists are: *Coniothyrium minitans*, *Trichoderma* spp., *Gliocladium* spp., *Sporidesmium sclerotivorum*, *Fusarium*, *Hormadendrum*, *Mucor*, *Penicillium*, *Aspergillus*, *Stahcybotrys*, and *Verticillium* (Adams & Ayers, 1979; Bedi, 1961; Makkonen & Pohjakallio, 1960). From them *C. minitans* and *Gliocladium virens* have shown a promising control of *S. sclerotiorum* (Budge et al., 1995). *Agrobacterium, Pseudomonas, Bacillus, Alcaligenes, Streptomyces* are among the common bacterial control agents (Liang et al., 1982; Weller, 1988; Gutterson, 1990; Dowling and OGara, 1994). *Pseudomonas* and *Bacillus* species have been successful against *S. sclerotiorum* (McLoughlin et al., 1992; Huang et al., 1993; Fernando et al., 2007).

2.3.4.1 Mechanisms of Control

The biocontrol mechanisms involved in control of plant pathogens include competition for an ecological niche or a substrate, parasitism specially by fungal BCA (Fravel, 1988), antibiosis and induction of systemic resistance (ISR) to a broad range of pathogens (Fravel, 1988; Glick, 1995; Bloemberg & Lugtenberg, 2001; Hass et al., 2002) and abiotic stresses (Nowak & Shulaev, 2003; Mayak et al., 2004).

Competition exerted by the introduced BCA is primarily important in effective colonization and survival in the target area. The effective control of plant pathogen by

BCA is strongly dependant on the efficiency of colonization of the applied area (Chin-A-Woeng, et al., 2000). In the soil environment, the pathogen and indigenous microflora as well as introduced BCA are attracted by the various root exudates present on the root surface, including organic acids, amino acids and specific sugars (Welbaum et al., 2004). Some exudates can act as antimicrobial agents, where only organisms having adequate enzymatic machinery to detoxify them will survive in the rhizosphere (Baise et al., 2004). The phyllosphere, in contrast to rhizosphere, is primarily carbon limited and secondarily nitrogen limited (Wilson & Lindow, 1995). In these situations the ability of the efficient use of available nutrients of the BCA is essential. Mari et al. (1996) report that the reduction of gray mold caused by *Botrytis cinerea* in pears by *Bacillus amyloliquefaciens* 2TOE, is due to competition for nutrients. In the phyllosphere, competition for nutrients mainly affects the spore germination of the pathogen (Yoshida et al., 2002). Most BCA achieve this goal by producing different kinds of secondary metabolites and enzymes, which have harmful effects on competing indigenous microflora. These include ion-chelating siderophores, lytic enzymes (by fungal BCA, Carsolio et al., 1999; Sanz et al., 2005), and antibiotics (Mazzola, et al., 1992). Under iron-limiting conditions bacterial antagonists such as *Pseudomonas putida* (Kojic et al., 1999) produce siderophores, which acquire ferric ion competitively (Whipps, 2001).

Hyperparasitism, using living fungi or other organisms as growth substrate, is one of the main mechanisms, by which fungal BCA control plant pathogens. *Coniothyrium minitans* Campbell (Campbell, 1947), *Trichoderma* spp., *Gliocladium roseum* (Link) Bainier (Mueller et al., 1985; Makkonen & Phojakallio, 1960; Huang, 1980a) have been effective as hyperparasites of sclerotia of *S. sclerotiorum*. Production of extracellular cell

wall degrading enzymes such as β-(1,3)-glucanases, chitinases, lipases, and proteases (Carsolio et al., 1999), have been observed with hyperparasitism by *Trichoderma* spp.. Although there is no published information on the occurrence of bacterial mycoparasitism, a newly defined genus of bacteria, *Collimonas*, has been demonstrated to grow on living hyphae in soil microcosms and further investigations on this are underway (Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO), 2007).

Antibiosis is the inhibition of plant pathogens through the production of antibiotics. Antibiotics are a "chemically heterogeneous group of organic, low-molecular weight compounds produced by microorganisms at low concentrations and deleterious to the growth or metabolic activities of other microorganisms" (Thomashow et al., 1997). Upon limited nutrients or high cell density bacteria start to produce secondary metabolites like antibiotics, which help them to remain competitive in the environment (Vining, 1990). Secondary metabolites are produced in the "idiophase", which is the growth phase that transits to stationary phase (Liao et al., 1995). Amphisin, 2,4-diacetylphloroglucinal (DAPG), oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, tropolone, and cyclic lipopeptides are some of the well studied antibiotics produced by Pseudomonads (Défago, 1993; Nielson et al., 2002; Raaijmakers et al., 2002; de Souza et al., 2003; Nielson & Sørensen, 2003;). *Bacillus* antagonists produce ribosomal compounds like subtilin (Zuber et al., 1993), subtilosin A (Babasaki, et al., 1985), TasA (Stover & Driks, 1999) and sublancin (Paik et al., 1998) also non-ribosomal lipopeptides belonging to the surfactin, iturin and fengycin families and aminopolyols like zwittermicin A (Milner et al., 1996). In many host-pathogen and pathogen-BCA systems antibiosis was found to be the major means of action (Raaijmakers and Weller, 1998;

Leclère et al., 2005). Some examples are: control of *S. sclerotiorum, Rhizoctonia solani, Alternaria brassicae* and *Leptosphaeria maculans* by fengicins produced by *Bacillus subtilis* strain LEV-006 (Hou et al., 2006; Ramarathnam et al., 2007); control of *Phytophthora medicaginis* in alfalfa by zwittermicin A produced by *B. cereus* UW85 (Silo-Suh et al., 1994); control of *Pythium ultimum* in sugar beets by xanthobaccins produced by *Stenotrophomonas* SB-K88 (Nakayama et al., 1999); control of take-all of wheat by phenazine-1-carboxylic acid (PCA) produced by *Pseudomonas fluorescens* strain 2-79 (Slininger & Shea-Wilbur, 1995; Slininger et al., 2000; Zhang et al., 2006); control of *Rhizoctonia solani* in cotton by pyrrolnitrin produced by *P. fluorescens* BL915 (Ligon et al., 2000); control of *Thielaviopsis basicola* and *P. ultimum* in cotton by pyoluteorin produced by *P. fluorescens* CHA0 (Maurhofer et al., 1992).

In addition inhibitory volatiles produced by bacteria are gaining interest. For example, ammonia produced by *Enterobacter cloacae* controlled preemergence damping-off caused by *Pythium* spp. (Howell et al., 1988); hydrogen cyanide produced by pseudomonads controlled root rot of tobacco (Voisard et al., 1989); three organic volatiles produced by *Pseudomonas chlororaphis* strain PA23 controlled *S. sclerotiorum* (Fernando et al., 2005); rhizobacterial isolates *Serratia plymuthica, Serratia odorifera, Stenotrophomonas maltophilia, Stenotrophomonas rhizophila, P. fluorescens,* and *Pseudomonas trivialis* inhibited the growth of *Rhizoctonia solani* (Kai et al. 2006); volatiles of *Stenotrophomonas, Serratia* and *Bacillus* inhibited growth of *Microdochium bolleyi*, *P. carneus*, *Phoma betae* and *S. sclerotiorum in vitro* (Vespermann et al., 2007). In addition, oxalate oxidase producing bacterial antagonists like *Pantoea agglomerans* (Savchuk, 2002) are important in controlling *S. sclerotiorum* since the main pathogenicity

factor is oxalate acid. These inhibitors weaken their competitors, so that they can reach the available resources effectively (Hou et al., 2006). Furthermore, enhanced inhibition of spore formation by the synergistic interaction between fungal lytic enzymes and antifungal compounds has been observed (Lorito et al., 1994).

The role of antibiotics in the control of plant pathogens has been demonstrated via different techniques. For example, demonstrating same levels of inhibition as shown by the antagonist using cell-free cultures or purified antibiotics (Kang et al., 1998; Nakayama et al., 1999); inactivation of antibiotic production through mutagenesis (Anjaiah et al, 1998; Ching-A-Woeng et al., 1998, Poritsanos, 2005) and restoring antibiotic production by isolating complemented mutants (Thomashow and Weller, 1988) are some of them. In addition, enhancement of biocontrol activity by genetic manipulation, for example, introduction of multiple copies of the regulatory genes (Fernando and Pierson, 1999; Ligon et al, 2000); heterologous expression of the antibiotic biosynthetic genes in non-producing strains using multi-copy plasmid vectors (Raaijmakers et al., 2002) or by introducing into the chromosome (Timms-Wilson et al., 2000) are also in use. *In situ* recovery and detection of antibiotic production (Raaijmakers et al., 2002) is another methods, however, in this method the detection is sometimes hindered by the instability of the compound, irreversible binding to the soil colloid or the organic matter, or microbial decomposition (Thomashow et al., 1997). Sensitive methods such as high performance liquid chromatography can overcome these problems (reviewed by Thomashow et al., 1997). In addition to these methods, PCR-based detection of antibiotics and antibiotic producing bacteria using specific primers and probes has proved to be a powerful tool, in the study of distribution and function of indigenous antibiotic

producing bacteria (Raaijmakers et al., 2002). Recently, Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF) was found to be an innovative, highly efficient technique that enables rapid analysis of the secondary metabolite spectra of microorganisms (Leenders et al., 1999). It is well suited for studying the production of natural compounds in both surface and suspension cultures and the detection limit is in the upper femtomolar to picomolar range (Vater et al., 2002). The accuracy of molecular mass determination is 0.01-0.02% (Vater et al., 2002). In addition to screening microbial secondary metabolites, it allows researchers to characterize their molecular structures (Vater et al., 2002). Many studies have used this technique to identify and characterize antibiotics produced by bacteria especially by *Bacillus* spp. (Vater et al., 2002; Moyne et al., 2001; Duitman et al., 1999; Thaniyavarn et al., 2003; Ramarathnam et al., 2007).

2.3.4.1.1 Biosynthetic Genes

With the advancement of the recombinant DNA technology cloning, expressing and detection of biosynthetic gene clusters was possible from differenct biological sources like plants, fungi and bacteria. Biosynthetic gene clusters are the gene clusters that encode the proteins catalyzing the synthesis of different bioactive products in organisms (Bechthold & Fernández, 1999). SpecificPCR primers have been extensively used in this regard. Recently the PCR based method has been widely used for the detection of biosynthetic genes of antifungal antibiotics produced by bacteria and for the selection and identification of antibiotic producting bacteria from natural environments.

2.3.4.1.1.1 *Pseudomonas* Species and Antibiotic Biosynthetic Genes

A PCR based method was first used for the detection of antibiotic producing genes from *Pseudomonas* spp. for the production of phenazine and 2,4-diacetylphloroglucinol (2,4-DAPG) by Raaijmakers et al (1997). Recently, specific primers have been developed for pyrrolnitrin (de Souza and Raaijmkers, 2003) and pyoluteorin (Mavrodi et al, 2001), which are another two antibiotics produced by *Pseudomonas* species. Sequencing of the gene regions responsible for above antibiotics (Hammer et al. 1997; Mavrodi et al., 1998; Bangera & Thomashow, 1999; Nowak-Thompson et al., 1999) has been done and enabled the designing of primers for PCR. Since then many researchers have used this method to detect antibiotic biosynthetic genes responsible for the above antibiotics by several *Pseudomonas* sp. (Raaijmakers et al. 1997; Bangera & Thomashow, 1999; Picard et al., 2000; McSpadden Gardener et al., 2001; de Souza & Raaijmakers, 2003). For example, Zhang et al. (2006) detected antibiotic biosynthetic genes encoding phenazine-1-carboxylic acid (PCA), pyrrolnitrin in *P.chlororaphis* PA23. Furthermore, the research has extended towards the quantification of these antibiotic producing bacteria in soils from different ecosystems through techniques like real time PCR (Garbeva et al., 2004).

2.3.4.1.1.1 *Bacillus* Species and Antibiotic Biosynthetic Genes

With the isolation and identification of *Bacillus* sp. inhibiting microbial growth, production of antibiotics has been found to be one of the main mechanisms involved. Rather than extracting antibiotics produced from these strains through biochemical methods, PCR based methods are being widely used. As for *Pseudomonas* species the biosynthetic genes for most of the antibiotics produced by *Bacillus* sp. have been sequenced, which has made this a success (Duitman et al., 1999, Tsuga et al., 2001). Many studies identified the presence of antibiotic biosynthetic genes responsible for a variety of lipopeptide antibiotics belonging to three main families, sufactin, iturin and fengycin, together with other lipopeptides (Duitaman et al., 1999; Lin et al., 1999; Koumoutsi et al., 2004; Ramarathnam et al., 2007) in *Bacillus* spp. Apart from that aminopolyols such as zwittermicin A have also been identified through molecular techiniques (Stohl et al., 1999).

Induced systemic resistance (ISR) is similar to systemic acquired resistance (SAR), where plants activate their defense mechanisms against a broad range of pathogens through a primary infection of a pathogen, except that in ISR the BCA does not cause visible symptoms such as a necrotic lesion at the initial infection site (van Loon et al., 1998). This has been common mostly in bacteria like *Pseudomonas* and *Bacillus* species (Beattie, 2006), free-living and endophytic respectively (Compant, et al., 2005). Plant growth promoting bacteria (PGPB)-elicited ISR was first noticed on carnation (*Dianthus caryophillus*) against Fusarium wilt (van Peer et al., 1991) and on cucumber against *Colletotrichum orbiculare* (Wei et al., 1991). ISR was observed by *P. fluorescens*

EPI and *P. fluorescens* 63-28 against red rot caused by *Colletotrichum falcatum* on sugarcane (Viswanathan & Samiyappan, 1999) and *Fusarium oxysporum* f. sp. *radicis-lycopersici* on tomato (M'Piga et al., 1997) respectively. *Bacillus pumilus* SE34 triggered ISR against *F. oxysporum* f. sp. *pisi* on pea roots (Benhamou et al., 1998). Flagellation, production of siderophores and lipopolysaccharides (Hoffland et al., 1995; Leeman et al., 1995; Leeman et al., 1996; van Wees et al., 1997; Van Loon et al., 1998) and volatile organic compounds (Ping & Boland, 2004; Ryu et al., 2004) produced by bacteria have been proposed to trigger ISR. Defense mechanisms triggered by ISR include thickening of cell wall, phenolic compound accumulation, papillae formation (Benhamou et al., 1996; M'Piga et al., 1997; Benhamou et al. 1998; Compant et al., 2005), accumulation of pathogen-related proteins (PR proteins) (Park & Kloepper, 2000; Jeun et al., 2004) and other compounds such as peroxidase, phenylalanine ammonialyase, phytoalexins, polyphenol oxidase, and chalcone synthase (Van Peer et al., 1991; Chen et al., 2000; Ongena et al., 2000; Ramamoorthy et al., 2001; Nakkeeran et al., 2006). However, the importance and application of ISR in most of the plant pathogen systems have yet to be discovered.

2.3.4.2 Biological control of *S. sclerotiorum*

Over 20 years numerous studies have demonstrated the success of using biocontrol against *S. sclerotiorum* through oxalate oxidase (Tu, 1985), hyperparasitism (Huang & Kokko, 1993, Gracia-Garza et al., 1997), nutrient competition (Yuen et al., 1991) and antibiosis (Oedjijono et al., 1993). The majority of work has been on the use of

fungal mycoparasites. As Masirevic & Gulya (1992) report there are over 30 species of fungi and bacteria that affect *Sclerotinia* species.

Among fungi, *Coniothyrium minitans* Campbell is one of the parasites that have been successful against *S. sclerotiorum* (Campbell, 1947; Budge & Whipps, 1991; Mcquicken & Whipps, 1995). Among other parasites, *Gliocladium catenulatum* Gilman and Abbott and *G. roseum* (Link) Bainier (Makkonen & Phojakallio, 1960; Huang, 1978; Mclaren et al., 1994), *Trichoderma viride* Pers. ex Fr. (Makkonen & Phojakallio, 1960; Jones & Watson, 1969; Knudsen & Eschen, 1991) and *Talaromyces flavus* (Klocker) Stolk and Samson (Su & Leu, 1980; McLaren et al., 1989, 1994) have shown promising results. *Laterispora brevirama* Uecker et al. and *Teratosperma oligocladum* Uecker et al. are dematiaceous hyphomycetes distributed worldwide (Uecker et al., 1980, 1982). *Sporidesmium sclerotiovorum* was one of the most promising on sunflower (Adams, 1989; Adams & Fravel, 1990) and has been found frequently in sunflower fields after *Sclerotinia* infection (Gulya et al., 1992).

In Canada, research on biocontrol of *Sclerotinia* diseases have gained interest over fast few decades mainly concentrating on white mold of bean, stem rot of canola and lettuce drop and sunflower wilt (Bardin & Huang, 2001). It was Huang (1980a) who first attempted to use a mycoparasite against sunflower wilt. *Coniothyrium minitans* Campbell, *Gliocladium catenulatum* Gilman and Abbott, and *Trichoderma viride* Pers. ex Fr. were the first used BCA. *Coniothyrium minitans* was the most effective one to reduce sclerotinia wilt of sunflower by parasitizing sclerotia (Huang, 1980a, McLaren et al., 1994). Spraying spore suspension of *C. minitans* was better in controlling white mold of dry bean (Huang & Kokko, 1993). In bean and pea crops *Talaromyces flavus* (Klöcker)

Stock and Samson reduced carpogenic germination of sclerotia (Huang & Erickson, 2000). In addition strains like *Trichothecium roseum* (Huang & Kokko, 1993), *Talaromyces flavus* and *Trichoderma virens* (Huang et al., 2000b) have been tested and under Canadian Prairie conditions and are the ones that have shown relatively consistent results. With the successful results *C. minitans* has been developed into a commercial biopesticide named "Contans" (Vrije et al., 2001).

The use of bacteria for the management of *S. sclerotiorum* is not very common (Willetts & Wong, 1980; Bardin & Huang, 2001) and remains to be explored (Fernando et al., 2004). However, so far a few *Bacillus* and *Pseudomonas* species have demonstrated antagonistic effect on *Sclerotinia* spp. (Wu, 1988; Fernando et al., 2007). *B. subtilis* Frankland and Frankland improved sunflower emergence by suppressing the sclerotial germination (McLoughlin et al., 1992) and reduced basal pod rot in pea (Huang et al., 1993). *P. fluorescens* reduced the *Sclerotinia* infection on seedlings (Expert & Digat, 1995). Organic volatiles produced by *P. chlororaphis* (PA23) were inhibitory to sclerotial and ascospore germination as well as to mycelial growth under *in vitro* conditions (Fernando, et al., 2005) and PA23 significantly controlled sclerotinia stem rot under field conditions (Fernando et al., 2007). Almost all studies have focused on destroying sclerotia in rhizosphere or non-rhizosphere regions, except in few situations. Yuen et al. (1991) evaluated the effect of *B. polymyxa* Mace and *Erwinia herbicola* Dye on *S. sclerotiorum* on the phyllosphere of bean. Savchuk & Fernando (2004) found that *Pseudomonas* spp. (DF41) and *P. chlororaphis* (PA23) were antagonistic to ascospore germination of *S. sclerotiorum* on senescing petals of canola. However, further research

is needed on the use of bacterial biocontrol agents on the phylloplane, which would be essential in management of diseases caused be ascospores of *S. sclerotiorum.*

In addition, hypovirulence has been observed in *S. sclerotiorum* (Boland, 1992) in Canada. A hypovirulent isolate is a *S. sclerotiorum* isolate that has a slower growth and atypical colonies in culture (Bardin and Huang, 2001). The mechanism is thought to be through the reduced or/and delayed production of oxalic acid when compared to virulent isolates, especially during the first 3-7 days of growth (Zhou and Boland, 1999). However, the use of hypovirulent isolates to control virulent isolates of *S. sclerotiorum* can be impractical because of the "high number of mycelial compatibility groups in this species" (Kohn, 1995) and it doesn't have an effect on reducing carpogenic germination.

2.3.4.3 Commercialization and Future Research Directions

Despite of the increasing number of biocontrol products, they still represent only about 1% of agricultural chemical sales (Fravel, 2005). The ultimate goal of biocontrol research is to use this as an additional tool for disease management. Commercialization comes into play when these tools go on to growers' hands. The first BCA that was registered with the United States Environmental Protection Agency (EPA) for the control of plant diseases was *Trichoderma harzianum* ATCC20476. Among EPA-registered organisms, 65% have been registered over the past 10 years and the technique is still emerging, overcoming many technological problems (Fravel, 2005). The important steps in the commercialization of BCA are discovering potential candidates, performance testing and the improving for the commercial use (Cook, 1993).

The ultimate success of the entire procedure depends on the searching and screening method for BCA. These methods vary depending on the target pathogen the crop and the cropping system (Fravel, 2005), most commonly accompanied by isolation and preliminary *in vitro* and *in vivo* testing. These performance tests will look at the ability of the BCA to control the target pathogen inoculum, to prevent plant infection and to limit the disease development after initial infection. Biocontrol agents that have shown greatest potential are advanced to performance testing, where they are subjected to replicated experiments under different natural conditions with natural or artificial inoculation (Cook, 1993). The final improvement step allows the mass production of the BCA with the correct formulation to meet the field production demand (Cook, 1993) and use with other agronomic practices including indigenous microflora (Duncan, 2003). Formulation is a very important step that directs the BCA toward commercialization through extending shelf-life. Many researchers over-looked this step until the performance testing stage, which can cause time and economic waste on a non-profitable target.

However, despite of the excellent performance of BCAs under laboratory, greenhouse and even under field condition, most of them fail to become products due to the following reasons. Overestimation of the power of environmental concerns as economic drivers, insufficient knowledge of grower needs, registrations strategy and competitive force, immature ideas about positioning and market strategy and underestimation of registration costs and difficulties as well as "insufficient cost-performance and poor shelf-life" (Lidert, 2001). As a solution for this, research should focus on the "product concept" early in the development process of a BCA (Harman &

Kubicek, 1998; Mintz & Walter, 1993) and will require more communication between public researchers and industry in the early stages of development (Fravel, 2005). At present biocontrol research is becoming oriented towards the industry's concerns and future research should aim at the production, formulation and delivery as well as rotating BCA with chemical pesticides through forecast models, which would greatly assist in commercialization of biocontrol agents. Continuing biocontrol research should continue in order to enhance the concept of "sustainable agriculture" and it will be an alternative path for disease control in case of unexpected failure or loss of other tools.

CHAPTER 3

3.0 Field Investigation of the Efficacy of Two Biocontrol Agents and a Fungicide Against Sclerotinia Diseases of Sunflower in Canada

3.1 Abstract

Sclerotinia sclerotiorum (Lib.) de Bary causes three main diseases; head rot, mid-stalk rot and wilt in sunflower. At present no adequate resistance identified in commercial hybrids and no chemical fungicide is registered in Canada for the control of these diseases. Thus, two bacterial biocontrol agents *(Psuedomonas chlororaphis* strain PA23, *Bacillus amyloliquefacience* strain BS6) and a fungicide Ronilan (vinclozolin) were tested in a field study at Morden, Manitoba, Canada against head rot and wilt disease in sunflower in 2006 and 2007. The bacteria (3000L/ha; 10^8 cfu/ml) and the fungicide (2 Kg/ha) were applied onto sunflower heads followed by inoculation of heads with ascospores (10^4 spores/ml) and sclerotinia-infected ground millet seeds twice during flowering period. In 2007, only ascospores were used as inoculum. In both years, none of the biocontrol agents and the fungicide showed significant reduction in the Disease Severity Index (DSI) and the Area Under the Disease Progress Curve (AUDPC) of head rot in comparison to the inoculated control. In the wilt experiment, the bacteria (10^8 CFU/seed in 2006 and 10^{11}CFU/seed in 2007) were applied as a seed treatment and soil drench while the fungicide (4g/1 Kg of seeds) was applied only as a seed treatment. The field was inoculated with sclerotinia-infected millet seed (10 g/3m row in 2006 and 5 g/3m row in 2007) at the time of seeding. In

both years only the fungicide treatment significantly (P=0.05) increased the seed emergence and yield in comparison to the inoculated control. In addition, in 2007, the growth promotion ability of PA23 and BS6 was investigated in Morden and Winnipeg. None of the data on plant height, leaf number, leaf length, leaf breadth, leaf area, and yield showed significant growth promotion compared to the control in both locations. The fungicide (Ronilan) seed treatment would be potential method to manage *Sclerotinia* wilt disease while further research is needed with the biocontrol agents.

3.2 Introduction

Sunflower (*Helianthus annuus* L.) has been grown commercially in Canada since the early 1940s and it is the only oilseed native to North America (Agriculture and Agri-food Canada, 2007). Canada is a competitive partner of the growing international market and there is a growing market for Canadian-grown sunflower seeds. For example, the birdseed market for Canadian-grown sunflower seeds is growing at an annual rate of 10% in North America (Agriculture and Agri-food Canada, 2007).

Sclerotinia sclerotiorum (Lib.) de Bary is a common and widespread pathogen of sunflower (Bisby, 1921) causing significant yield reductions in the sunflower growing regions of the world (Gulya et al., 1997). Since 1921, sclerotinia diseases have been common and destructive to sunflower in Canada and the United States. In terms of yield losses, sclerotinia wilt is considered the most important sunflower disease in North America (Gulya, 1985, 1996). In Manitoba, wilt is reported every year and is considered to be one of the limiting factors affecting sunflower production (Putt, 1958; Hoes & Huang, 1976). Early

infections of the plant may result in a total yield loss by killing the plant before flowering and up to 80% of yield loss has been reported (Rashid & Seiler, 2005). The loss caused by *Sclerotinia* is remarkable since the damage is both quantitative and qualitative (Putt, 1997).

Management of *S. sclerotiorum* in sunflower is rather limited when compared to other crops and no chemical fungicide is currently registered for this pathogen in sunflower in Canada due to the practical and economical difficulties (Mestries et al., 1998; Rashid & Dedio, 1992). Although breeding for sclerotinia-resistance is the most practical way to manage the disease, it is hindered due to the lack of resistant germplasm among sunflower varieties to this pathogen. Resistance is not always consistent from year to year (Rashid & Dedio, 1992). Furthermore, the hybrids with resistance to wilt are not always resistant to head rot due to the association of different genetic factors (Rashid & Dedio, 1992). Cultural practices including crop rotation do not always be successful since this disease is highly dependant on the prevailing environmental conditions.

Biocontrol using hyperparasites has been another way that proved to be effective against *Sclerotinia* by destroying sclerotia in soil (Huang, 1980a). Biocontrol is an environmentally-friendly way of combating plant diseases, which also provides safe and long-term protection to the crop (Fernando et al., 2005). There are over 30 species of fungi and bacteria that parasitize the sclerotia (Masirevic & Gulya, 1992). *Coniothyrium minitans* Campbell, *Gliocladium catenulatum* Gilman and Abbott and *Trichoderma viride* Pers. ex Fr. have been experimentally successful against sclerotinia wilt of sunflower under Canadian Prairie conditions (Huang, 1980a). However, use of antagonistic bacteria against *Sclerotinia* is rare in Canada, though there are a few situations where bacterial species like *Bacillus cereus* Frankland and Frankland, strain alf-87A, *Bacillus subtilis* (Ehrenberg) *Pseudomonas*

chlororaphis Guignard and Sauvageau strain PA23 and *Bacillus amyloliquefaciens* n. sp. Fukumoto strain BS6 have been tested against basal pod rot of pea (Huang et al., 1993), white mold of bean (Boland, 1997) and stem rot of canola (Savchuk & Fernando, 2004; Zhang, 2004) respectively. Furthermore, most of the studies have focused on destroying sclerotia through mycoparasitism. The use of bacterial antagonists to control ascospore infections has been attempted in a study carried out by Duncan et al. (2002), where *P. chlororaphis* strain PA23 and *Pseudomonas* spp strain DF 41 have been tested. Control of ascospore infection is important because the head rot disease is caused by ascospores produced by apothecia present in the same field or adjacent fields since ascospores can travel long distances. Using bacterial biocontrol agents is advantageous over fungal antagonists since the inoculum preparation is less time consuming and easy, which is very important when it comes to commercial applications.

The most appropriate way to control sclerotinia wilt disease is through the destruction of sclerotia in soil since root infections are caused by the mycelial fragments formed through the myceliogenic germination of sclerotia. In this case volatile antimicrobial compounds are more important over non-volatile antimicrobial compounds since they can easily diffuse through the soil and kill the pathogen (Fernando et al., 2005) without a contact between antagonist and sclerotia. Previous studies identified that *P. chlororaphis* PA23 produces three organic volatiles; nonanal, 2-ethyl-1-hexanol and benzothiazole, together with HCN, which are inhibitory to mycelial, sclerotial and ascospore germination of *S. sclerotiorum* under *in vitro* conditions (Fernando et al., 2005; Poritsanos et al., 2006). In the control of ascospore infection on the head, non-volatile antibiotics and competition play a more vital role than volatile antibiotics. In both situations, the degree of control under *in vivo* conditions

is strongly affected by the survival of antagonist in soils or on heads at an effective level (Yuen et al., 1991). The isolation and determination of survival of antagonist in rhizosphere and on heads is important in this regard.

Therefore, this study used two bacteria *P. chlororaphis* strain PA23 and *Bacillus amylolquefaciens* strain BS6 against *Sclerotinia* head rot and wilt diseases on an oil-seed sunflower variety IS 6111 in a two year field study. PA23 was proven a significant biocontrol agent against stem rot of canola both under green house and field conditions (Zhang, 2004) and against head rot of sunflower under field conditions (Duncan et al., 2002). It also produces antibiotics phenazine-1-carboxylic acid (PCA), 2-hydroxyphenazine and pyrollnitrin (Zhang et al, 2006) and several additional secondary metabolites including protease, lipase, hydrogen cyanide and siderophores (Poritsanos et al., 2006). BS6 has also successfully controlled *S. sclerotiorum* mycelial growth *in vitro* and stem rot of canola in the field (Fernando et al., 2007). It also produces the antibiotics surfactin, zwittermicin A and bacillomycin D (Ramarathnam, 2007). The efficacy of these two bacteria was compared with an experimental fungicide Ronilan (active ingredient vinclozolin). Furthermore, several studies have shown that *Bacillus* sp. (Algam et al., 2004; Joo et al., 2004; Chakraborty et al., 2006) and *Pseudomonas* sp. (Kurek et al., 2003; Urashima et al., 2005; Choi et al., 2007) promoted growth of plants. While several factors may contribute to this, bacterial volatiles were also found to play a major role (Ryu et al., 2003). Since both PA23 and BS6 produce volatiles (Fernando et al., 2005; Zhang, 2004) a separate field study was carried out in University of Manitoba Field Research Station in 2007 to investigate the growth promotion of sunflower by these two strains.

3.3. Materials and Methods

3.3.1 Experimental Site, Design and Agronomy

Head rot and wilt trials were conducted at the Agriculture and Agri-Food Canada Research Station in Morden, Manitoba in 2006 and 2007. In 2007 an additional experimental site was added at the University of Manitoba Field Research Station, Winnipeg, Manitoba to study the growth promotion by the two bacterial species used in the study. Each plot contained four 3 m rows with 75 cm spacing between rows. In 2006 both head rot and wilt trials had 7 treatments in a randomized complete block design (RCBD) with four replicates; 1. healthy control, 2. pathogen-inoculated control, 3. fungicide and *S. sclerotiorum* application, 4. PA23 and *S. sclerotiorum* application, 5. BS6 and *S. sclerotiorum* application, 6. PA23 alone, 7. BS6 alone. In 2007 another two treatments (PA23 + BS6 + *S. sclerotiorum*, and PA23 + BS6) were added to both head rot and wilt trials. Treatments and replicates were arranged in a randomized complete block design (RCBD). In 2006 wilt and head rot trials were seeded using a Sunflower 2-Row Cone Planter (John Deere, Ontario, Canada) on the 11th of May and the 15th of May respectively, while in 2007 the head rot trial was seeded on the 15th of May and wilt trial was seeded on the 16th of May. A separate experiment containing only 4 treatments; 1. control (without bacteria), 2. PA23 only 3. BS6 only 4. PA23 + BS6, was conducted at the University of Manitoba field research station, Winnipeg in 2007 to monitor the growth promotion ability of the strains. It was hand seeded on the 22nd of June 2007.

3.3.2 Biocontrol Agents and Culture Conditions

Rifampicin resistant strains (Zhang, 2004) of two bacterial strains namely *P. chlororaphis* (PA23) and *B. amyloliquefaciens* (BS6) were used for both head rot and wilt experiments. They were retrieved from –80 °C storage and cultured on Luria Bertani Agar [LBA, 15.0 g technical agar (Difco Laboratories, Detroit, Mich., USA), 10.0 g tryptone peptone (Difo Laboratories, Detroit, Mich., USA), 5.0 g yeast extract (Difco Laboratories, Detroit, Mich., USA) and 5.0 g NaCl] at 28 °C for 48 h.

3.3.3 Head Rot Trial

3.3.3.1 Preparation of Biocontrol Agents

A loop full of bacteria from a 48 h fresh culture was transferred to LB broth (same formulation as LBA, but without 15.0 g of technical agar) and incubated at 30 °C for 16-18 h in an incubator shaker at 180 rpm. The bacterial suspension was diluted with a potassium phosphate buffer solution (0.1 M, pH 7.0) with 0.2% Tween 20 (ICI Americas, Inc., SIGMA, St Louis, MO., USA) added as a surfactant, so that the concentration was log 8 cfu/ml.

3.3.3.2 Preparation of Pathogen Inoculum

Two types of inocula, ascospores and sclerotinia-infected ground millet seed (*Pennisetum glaucum* (L.) R. Br.) were used in the 2006 trials while only ascospores were used in the 2007 trial. The ascospore suspension (with $8x10^4$ spores/ml) was prepared in

water with the addition of an appropriate amount of glucose (3 g/L). The millet seed was autoclaved twice (120 °C for 25 min) in closed containers followed by inoculating with potato dextrose agar (Becton/Dickinson, Sparks, MD, USA) plugs infected with *S. sclerotiorum* and incubated for 14 days at room temperature. Sclerotinia-infected millet seeds were then ground into a powder for application.

3.3.3.3 Application of Fungicide and Biocontrol Agent

The fungicide used was the commercial product (BASF Canada) Ronilan in which 50% active ingredient is vinclozolin. The fungicide and BCA treatments were applied twice in the 2006 and 2007 trials. In 2006, the first application of fungicide was done at the late flowering stage (19th of July) and the second application was done on the 1st of August (just after the ray florets had fallen) at a rate of 2 kg of product per hectare. The first application of BCA was carried out on the 20th of July while the second application was done on the 2nd of August at a rate of 3000L per hectare. Both the fungicide and the biocontrol agents were sprayed on to both sides of individual heads. In 2007, the first application of fungicide was done on the 1st of August while the 2nd application was done on the 14th of August. The BCA was sprayed onto both sides of individual heads on the 3rd and 15th of August.

3.3.3.4 Inoculation of the Pathogen

In the 2006 trial, both ascospores and ground millet seed inoculum were applied twice, the first application being on the 20th of July and the second application was on the 1st

of August. Ascospore suspension was sprayed on to both sides of the head followed by sprinkling of ground millet inoculum over the heads. An overhead misting system was programmed to mist water for 5 minutes every half an hour to enhance the disease development and to increase the longevity of the BCA. In 2007, the heads were spray inoculated only with ascospore suspension and misted as in 2006. A manually operated backpack sprayer with a hand held nozzle was used for all spraying.

3.3.3.5 Survival of Biocontrol Agents

Ten ray florets and ten sepals were collected from each plot sprayed with BCA and from the healthy control. Standard dilution plating was performed by suspending 10 ray florets and 10 sepals in 100 mL of sterile distilled water. Nutrient agar (NA) plates amended with appropriate amounts of antibiotics (rifampicin: 100 μg/mL) were used to determine the introduced bacterial count and NA plates without antibiotics were used for the total bacterial count.

3.3.3.6 Disease Assessment and Data Analysis

Visual assessment of disease development was done according to the scale described by Rashid et al., 2002 (0 = no lesion, 1 = 1% to 5% head area infected (HAI), 2 = 5 % to 20 % HAI, 3 = 20 % to 40 % HAI, 4 = 40 % to 60 % HAI, 5 = greater than 60% HAI) in single heads selecting 10 randomly tagged sunflower plants per plot. Disease severity index (DSI) was calculated for each treatment based on above rating using the modified formula, DSI =

(sum of individual plant ratings / 5 X number of plants rated) X 100 (Cober et al., 2003). The area under the disease progress curve (AUDPC) was also calculated (Shaner & Finney, 1977) using the above DSI values. The yield of each treatment was also compared. The results were analyzed using analysis of variance (ANOVA) and mean separation test was done using Fisher's Least Significant Difference test at P = 0.05, using SAS version 8.1 (SAS Institute, Cary, NC).

3.3.4 Wilt trial

3.3.4.1 Application of Biocontrol Agent and the Fungicide

Bacteria were applied both as a seed treatment and soil drench. For the seed treatment, bacteria were grown in LBB for 16-18 h in an incubator shaker (180 rpm, at 30 °C) and centrifuged at 17,000 g for 15 minutes. The pellet was washed three times with sterile distilled water and resuspended in 10% methylcellulose in potassium phosphate buffer (0.1 M, pH 7.0) so that the concentration of the suspension was 10^8 cfu/mL (in 2006) and 10^{11} cfu/mL in 2007. The seeds were surface sterilized by dipping them in 4% NaOCl (Clorox) solution for 3 minutes followed by washing three times with sterile distilled water. Seeds were dried under a sterile air stream overnight. Then they were soaked in the methylcellulose solution for 1 h and dried overnight under a sterile air stream. The initial bacterial count on treated seeds was determined. Ten seeds were suspended in 100 mL of phosphate buffer (0.1 M, pH 7.0) sonicated for 30 sec. followed by vortexing for 5 sec. A dilution series was prepared and appropriate dilutions were plated onto nutrient agar amended with rifampicin (100 µg/mL; Sigma®, St. Louis, MO, USA). After 3 days of

incubation at room temperature colony count was taken. For the soil drench treatment, bacteria were grown in LBB for 16-18 h at 30 °C with shaking at 180 rpm. Concentration was adjusted to 10^8 cfu/mL in sterile distilled water. Biocontrol agent (10^8 cfu/ml) was added to field soil at a rate of 150 mL/row in 2006 and 500 mL/row in 2007. For the fungicide application, seeds were mixed with slightly wetted fungicide Ronilan (4 g per 1 kg of seeds) and dried overnight at room temperature.

3.3.4.2 Application of the Pathogen

The pathogen-treated plots were inoculated with sclerotinia-infected millet seed inoculum at the time of seeding. Infected millet seeds were drilled into furrows with the seeds at seeding and applied to the field (10 g per row in 2006 and 5 g per 3 m row in 2007).

3.3.4.3 Survival of the Biocontrol Agents

Survival of biocontrol agent in the treated field was determined 4 weeks after seeding. Two seedlings were collected randomly from each of 4 replicates of bacterial treated plots. The seedlings were shaken gently to remove soil so that only the most tightly adhering rhizosphere soil remained. Seminal root from both seedlings were pooled and 0.1 g of root measured from the pooled root sample was suspended in 10 mL of sterile distilled water. The root suspension was sonicated for 30 s followed by vortexing for 5 s. A dilution series was prepared and appropriate dilutions were plated onto both nutrient agar amended with rifampicin (100 µg/mL; Sigma®, St. Louis, MO, USA) to get the introduced bacterial count

and nutrient agar without antibiotics to get the total bacterial count. After 3 days of incubation at room temperature, colony counts were determined. 10 g of removed soil (non-rhizosphere) was measured and suspended in 99 mL of sterile distilled water. Dilution series and plating were done as described above. Bacterial colonies were counted after 3 days of incubation at room temperature.

3.3.4.4 Disease Assessment and Data Analysis

The number of emerged seedlings was determined and the percentage seedling emergence was calculated in both 2006 and 2007. At harvest, seed yield of each treatment was taken. Data from 2006 and 2007 trial was analyzed separately using analysis of variance with Statistical Analysis System (SAS Institute, Cary, NC, USA) and Fisher's LSD mean separation test at 0.05 significant level.

3.3.5 Growth Promotion Trial

Seed treatment and seeding was done in a similar manner to that of the Morden 2007 wilt trial. Five days after seeding, a 1 m area was marked along the 2 middle rows in each plot randomly and the number of emerged seedlings was counted each day starting from 5 days after seeding until 100% emergence was completed. The days taken by each plot to attain 50% emergence was calculated using the equation; (50 - percentage lower than 50% (PE)) / {(percentage immediately higher than 50% - (PE)) + no. of days showing % immediately lower than 50%}. Plant height and number of leaves were counted in randomly

tagged 10 plants from the two middle rows in each plot. Twenty leaves were collected from the middle area of 10 tagged plants (2 leaves per plant) from each plot and leaf length and width were measured. In addition leaf area was calculated using the equation a + b(W*W) where W is leaf width and a and b are constants; 6.72 and 0.65 respectively. The whole procedure was done at two different growth stages (V-10 and R-3 stages) of sunflower plants. In 2007, same growth measurements, as in University of Manitoba field trial, were taken from the treatments; healthy control, PA23 only, BS6 only and PA23 + BS6 in Morden 2007 wilt trial. Data from University of Manitoba field and Morden was analyzed separately using analysis of variance with Statistical Analysis System (SAS Institute, Cary, NC, USA) and Fisher's LSD mean separation test at 0.05 significant level.

3.4 Results

3.4.1 Head Rot Trial

In the Morden trial of 2006, DSI and AUDPC values for the treatments inoculated control, Fung+Scl, PA23 + Scl and BS6 + Scl were significantly higher than treatments PA23, BS6 and healthy control. There was no significant difference between AUDPC values of inoculated control and BS6+Scl but that of BS6+Scl was higher than inoculated control and PA23+Scl (Table 3.1). However, the healthy control, BS6 and PA23 treatments showed significantly lower AUDPC and DSI values than those of other treatments. Although Fung+Scl treatment showed a significant lower AUDPC value than BS6+Scl, the reduction was not significant compared to the other sclerotinia containing treatments. The yields obtained from the treatments Fung+Scl, PA23+Scl, BS6+Scl and the inoculated control were

not significant. In the Morden trial of 2007, no significant difference (P=0.05) was observed among DSI in all treatments (Table 3.2). Similarly, significant differences (P=0.05) were observed among AUDPC values in all treatments except between PA23+BS6+Scl and Fung+Scl as well as between BS6 and the healthy control (Table 3.2). PA23+BS6+Scl showed lower DSI and AUDPC compared to those of PA23+Scl and BS6+Scl although the differences are not significant (Table 3.2). AUDPC of PA23+Scl, BS6+Scl, PA23+BS6+Scl and Fung+Scl were 1.2, 1.25, 1.46 and 1.49 times lower than that of inoculated control respectively. Similarly, DSI of Fung+Scl, PA23+Scl, BS6+Scl and PA23+BS6+Scl were 1.1, 1.1, 1.2 and 1.4 times lower than that of the inoculated control. However, there was no significant yield increase by the treatment PA23+Scl, BS6+ Scl, PA23+BS6+Scl and the fungicide compared to the inoculated control (Table 3.2).

3.4.2 Wilt Trial

In the Morden trial of 2006, the percent seedling emergence shown by treatments PA23+Scl, BS6+Scl and inoculated control were not significantly different from each other and were lower than those of other treatments. However, Fung+Scl was significantly (P=0.05) higher than inoculated control (Table 3.1). Percent emergence of BS6, PA23 and healthy control (HC) was not significantly different from each other however was significantly different from other treatments. The yields obtained from Fung+Scl treatment was significantly higher (P=0.05) than IC, PA23+Scl and BS6+Scl and also significantly similar (P=0.05) to that of HC and other treatments. In 2007 similar trends were observed. There was no significant difference between percent emergence values of treatments PA23+Scl, BS6+Scl, PA23+ BS6

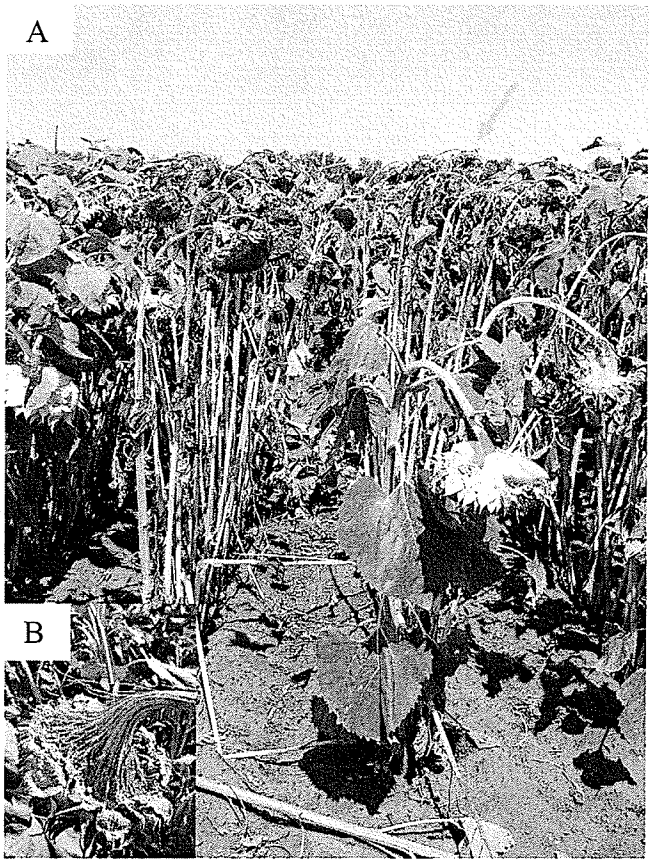


Figure 3.1. *Sclerotinia sclerotiorum* infection on heads of sunflower in Morden 2006**.** Severe disease pressure prevailed in the 2006 Morden trial, Manitoba (A). Infected sunflower head with decaying tissue (B). In each plot the middle row was treated with the biocontrol agent or/and sclerotinia-infected ground millet seeds and ascospores. Ten plants from the middle row were tagged (red strips) and the head area infected was rated in each head. The untreated edge row also showed high disease (arrow).

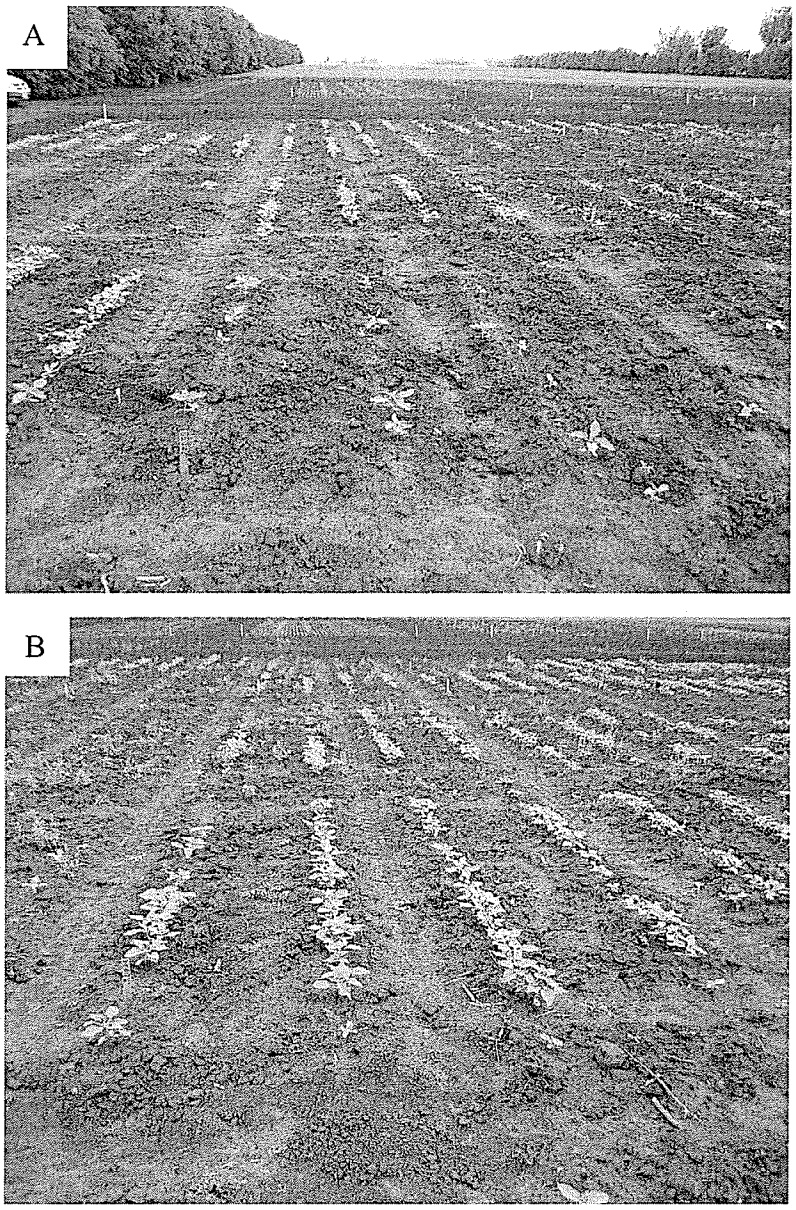


Figure 3.2. Sunflower seedling emergence in the Sclerotinia-infested 2006 Morden wilt trial. Each plot contained four rows and each row was treated with the biocontrol agent or/and sclerotinia according to the treatments. Very low numbers of seedlings emerged in the treatment PA23+Scl (A) in comparison to the number of emerged seedlings in the treatment PA23 (B).

Table 3.1. Disease severity index (DSI), AUDPC and yield from 2006 head rot trial and percent emergence from wilt trial carried out at Morden Research Centre, MB, Canada.

Treatment	Head Rot			Wilt	
	DSI	**AUDPC**	**Yield (g/plot)**	**Percent Emergence**	**Yield (g/plot)**
Healthy Control	47.5c	537.5c	1060.6a	26.3a	2193.3a
Inoculated Control + Scl	100a	2740ab	335.9b	2.1c	654.5b
Fung +Scl	88a	2165b	633b	20.1b	2187.2a
PA23 + Scl	98a	2830ab	427.6b	2.1c	645.5b
BS6 + Scl	100a	2945a	415.2b	1.3c	328.6b
PA23	63b	617.5c	1160.9a	25.9a	2070.8a
BS6	60.5bc	630c	1004a	27.4a	2116.6a
LSD=(P=5%)	**15.48**	**697.03**	**328.76**	**3.1257**	**563.54**

The values followed by the same letter are not significantly different at P=0.05 according to Fisher's Least Significant Difference test.

Table 3.2. Disease severity index (DSI), AUDPC and yield from 2007 head rot trial and percent emergence from wilt trial carried out at Morden Research Centre, MB, Canada.

Treatment	Head Rot			Wilt	
	DSI	AUDPC	Yield (g/plot)	Percent Emergence	Yield (g/plot)
Healthy Control	24.2d	612.4bcd	675.9a	55.6a	901.4ab
Inoculated Control+scl	65.4a	1704.1a	558.8ab	4.2c	152.1c
Fung+Scl	61.2ab	1141.4abc	620.7ab	40.0b	1050.9a
PA23+Scl	61.2ab	1416.8a	475.9b	12.8c	492.8bc
BS6+Scl	54.4abc	1364.5ab	560.1ab	11.6c	357.1c
PA23+BS6+Scl	47.2abcd	1166.1abc	521.7ab	8.6c	202.6c
PA23	26.5d	298.9d	643.3ab	50.8a	944.6a
BS6	39.6bcd	644.3bcd	572.3ab	53.1a	513.4bc
PA23+BS6	32.0cd	427.9dc	718.0a	57.2a	895.1ab
LSD (P=5%)	**24.547**	**762.83**	**198.46**	**9.7748**	**430.8**

The values followed by the same letter are not significantly different at P=0.05 according to Fisher's Least Significant Difference test.

+Scl and IC. PA23+BS6+Scl showed lower emergence than single application of the two strains although the reduction was not significant (Table 3.2). Fung+Scl was significantly higher than IC and all sclerotinia-inoculated treatments as in the results obtained in 2006. All treatments without sclerotinia including healthy control showed relatively higher percentage emergence and were not significantly different from each other. However, treatment PA23+BS6 showed the highest emergence although it was not significantly different from other treatments that do not contain sclerotinia. The yield of plots treated with the fungicide was significantly higher (P=0.05) than other sclerotinia inoculated treatments (Table 3.2).

3.4.3 Growth Promotion Trial

In the Morden trial of 2007 there was no significant difference among the treatments with regards to any of the growth measurements taken (height, leaf number, leaf length, leaf width, and leaf area) on the 1st sampling date, while there was a significant difference between leaf lengths measured on the 2nd sampling date (Table 3.3). The leaf length of PA23+BS6 treatment on the 2nd sampling date was significantly higher than that of BS6 but not significantly different from other treatments. In the University of representing one separate treatment. A plot had four 3m rows having 0.75m between them. Manitoba trial in 2007, significant differences were observed in rate of seedling emergence, height, leaf breadth and area in the 1st data set. The percentage emergence of PA23+BS6 was significantly lower than that of other treatments. There was a significant increase in height due to BS6 and PA23+BS6 treatments compared to the control but the difference

Figure 3.3. Sunflower growth promotion trial at the University of Manitoba Research Field (Point), Winnipeg, Manitoba, Canada. The experiment was designed according to a randomized complete block design. The picture shows one block containing 4 plots each representing one separate treatment. A plot had four 3m rows having 0.75m between them.

Table 3.3. Average growth parameters measured from 2007 trial carried out at Morden Research Centre, MB, Canada.

Treatment	44 Days*					65 Days**					Yield
	Plant Height (cm)	Leaf #	Leaf Length (cm)	Leaf Width (cm)	Leaf Area (cm^2)	Plant Height (cm)	Leaf #	Leaf Length (cm)	Leaf Width (cm)	Leaf Area (cm^2)	
Control	41.1a	10.7a	15.2a	13.8a	130.5a	131.9a	23.5a	17.7ab	17.2a	234.7a	901.4a
PA23	42.3a	10.3a	15.3a	13.8a	130.3a	119.7a	24.2a	17.9ab	17.5a	228.2a	944.6a
BS6	35.4a	10.5a	15.0a	13.6a	127.8a	95.6a	22.5a	14.5b	13.8a	151.0a	513.4a
PA23 + BS6	43.1a	10.4a	15.7a	14.1a	136.1a	126.4a	25.3a	19.1a	18.6a	258.7a	895.1a
LSD (P=5%)	**7.9229**	**0.8838**	**1.209**	**5.327**	**33.563**	**41.676**	**3.3349**	**4.35**	**5.33**	**117.14**	**605.25**

*Data was collected after 44 days from seeding (V-10 stage).

**Data was collected after 65 days from seeding (mature floral bud observed - R-3 stage).

The values followed by the same letter are not significantly different at P=0.05 according to Fisher's Least Significant Difference test.

Table 3.4. Average growth parameters measured from 2007 trial carried out at University of Manitoba Research Fields (Point), Winnipeg, MB, Canada.

Treatment	Emerg. Speed	31 Days*					47 Days**					Yield
		Plant Height (cm)	Leaf #	Leaf Length (cm)	Leaf Width (cm)	Leaf Area (cm^2)	Plant Height (cm)	Leaf #	Leaf Length (cm)	Leaf Width (cm)	Leaf Area (cm^2)	
Control	6.6a	62.5b	11.2a	14.8a	12.8b	110.8b	147.5a	25.8a	17.5a	17.5a	2052.1a	285.3a
PA23 (P)	6.4a	66.6ab	11.3a	15.0a	12.8b	110.3b	153.2a	25.6a	16.8a	17.0a	1899.9a	244.4a
BS6 (B)	6.4a	67.7a	11.6a	15.3a	13.7a	124.0a	152.8a	25.4a	17.1a	17.2a	1962.2a	263.0a
PA23+BS6	6.1b	68.4a	11.5a	14.7a	13.3ab	119.8ab	152.2a	25.8a	17.5a	16.8a	1889.3a	278.6a
LSD (P=5%)	**0.227**	**4.717**	**0.530**	**0.81**	**0.779**	**12.103**	**8.659**	**1.173**	**11.3**	**8.989**	**224.6**	**46.477**

*Data was collected after 31 days from seeding (V-11 stage).

**Data was collected after 47 days from seeding (mature floral bud observed - R-3 stage).

The values followed by the same letter are not significantly different at P=0.05 according to Fisher's Least Significant Difference test.

was not significant compared to PA23 in the 1st data set. Leaf width and area of the treatments BS6 in the 1st data set were significantly higher than that of control and PA23 while the difference was significant in comparison to PA23+BS6 (Table 3.4). In the 2nd data set there was no significant differences observed among any of the parameters tested (Table 3.4). Also the yields from both Morden and Winnipeg trials were not significantly different among treatments.

3.4.4 Survival of Antagonists at the Sites of Inoculation

The bacterial density on sunflower petals 24 h after spraying of antagonists in the 2007 Morden head rot trial varied between log 2.8 – 3.6 (Figure 3.4A). The bacterial density on sunflower seedling roots from the 2007 Morden wilt trial varied between log 6.2 to 8.3 (Figure 3.4B). In 2007 Winnipeg trial the bacterial density on the seedling roots were in the range of log 5.1 – 5.9 (Figure 3.4C).

3.4 Discussion

The results of this study show that the effect of PA23 and BS6 on sclerotinia wilt and head rot disease control is relatively variable between the two years 2006 and 2007. In 2006, none of the data collected was significant over the inoculated control in both wilt and head rot trials. The sclerotinia inoculum density would have been too much since 10 g of millet seed inoculum were added per row (3 m) for the wilt trial while two sources of

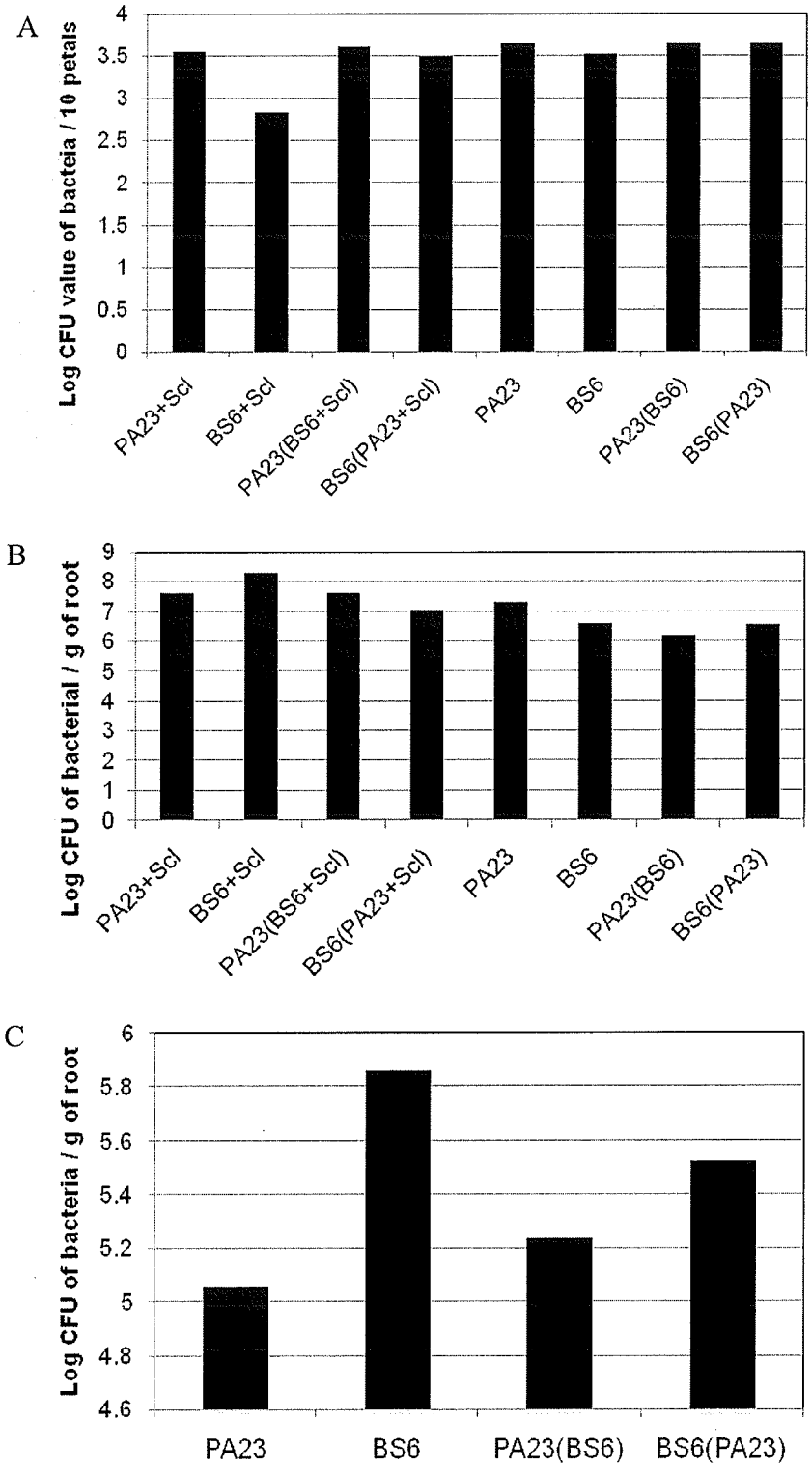


Figure 3.4. Survival of introduced bacteria (A) 24 h post inoculation in 2007 Morden head rot trial, (B) 23 d after seeding in 2007 Morden wilt trial, (C) 23 d after seeding in 2007 Winnipeg growth promotion trial.

inocula, ascospores and sclerotinia-infected ground millet seeds were used for the head rot trial to get the adequate amount of disease on the heads. Furthermore, sclerotinia-infected ground millet seeds do not behave as natural sclerotia so that the infection process of wilt was also not natural. The healthy control in the head rot trial also showed a considerable DSI (approximately 50%), which shows the severity of the disease pressure prevailed in the plot. Furthermore, due to practical problems and changing weather conditions the pathogen and the BCA were applied on the same day. For the wilt trial, millet seed inocula was applied with seeding while for the head rot, trial ascospores and ground millet seeds were applied about 1 hour after BCA application. Therefore, the time for BCA to grow and colonize the seed-adjacent soil and sunflower petals might not be sufficient. At least a 24 h period is recommended in between BCA and pathogen application (Zhang, 2004). In addition, the bacterial density on seeds might have not been adequate for such a high inoculum strength. Considering these factors, in 2007 the two experiments were designed so that the amount of millet seed inoculum added for each row in wilt trial was 5 g and the seeds were treated with bacterial suspensions with 10^{11} cfu/ml strength and 500 ml of bacterial suspension was added per row instead of 150 ml done in 2006. Only ascospores were sprayed on to flowers in the head rot trial so that the natural infection conditions are represented. In addition, ascospores of *S. sclerotiorum* were applied 24 h after BCA application. But due to practical difficulties in 2007, in the wilt trial millet seeds were used instead of sclerotia and they were applied into rows with seeds. An additional two treatments were included (PA23+BS6+Sclerotinia and PA23+BS6) to determine positive or negative interaction between PA23 and BS6 and their effect on wilt and head rot disease reduction ability of these strains.

As expected in the 2007 head rot trial, there was a reduction of DSI and AUDPC by both PA23 and BS6 over the inoculated control, however, the reduction was not statistically significant. However, as in 2006 the healthy control and other treatments which did not contain *Sclerotinia* application showed some degree of infection (DSI<40%). This may have been caused by natural sources of ascospores or the contamination between treatments due to high winds which prevailed during that particular period of the year. A previous study done at the same research station, Morden, MB in 2001 by Duncan (2003) showed that the natural head rot infection rates were as small as 7.5% and 15%. The wet weather in 2007 may have enhanced the apothecial production by sclerotia present in nearby fields thereby increasing the natural infection level. Interestingly, the PA23 and BS6 combined treatment showed significantly higher disease reduction than strains applied alone indicating possible synergistic effects between the two strains. Since both strains are capable of producing antibiotics (Zhang et al., 2006; Ramarathnam, 2007; data from current study - chapter 5), the possible mechanism of control might be antibiosis rather than competition. In addition, the fungicide treatment (Ronilan) also showed reduction (not significant) in DSI and AUDPC compared to inoculated control and sometimes the performance was better than that of the two bacterial strains. However, the biological and economical significance of such a small reduction in DSI (4.4%, 14% and 4.2%) and AUDPC (387.3, 339. 6 and 562.7), caused by PA23, BS6 and the fungicide is questionable since it was not able to increase the yield significantly in those treatments (Table 3.2). However, it is interesting that the density of the two strains on flowers was around log 2.8 – 3.6 (Figure 3.4) at the time of the application of *S. sclerotiorum*. This indicates that such a small density is enough for a

considerable control of the disease. However, the reduction in DSI and AUDPC values caused by PA23 and BS6 compared to inoculated control indicates the potential use of these strains against head rot disease in sunflower upon enhanced formulations and delivery systems which increase the survival and performance of BCA. Because of the hairy nature of the sunflower head, applied BCAs might have trapped on the hairs on the receptacle rather than on the surface. This might have blocked the BCAs from taking nutrients from the head surface and also PA23 from forming the biofilms, which is found to be important in biocontrol action. Therefore, most of the applied BCAs might have dried out on hairs and died making the biocontrol unsuccessful. A strong surfactant, which enhances the adherence of BCA to the surface, might be a possible solution for this. Plants all over the research plot including tagged ones suddenly became necrotic, dry and dead producing very small heads and thin stems, most probably due to the effect of another biotic or abiotic disease agent. This might have caused insignificant results in DSI, AUDPC and yield values. Even though the performance of PA23+BS6+Scl is relatively better than PA23+Scl and BS6+Scl, the total bacterial density (PA23 and BS6) on the flowers treated with PA23+BS6+Scl is more or less similar to that of PA23+Scl and BS6+Scl. This supports the finding of Zhang (2004), where a significant disease suppression of canola stem rot was observed under field condition, when the PA23 and BS6 populations were very low on the blossoms and strengthens her idea that induce systemic resistance (ISR) might be another mode of action. Although the weather was completely different between the two years, 2006 being dry and 2007 wet, disease development was not influenced by this since a misting system was used in both years after *S. sclerotiorum* inoculation. In contrast to the head rot trial in both years PA23 and

BS6 were not successful against sclerotinia wilt even though the inoculum density and BCA density were changed.

Both phyllosphere and rhizosphere are very complex environments, even though, the population and diversity of indigenous microflora as well as the fluctuation of physical and biochemical parameters differ between them. Among the abiotic and biotic factors that influence the production of antibiotics by a BCA on the rhizosphere are oxygen, temperature, specific carbon and nitrogen sources, and microelements (Duffy & Défago, 1997; Howie & Suslow, 1991; Ownley et al., 1992; Shanahan et al., 1992; Slininger & Jackson, 1992), plant host (Georgakopoulos et al., 1994; Maurhofer et al., 1995), the pathogen (Duffy & Défago, 1997), the indigenous microflora (Wood & Pierson, 1994), and the cell density (Raaijmakers et al., 2002) of the producing strain. Phyllosphere microbial residents have limited resources available (Mercier & Lindow, 1999; Hirano & Upper, 2000) and the survival depends on the ability of organisms to withstand varied environmental stress conditions, including fluctuating water availability, heat, osmotic stress, and exposure to solar UV radiation (UVR) (Janette & George, 2001). For example, biocontrol efficacy of nonpathogenic *Fusarium oxysporum* was significantly affected by light and temperature (Larkin & Fravel, 2002) and organic matter favored the pathogen and higher pH values favored the biocontrol of mycoparasites against *Rosellinia* (Garcia et al., 2003). Under laboratory conditions growth and antibiotic production of PA23 was observed to be highly variable depending on the temperature and other unknown factors (Carrie Selin, personal communication). Therefore, as introduced organisms to the phyllosphere and the rhizosphere, PA23 and BS6 might have been affected by variations in these factors which could have negatively affected survival and antibiotic production

thereby their biocontrol activity. However, the cell densities of PA23 and BS6 on roots of sunflower seedlings in the 2007 trial in Morden were around log 6.6 – 8.3, which is relatively high. When reisolated on some agar plates the typical bright orange colony color could not be observed in PA23. This indicates that the cell density of BCA was not the problem but may be the reduction in antibiotic production caused by various soil factors. The bacterial density of BS6 (log 8.3) on roots was relatively higher than that of PA23 (log 7.6) in sclerotinia inoculated treatments while in treatments without sclerotinia it was the other way around (Figure 3.1B). This may be due to the ability of BS6 to produce endospores which are resistant to adverse environmental conditions especially in the presence of an aggressive organism like *S. sclerotiorum*. In 2006, cell density might also have been a problem due to the very dry and hot soil conditions that prevailed during the growing season. However, this idea can not be confirmed since bacterial survival was not studied in 2006. Burr et al. (1978) reported that survival of fluorescent pseudomonads TL-3 on potato was low in soil at low soil water potentials. Furthermore, *S. sclerotiorum* was added to soil with seeds. Sclerotinia–infected millet seed inocula contain actively growing mycelia fragments of *S. sclerotiorum*, which could have started growth just after a few hours from application where BCA could not due to the competition exerted by aggressive growth of *S. sclerotiorum*. Therefore, by the time BCA start to proliferate the infection might already have taken place. The germination of sclerotia even on a nutrient rich media takes 2-3 days, which might be enough time for the BCA to proliferate and protect the growing roots and millet seeds do not mimic the natural process of infection of *S. sclerotiorum*. Therefore, if sclerotia were used or soil was treated with the BCAs prior to seeding the results might have been different from the current study. Simon

(1989) observed a 95% reduction of disease by *Trichoderma koningii* when the soil was inoculated with the pathogen and antagonist 2 weeks before sowing and it was only a 52% reduction in disease when soil was inoculated just before sowing. Ellis et al., (1999) found that the initial interactions in the soil were significant for disease suppression of damping-off by *P. fluorescens* 54/96. Furthermore, the number of surviving plants was not changed throughout the season from the initial emergence count which is a similar observation to that made by McLoughlin et al. (1992).

Although there were certain significant observations made in the 2007 Winnipeg and Morden growth promotion trials in some of the parameters tested, their economical significance is unconvincing. Especially, in the Winnipeg trial the significant increases in height, leaf width and area observed in the early growing season disappeared by the late growing season, which is the important phase in terms of yield. Thus, this study does not clarify the growth promoting ability of PA23 and BS6 in sunflower. In the Winnipeg trial, the bacterial density on roots was relatively lower (log 5 - 5.9) than that of Morden (log 6.2 – 7.3). The bacterial density should not be a problem to induce growth because unlike what is observed for biocontrol activity via antibiotics, proper colonization and coverage of root is not essential for the induction of growth promotion.

Furthermore, PA23 produces volatile antibiotics which can be more effective against sclerotia since they can easily penetrate through soil (Fernando et al., 2005). With BS6 around 100% *in vitro* inhibition of *S. sclerotiorum* mycelia and sclerotial germination was observed (data not shown) in volatile plate assays even though the identification of volatiles has not yet been done. Induction of systemic resistance and growth promotion by *B. amyloliquefaciens* IN937a and *B. subtilis* GB03 has been observed in *Arabidopsis*

(Ryu et al., 2003, 2004). Therefore, volatile antibiotics might play a role in growth promotion and also wilt control. However, 2007 was extremely wet during the growing season. Under wet soil conditions the volatiles would not be effective.

When all these factors are considered, it is clear that the performance of a BCA is governed by a number of factors such as the environment, the aggressiveness of the pathogen, and the physiology of the host. Weller et al. (1985) stated that in order to be effective, the strains should be isolated from the same environment that they will be used, however, the ecological niche for the pathogen and BCA can be different (Nakkeeran, et al., 2005). Many studies report that potential BCAs are sometimes totally ineffective at controlling plant diseases under field conditions (Duffy et al., 1996). The results of the wilt and growth promotion trials in the current study further support this idea. This study highlighted the need for effective formulations of PA23 and BS6 providing optimal conditions that support the survival and performance after their introduction. The fungicide Ronilan has the potential to be used as a seed treatment against sclerotinia wilt disease in sunflower.

CHAPTER 4

4.0 *Pseudomonas chlororaphis* Strain PA23 Antagonistic to Soil-Borne *Sclerotinia sclerotiorum* and the Role of Volatile and Non-Volatile Antibiotics Production in Its Root Colonization and Biocontrol Ability

4.1 Abstract

Pseudomonas chlororaphis strain PA23 has been a successful biocontrol agent in canola phyllosphere and found to produce the non-volatile antibiotics phenazine and pyrrolnitrin as well as the volatile antibiotics nonanal, benzothiazole and 2-ethyl–1-hexanol. Laboratory, gas chromatographic and mass spectrometric experiments were conducted to study the effect of different PA23-mutations on the production of 3 organic volatile antibiotics by PA23. Greenhouse experiments investigated the role of the non-volatile antibiotics on root colonization and biocontrol ability of PA23 using different phenazine and pyrrolnitrin deficient *Tn* mutants of PA23. In the first experiment, there were no differences observed among mutants in the production of the three volatile antibiotics, however, the differences in concentrations should be investigated. In the second experiment, each mutant was applied alone and in combination with wild type PA23 as a seed treatment. Introduced and total bacteria colonizing the roots were counted at 2, 4 and 6 weeks after seeding. There was a gradual reduction in the number of both introduced and total bacteria during the sampling period. On all sampling dates, PA23 applied alone or in combination with mutants showed significantly high (P=0.05) root bacterial number and decline of population seemed to be correlated with the number of

mutations on PA23 but not the type of mutation. However, by 6 weeks after seeding there was a rapid and significant (P=0.05) increase in the proportion of introduced bacteria which is capable of producing at least one antibiotic. PA23-314 showed a competitive colonization in comparison to other mutants in most sampling dates. Strain PA23 showed a significant sclerotinia disease reduction (P=0.05) than the pathogen-inoculated control, mutants and combined applications. However, the superior effect of phenazine over pyrrolnitrin or vise versa in root colonization or in controlling wilt was not clear. The antibiotic production by PA23 seemed to be triggered by the population density and nutrient availability in the rhizosphere. The results of this study highlight the need of further research into these objectives.

4.2 Introduction

Strains of *Pseudomonas* spp. especially fluorescent pseudomonads are among the most studied bacterial antagonists. When applied to planting material or soil, certain strains of pseudomonads have been proved to control soil-borne and air-borne plant pathogens (Cook & Rovira, 1976; Weller & Cook, 1986; Gamliel & Katan, 1993; Radja Commare et al., 2002; Zhang, 2004;), induce systemic resistance (Van Peer et al., 1991; Hoffland et al., 1996; Viswanathan & Samiyappan, 1999; Chen et al., 2000; Ramamoorthy et al., 2001) and enhance crop growth (Nandakumar et al., 2001; Vivekananthan et al., 2004). Antibiotics produced by these strains such as phenazine-1-carboxylic acid (PCA) (Delaney et al., 2001), 2,4-diacetylphloroglucinol (2,4-DAPG) (Nowak Thompson et al., 1994; Mavrodi et al., 2001), pyoluteorin (Plt) (de Souza &

Raaijmakers, 2003), pyrrolonitrin (Prn) (Chernin et al., 1996; Kirner et al., 1998), Oomycin A (Howie & Suslow, 1991), hydrogen cyanide (HCN) (Flaishman et al., 1996) etc. have been found to be contributing to the biocontrol ability of these *Pseudomonas* spp.

P. chlororaphis strain PA23, originally isolated from the root tips of soybean plants (Savchuk, 2002), is another *Pseudomonas* spp. that had been successful as a biocontrol agent of *S. sclerotiorum* (Lib.) de Bary both in greenhouse and field studies (Zhang 2004; Savchuk & Fernando 2004; Fernando et al., 2007). The production of non-volatile organic compounds such as PCA, 2-hydroxyphenazine and Prn (Zhang et al., 2006) and several additional metabolites including protease, lipase, HCN and siderophores (Poritsanos 2005) has been established. In addition, this bacterium produces three volatile organic compounds, including nonanal, benzothiazole and 2-ethyl–1-hexanol, which showed 100 per cent inhibition of mycelial and sclerotial germination of *S. sclerotiorum* (Fernando et al., 2005). Six mutants of PA23 have been isolated using transposon mutagenesis (Poritsanos et al, 2006). PA23-314 (mutation in *gacS*) and PA23-443 (mutation in LysR-type transcriptional regulatory gene *ptrA*) are no longer capable of inhibiting fungal growth and are deficient in the production of non-volatile antibiotics and other putative antifungal metabolites including HCN (Poritsanos et al, 2006). As expected, addition of the wild-type gene *in trans* restored the biocontrol ability of PA23-314 (*gacS*) (PA23-314 complemented strain) and PA23-443 (*ptrA*) (PA23-443 complemented strain). The ability of mutants PA23-63 (*phzE*-deficient) and PA23-754 (*phzC*-deficient) to inhibit fungal growth of *S. sclerotiorum* remained unchanged. The mutant PA23-1 produces phenazine but is deficient in producing

pyrrolnitrin, while the mutant 63-1 does not produce any of those two compounds (personal communication, R. Habibian).

When considering root borne diseases, colonization of root by the BCA is one of the major factors that influences the efficacy of the control of target plant pathogens. Successful colonization of rhizosphere by the introduced bacteria requires high level of adaptation and selective advantage over the indigenous microorganism (Mazzola et al., 1992). Antibiotics produced by the BCA are found to play an important role in this (Bruehl et al., 1969; Atlas & Bartha, 1987; Mazzola et al., 1992), although, this concept has been equivocal in several occasions (Gottlieb, 1976; Williams, 1982; Williams & Vickers, 1986). Antibiotic production is supported by the presence of carbon sources (Mazzola et al., 1992). The rhizosphere is the primary site of interaction among soil microorganisms and provides sufficient nutrients for the growth and antibiotic production of microbes. Antibiotic production is also strongly affected by the plant species and cultivars (Neal et al. 1973; Azad et al., 1985; Handelsman & Stabb, 1996). Kraus & Loper (1995) reported that induction of pyoluteorin biosynthesis varied with the plant species. The effect of the different antibiotics produced by BCAs on root colonization ability and the effect of different plant species on antibiotic production have been studied (Howie & Suslow, 1991; Thomashow et al., 1990; Mazzola et al., 1992). However, the involvement of bacterial antibiotics in the root colonization and thereby biocontrol of root diseases have not been studies for the sunflower host system. There is a need to study the colonization of BCA on sunflower roots and their effects on sclerotinia wilt which is a root disease to develop environmental-friendly management strategies for this disease. Understanding the genetic regulation of volatile antibiotic production is important when

controlling soil-borne diseases since volatiles can easily reach the pathogen and do not require direct contact with the pathogen like in the case of non-volatile antibiotics.

In this study we investigated the impact of different mutations on PA23 production of antimicrobial volatile compounds. In addition, the effect of phenazine and pyrrolnitrin on root colonization and biocontrol ability of *P. chlororaphis* PA23 was also investigated. This study is important for understanding how volatile organic antimicrobial compounds are regulated and what impact the production of particular antibiotics has on the rhizosphere colonization ability of PA23. Furthermore, better understanding of the gene regulation of biocontrol mechanisms and population dynamics will facilitate the development of rational strategies of application of antagonists and gathering desirable characteristics through genetic engineering.

4.3 Materials and Methods

4.3.1 Bacterial Strains, Fungal Strain and Culture Conditions

New cultures of all bacterial strains were started from stock cultures maintained in Luria Bertani both (LBB) amended with 20% glycerol at –80 °C by streaking the bacteria onto Luria Bertani agar ((LBA) Difco Laboratories, Detroit, MI) amended with appropriate antibiotics for each mutant (Table 4.1). The fungal strain *S. sclerotiorum* SS33 was used in all the experiments. Fresh cultures were started from surface sterilized sclerotia on potato dextrose agar ((PDA) Difco Laboratories, Detroit, MI) and incubated at room temperature.

4.3.2 Laboratory Experiment

Laboratory experiments were carried out with strains PA23, PA23-314, PA23-314 *gacS*, PA23-63, PA23-754, PA23-443 and PA23-443 *ptrA* according to the procedures described by Fernando et al., 2005 with slight modifications.

Table 4.1. List of bacterial strains used in the study and type and amount of antibiotics added for each strain.

Strain Name		Antibiotic: amount (μg/mL)
PA23		Rifampicin: 25
PA23-314		Tetracycline: 15
PA23-314	*gacS*	Gentamycin: 20
PA23-63		Tetracycline: 15
PA23-754		Tetracycline: 15
PA23-443		Tetracycline: 15
PA23-443	*ptrA*	Gentamycin: 20
PA23-1		Gentamycin: 20
PA23-63-1		Gentamycin:10 and Tetracyclin: 10

4.3.2.1 Effect of Bacterial Volatiles on Mycelial Growth of *S. sclerotiorum* (divided plate method)

The bacteria were streaked on to one half of the divided plate containing tryptic soy agar ((TSA) Difco Laboratories, Detroit, MI) amended with appropriate antibiotics. The plate was immediately wrapped with Parafilm® (Pechinery Plastic Packaging, Menasha, WI) to trap the volatiles produced by bacteria and incubated for 5 days at 28 °C. Following the incubation period a 5mm mycelial plug of *S. sclerotiorum* was placed

on the other half of the divided plate containing PDA and the plates were resealed. Measurements of radial mycelial growth were taken after 24 h intervals post-inoculation for 5 days. The percentage inhibition (PI) was calculated using the formula, ((Radial mycelial growth in treatment-Radial mycelial growth in control)/Radial mycelial growth in control) X 100, when the mycelia reached the periphery just opposite to the bacterial culture in the control plate. There were ten replicates for each treatment and the experiment was repeated twice.

4.3.2.2 Effect of Bacterial Volatiles on Sclerotial Germination of *S. sclerotiorum*

The method previously described was used except that following the 5 d bacterial incubation period cut sclerotia (approximately 2mm in diameter) were placed on the other half of the divided plate containing PDA. The sclerotia were placed on a PDA plate to start germination prior to placing them on PDA in the divided plate to ensure that all the used sclerotia were viable. There were ten replicates for each treatment and the experiment was repeated twice.

4.3.2.3 Effect of Bacterial Volatiles on Ascospore Germination of *S. sclerotiorum*

A twenty microliter aliquot of ascospore suspension (5 X 10^4 spores/mL of 0.1 M phosphate buffer pH 7.0) was placed on a cavity slide. The slide was then placed inside the bottom dish of a sterile Petri plate. Another bottom dish containing 24 h old bacterial culture on TSA was inverted on the dish containing the cavity slide and the two dishes were sealed together using parafilm. After 24 and 48 h of incubation at room temperature

the slides were observed for spore germination under a microscope. Ten microscopic fields were selected (10 x 45x) and in each field the number of germinated spores was counted out of 10 selected ascospores. The percentage inhibition of spore germination was calculated compared to the control. There were four replicates for each bacterium and the experiment was repeated once.

4.3.2.4 Collection of Volatile Organic Compounds

Headspace volatiles produced by each bacterium were collected using a setup described by DeMilo et al. (1996) with slight modifications. Bacteria were grown in 100ml of tryptic soy broth ((TSB) Difco Laboratories, Detroit, MI) and M9 minimal medium per litre: (16 g $NaHPO_4$-$7H_2O$, 3.75 g KH_2PO_4, 0.625 g NaCl, 1.25 g NH_4Cl, 0.24 g 1M $MgSO_4$, 0.01 g 1M $CaCl_2$ and 20 ml of 20% glucose as the carbon source) for 5 days and the headspace volatiles were collected into a volatile trap (7cm length and 0.4 cm diam) containing 150 mg of activated charcoal (Darco®. 20-40 mesh, Aldrich, Milwaukee, WI) by using a stream of dry nitrogen (300 ml/min) for 24-48 h. Volatiles in the trap were extracted into glass vials with 500µL of methylene chloride and analysed through gas chromatography and mass spectrometry.

4.3.2.5 GC-MS Analysis of Volatiles

Gas chromatography was performed in the Department of Chemistry, University of Manitoba, Winnipeg using a Varian Star 3400 CX series GC with a flame ionization

detector. The Column was a 15 m DB-1 megabore column of 100% dimethylpolysiloxane (Shojania et al., 1999). The absorption of the trap contents was done at 22 °C from a unijector (SGE) fused on to a silica column (BPI, 25 m length X 0.22 mm i.d., 0.25 μm film thickness). The silica column was cryogenically focused with dry ice and acetone for 2 min. The detector was a Hewlett-Packard mass selective detector, which was attached to the Hewlett-Packard 5890 Gas chromatograph. The carrier gas was helium which was maintained at a rate of 1 ml/min. Column temperature was programmed from 35 to 200 °C at 10 °C/min ramp rate. The mass spectra of the compounds observed in the samples were identified by comparing them with those in the NIST/EPA/NIH Mass Spec. Library (Version 2.0). The collection and GS-MS analysis of organic volatiles was repeated once with both types of media.

4.3.3 Growth Room Experiment

4.3.3.1 Root Colonization Study

Two separate experiments, which were carried out simultaneously, were set up to understand the relative importance of phenazine and pyrrolnitrin on the root colonization ability of PA23. In the first experiment, the *gacS*-deficient and complemented strains were used while in the second experiment the *phzE*-deficient, *prnB-C* deficient and the *phzE/prnB-C* double mutant were used. The experiment was carried out in a standard 2:1:1 soil mix: 2 parts soil, 1 part sand, 1 part peat. Ten pots were used per treatment and each experiment was repeated twice. The first experiment included the following: (i) control (no bacteria) (ii) PA23 (iii) PA23-314 (iv) PA23-314 *gacS* (v) PA23+PA23 314

(vi) PA23+PA23-314 *gacS*. The second experiment included the following: (i) control (no bacteria) (ii) PA23 (iii) PA23-63 (*phz*$^-$) (iv) PA23-1 (*prn*$^-$) (v) PA23-63-1 (*phz*$^-$, *prn*$^-$) (vi) PA23+PA23-63 (vii) PA23+PA23-1 (viii) PA23+PA23-63-1. Sunflower seeds were surface sterilized with diluted NaOCl (4:1; Chlorox) for 3 min followed by three washings with sterile distilled water. Seeds were air dried overnight under a sterile air flow. Seeds were then soaked in bacterial suspensions (10^8 CFU/ml) in 1% methylcellulose (Sigma®, Sigma Chemical Co., St. Louis, MO, USA) in 0.1 M sodium phosphate buffer (pH 7.0) for 1 h and air dried overnight under a sterile air flow. The initial bacterial count on seeds was determined using a standard dilution plate method (10 seeds suspended in 100 ml sterile sodium phosphate buffer pH 7.0). One seed was planted per pot to a depth of 1.5 cm and pots were placed in the growth room for 10 weeks at 21 °C and 19 °C (day and night temperatures with 16 h photoperiod) and watered daily. Root bacterial counts were determined every two weeks for 6 weeks starting from the 14th day after planting. Two seedlings were collected randomly from each treatment. The seedlings were shaken gently to remove soil so that only the most tightly adhering rhizosphere soil remained. Roots from both seedlings were pooled and standard dilution plating was performed with 0.1 g of root (at seedling stages) and 1g of root (at mature stages) by suspending the roots in 10 mL of sterile distilled water. Root suspension was sonicated for 60 s followed by vortexing for 5 s. For each treatment dilutions were plated on nutrient agar (Difco Laboratories, Detroit, MI) amended with appropriate antibiotics to get the introduced bacterial count and then on nutrient agar without antibiotics to get the total bacterial count on roots.

4.3.3.2 Early Wilt Disease Incidence

The growth conditions in this experiment are the same as in the root colonization study and were carried out simultaneously. There were two different experiments as above. For the fungicide, the seeds were mixed with slightly wetted fungicide (4g/1 kg of seeds) and air dried for overnight. The bacterial strains used were the same and seeds were treated in the same way as previously described. The only difference is that the pots were filled ¾ with soil mix and each pot was sprinkled with 1g of sclerotinia-infected pearl millet seeds, which mimicked sclerotia, prior to seeding. One seed was placed on the millet seed sprinkled soil surface and was covered with soil mix to a thickness of 1.5 cm. Each treatment was composed of 10 pots. Pots were placed in a growth room for 3 weeks and watered daily. The percent emergence of the seedlings was determined at 14 days after planting for both experiments. The whole experiment was repeated twice.

4.3.4 Data Analysis

Data was analyzed using analysis of variance (ANOVA) and Fisher's Least Significant Difference test at P=0.05 using the Analyst procedure of SAS, Version 8.1(SAS Institute, Carry, NC, USA).

4.4 Results

4.4.1 Laboratory Experiment

4.4.1.1 Effects of Bacterial Volatiles on Mycelial Growth of *S. sclerotiorum*

Seventy two hours post inoculation of the mycelial plugs, mutants PA23-314 *gacS*, PA23-63, PA23-754, PA23-443 *ptrA* showed a significant (P=0.05) inhibition in mycelial growth of *S. sclerotiorum* compared to control. PA23-754 exhibited the best inhibition at 25.56% (Figure 4.1 & 4.2). The mutants PA23-314, PA23-314 pUCP23 and PA23-443 did not show any inhibitory effect. However, the inhibition done by PA23-443, PA23-63 and PA23-754 is greater than that done by PA23-314. These results are comparable with those of studies that have been done with radial diffusion assay using these mutants (Poritsanos et al, 2006). Control plates showed normal mycelial growth.

3.4.1.2 Effect of Bacterial Volatiles on Sclerotial Germination of *S. sclerotiorum*

Mutants PA23-314 *gacS*, PA23-63, PA23-754, PA23-443 *ptrA* showed a significant (P=0.05) inhibition of sclerotial germination of *S. sclerotiorum* compared to control 72 h post inoculation of sclerotia. Similar to mycelial inhibition study, PA23-754 showed the best inhibition having 98.73% of percentage inhibition of sclerotial germination (Figure 4.1 & 4.2). In contrast to the mycelial inhibition study, the strains PA23-314, PA23-314 pUCP23 and PA23-443 showed a considerable amount of inhibition of sclerotial germination, which was not statistically significant (P=0.05) when compared to control and other inhibitory strains. However, the inhibition done by PA23-443, PA23-63 and

PA23-754 is greater than that done by PA23-314. The inhibition showed by strain PA23-314 and PA23-314 pUCP23 is more or less similar and also statistically not different. This indicates that there is no effect of the plasmid inserted into PA23-314 on its biocontrol behavior and it is not needed to include the strain having the plasmid and strain not having the plasmid into further experimentations. In general, all strains exhibited better inhibition of sclerotial germination than mycelial growth of *S. sclerotiorum*.

4.4.1.3 Effect of Bacterial Volatiles on Ascospore Germination of *S. sclerotiorum*

Except for strains PA23-314 and PA23-314 pUCP23, all other mutants completely (100%) inhibited ascospore germination of *S. sclerotiorum* after 48 h of incubation at room temperature (Figure 4.1). PA23-314 and PA23-314 pUCP23 showed 28.25% and 31% of ascospore inhibition, which were significantly different (P=0.05) from other treatments and the control.

4.4.1.4 GC-MS Analysis of Volatiles

The chromatograms obtained from each strain revealed that all mutants produce the three organic volatile compounds, nonanal, benzothiazole, and 2-ethyl-1-hexanol, as wild type (PA23) (Figure 4.3) regardless of the medium used.

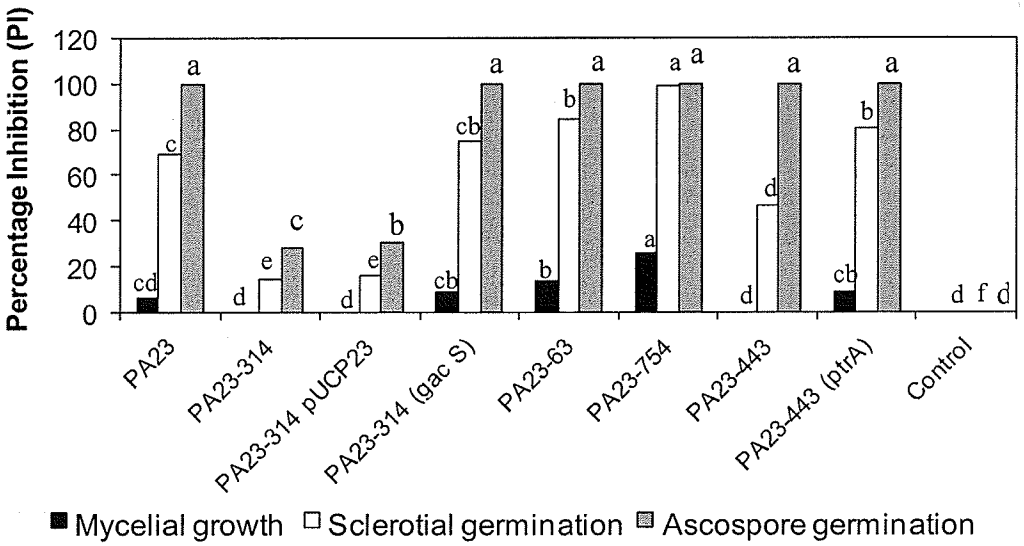


Figure 4.1. Percentage inhibition (PI) shown by each bacterial strain against mycelial growth, sclerotial germination and ascospore germination. Mycelial plugs and half cut partially germinated sclerotia were inoculated onto the other half of the divided Petri plate containing PDA after 5 days of incubation of each bacterium at 28 °C. Ascospores of *S. sclerotiorum* were introduced into the experimental set up after 24 h of incubation of each bacterium at 28 °C. The graph shows the PI of mycelial growth and sclerotial germination 72 h post inoculation of *S. sclerotiorum* and PI of ascospore germination 48 h post inoculation of ascospores of *S. scleotiorum.* Series of bars sharing a single colour followed by the same letter are not significantly different, P=0.05, using the Fisher's least significant difference test. LSD: ■ 6.498 □ 10.767 ▨ 2.71.

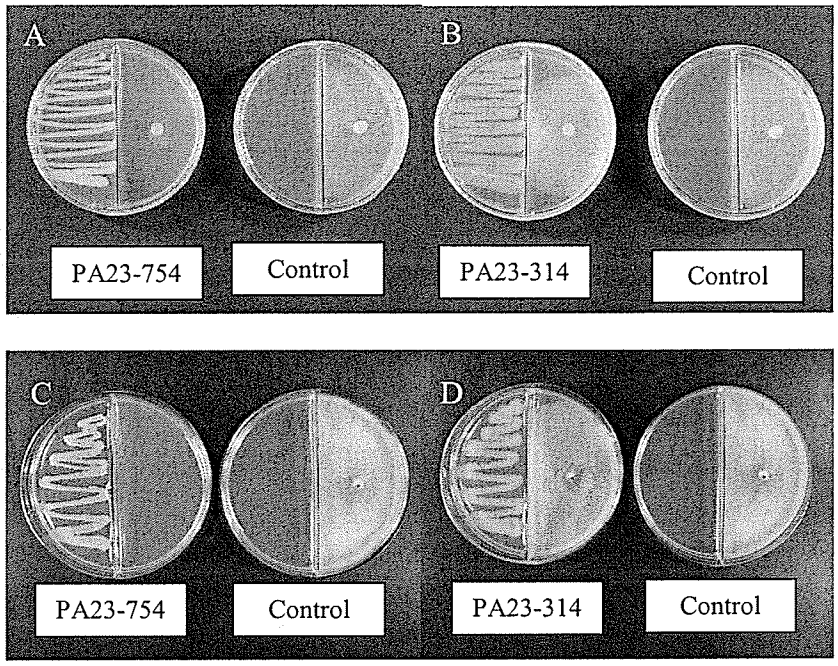


Figure 4.2. Antifungal-volatile activity in divided plates 72 h post inoculation of mycelial plugs and sclerotia. (A) Mycelial growth inhibition in the presence of PA23-754. (B) Normal mycelial growth in the presence of PA23-314. (C) Sclerotial germination inhibition in the presence of PA23-754. (D) Normal sclerotial germination in the presence of PA23-314. Mycelial plugs and half cut partially germinated sclerotia were inoculated on to other half of the divided Petri plate containing PDA after 5 days of incubation of each bacterium at 28 °C.

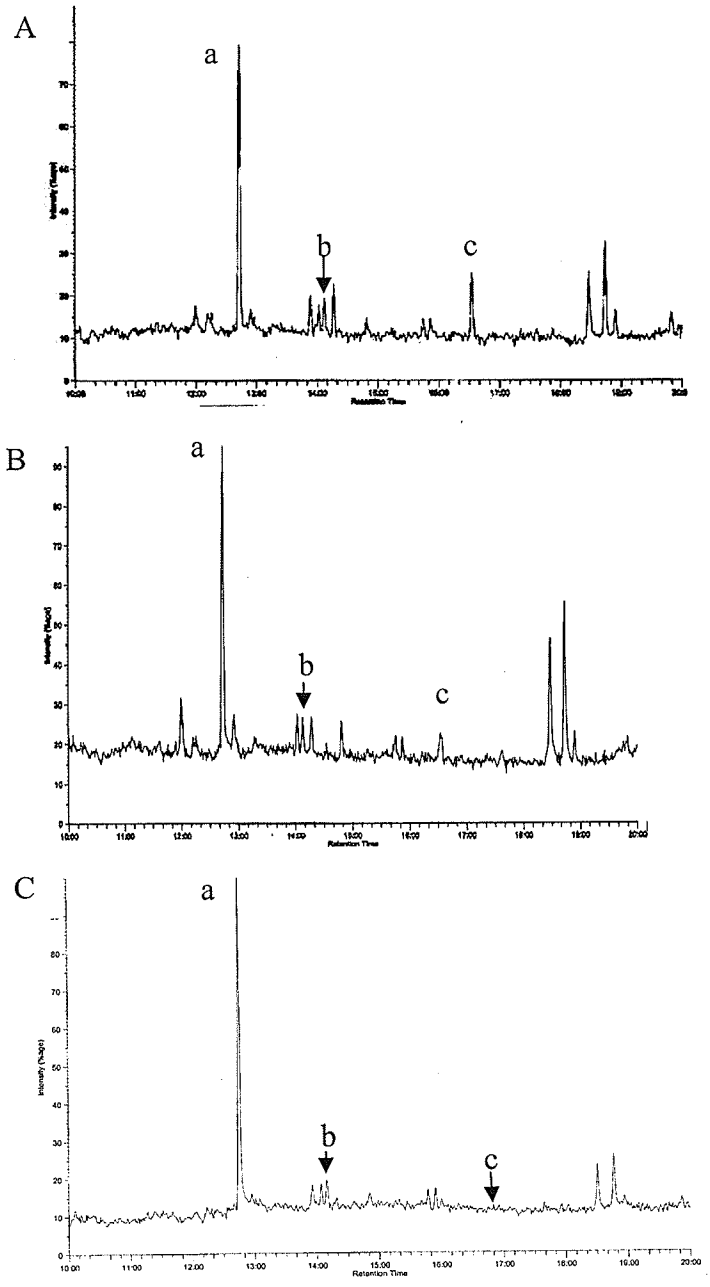


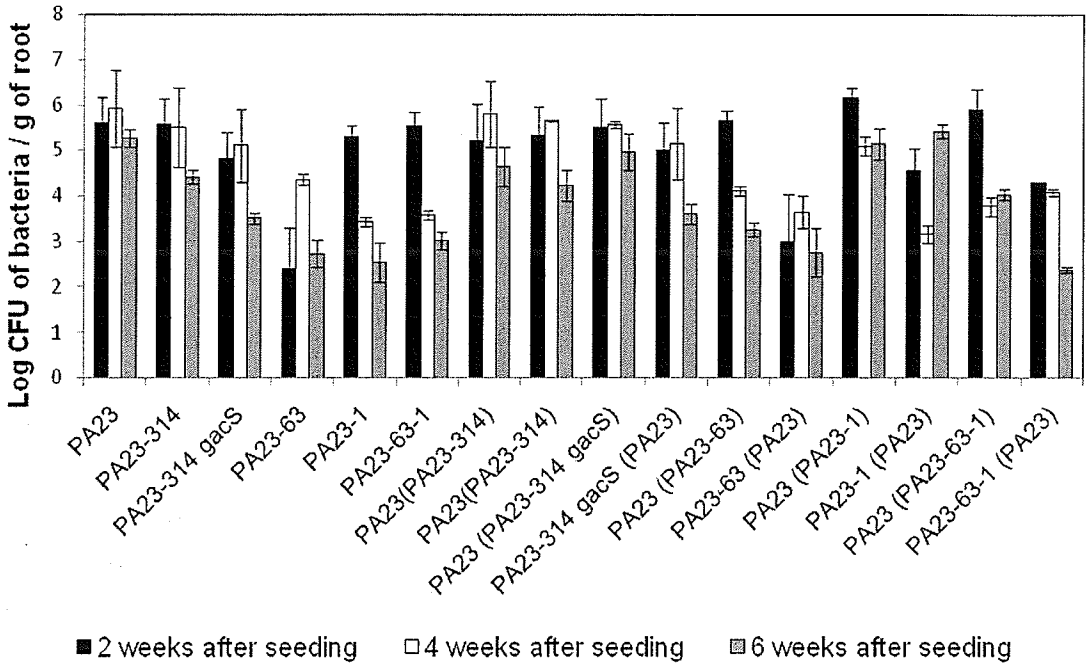
Figure 4.3. Chromatograms of volatiles collected from PA23 (A) and PA23-314 (B) (C) PA23-314-g*acS* in M9 medium; (a) 2-ethyl-1-hexanol, (b) nonanal, (c) benzothiozole. Volatiles were trapped into activated charcoal and extracted with mehthyl chloride and subjected to GC-MS analysis. X axis represents the retention time, Y axis represents the peak intensity (percentage).

4.4.2 Growth Room Experiment

4.4.2.1 Introduced Bacterial Count

Although two separate experiments were carried out for two different mutation systems, the results were analyzed by considering them as one experiment since they were done simultaneously under similar growth conditions. Results of each sampling date were analyzed separately. Initial bacterial density on seeds was not significantly different (P=0.05) among strains and ranged from log 5.6 to log 4.46 (data not shown). However, the highest density was shown by PA23-1 strain applied alone while the lowest density was shown by PA23- 63-1 strain applied in combination with PA23. In general, wild type exhibits better colonization on seeds whether it was applied alone or in combination with other strains and strains that can produce at least one antibiotic from phenazine and pyrrolnitrin maintained a comparatively higher bacterial number above log 5, except PA23-314 *gacS*, PA23-63 and PA23-1 both applied in combination with PA23. Despite the fact that in combined applications both the PA23 and mutant applied in same concentration, it was observed that most of the time mutants shows slightly poor colonization than PA23. Especially, strain PA23-1 showed the highest bacterial number when applied alone while it was the third lowest in colonization when applied in combination with PA23.

Two weeks after seeding the bacterial numbers on root ranged from log 6.17 to log 2.39 and there was a significant difference (P=0.05) between bacterial numbers shown by different strains (Figure 4.4). Interestingly, PA23 63 (producing pyrrolnitrin but phenazine) showed the lowest bacterial number. Interestingly, PA23-314 which is



	2 wks	4 wks	6 wks
PA23	ab	a	ab
PA23-314	ab	c	bcde
PA23-314 *gacS*	ab	d	efgh
PA23-63	b	e	ghi
PA23-1	ab	h	hi
PA23-63-1	ab	h	ghi
PA23(PA23-314)	ab	ab	abcd
PA23-314(PA23)	ab	bc	cdef

	2 wks	4 wks	6 wks
PA23(PA23-314 *gacS*)	ab	c	abcd
PA23-314 *gacS*(PA23)	ab	d	efg
PA23 (PA23-63)	ab	f	fghi
PA23-63 (PA23)	ab	g	ghi
PA23(PA23-1)	a	d	abc
PA23-1 (PA23)	ab	i	a
PA23 (PA23-63-1)	ab	g	def
PA23-63-1 (PA23)	ab	f	i

Figure 4.4. Number of colony forming units of introduced bacteria on 1 g of seminal roots of sunflower plants treated with different kinds of bacterial strains or strain combinations over a period of 6 weeks from seeding. The bacteria were isolated in NA supplemented with specific antibiotics for a particular bacterial strain. The table shows the mean comparison according to Fisher's least significant difference test. Treatments followed by the same letter are not significantly different at P=0.05 significant level. Error bars represent the standard error of mean (SEM) of log CFU of bacteria / g of root LSD: ■ 3.7472 □ 0.2039 ▩ 0.9921.

phenazine deficient and PA23-63-1, which is both phenazine and pyrrolnitrin deficient, showed relatively high bacterial numbers, log 5.57 and log 5.54 respectively (Figure 4.4). PA23-314 *gacS* (producing phenazine) applied alone and with PA23 showed comparatively low bacterial numbers. The wild type maintained relatively the same numbers as initially, where that applied either alone or in combination with mutants had bacterial numbers relatively higher than others. The high bacterial numbers shown by strains producing at least one antibiotic was not consistent among the strains as observed in initial colonization.

Four weeks after seeding, there was a significant difference between treatments (Figure 4.4). As observed, wild type strain had the highest bacterial count, which was significantly different from all other treatments. PA23-314 had been able to maintain its root colonization almost at the same level. An increase in bacterial number in the strains PA23, PA23-314 *gacS* was observed by fourth week. A similar trend was observed in strains PA23 applied with PA23-314 and PA23-314 applied with PA23 as well as in PA23-314 *gacS* applied with PA23. In PA23-314 and PA23 applied with PA23-314 *gacS* the bacterial numbers maintained at a level more or less similar to that of in week 2. In other strains the bacterial numbers were lower than in week 2. There are some statistical comparisons that are noteworthy. There was no significant difference between bacterial numbers of PA23 applied with PA23-314 *gacS* and PA23-314. Similarly bacterial numbers of PA23-314 *gacS* applied with PA23 and PA23-314 *gacS* applied alone as well as that of PA23-1 applied with PA23 are not statistically different. PA23-314 showed a competitive colonization of root more or less similar to that of PA23 and PA23-314 *gacS* (Figure 4.4).

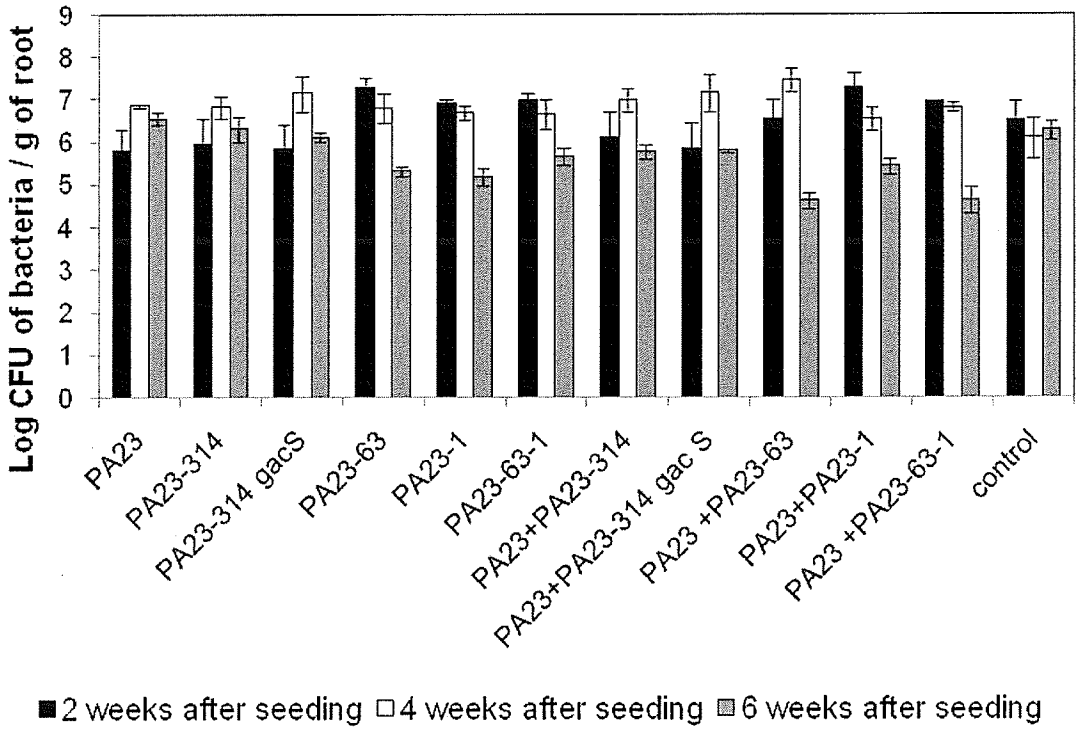
Six weeks after seeding, there were a number of statistically different groups that could be observed among the strains (Figure 4.4). However, a similar trend was observed as in week 2 and 4 where PA23 showed a high bacterial number regardless of combinations and PA23-314 showed a relatively higher number as well. Even though, PA23-314 *gacS* and PA23-63 produce antibiotics the numbers had drastically decreased to log 3 and 2 respectively. As observed in most sampling dates, PA23-63-1 applied with PA23 showed the lowest colonization which was significantly different from all other treatments.

In general, the results among different sampling dates are quite variable, however, there are some remarkable trends that can be observed. Whether it is alone or combined, PA23 wild type strain showed relatively higher root colonization compared to others. In most of the dates, PA23-314 seemed to have a superior colonization, which on occasion was comparable to wild type and its complemented strain (Figure 4.4). Colonization patterns of other mutants were not consistent over the sampling dates In most of the sampling dates PA23-63-1 applied with PA23 showed the lowest level of root colonization (Figure 4.4). In most strains the bacterial counts increased from initial colonization up to 2 weeks and started to decrease by week 4 having very low bacterial counts by week 6 compared to initial bacterial counts on seeds. In other strains, the bacterial numbers continuously increased up to 4 weeks and showed drastic reduction by week 6.

4.4.2.2 Total Bacterial Count

Similar to introduced bacterial count the results were analyzed by considering two separate experiments as one since they were done simultaneously under similar growth conditions. Results of each sampling date were analyzed separately. There was no significant difference (P=0.05) among bacterial counts among introduced strains 2 weeks after seeding. Bacterial numbers on root varied from log 7.29 to log 5.79 (Figure 4.5). Total bacterial count on roots treated with PA23 was the lowest. Four weeks after seeding, the bacterial numbers on roots varied from 7.46 to 6.1 with roots treated with PA23+PA23-63 showing the highest total bacterial count (Figure 4.5). Control treatment with no bacteria introduced had the lowest count. In the period from 2 to 4 weeks the bacterial numbers had slightly increased on roots treated with PA23, PA23-314 *gacS*, PA23+ PA23-314 *gacS* and PA23+PA23-63, while on those treated with PA23-63 and PA23+PA23-1 the numbers had decreased (Figure 4.5). Other strains maintained comparatively equivalent numbers. However, there was no significant difference observed among treatments by 4^{th} week.

Except in the control treatment, in all other treatments bacterial counts had reduced by 6^{th} week when compared to numbers in 4^{th} week. In PA23-314 *gacS*, PA23+ PA23-314 *gacS*, PA23+PA23-63 and PA23+PA23-63-1 there was a strong reduction in bacterial numbers while in others the drop was not remarkable (Figure 4.5). Over the sampling period, variation pattern shown by total bacterial count followed that of introduced bacteria whether or not a particular strain produces one or more antibiotics or no antibiotics.



	2 wks	4 wks	6 wks
PA23	a	ab	a
PA23-314	a	ab	ab
PA23-314 *gacS*	a	ab	bc
PA23-63	a	ab	ef
PA23-1	a	ab	f
PA23-63-1	a	ab	de

	2 wks	4 wks	6 wks
PA23+PA23-314	a	ab	cd
PA23+PA23-314*gacS*	a	ab	cd
PA23+PA23-63	a	a	g
PA23+PA23-1	a	ab	def
PA23+PA23-63-1	a	ab	g
Control	a	b	ab

Figure 4.5. Number of colony forming units of total bacteria (introduced and indigenous) on 1 g of seminal roots of sunflower plants treated with different kinds of bacterial strains or strain combinations over a period of 6 weeks from seeding. The bacteria were isolated on NA without any antibiotic added. The table shows the mean comparison according to Fisher's least significant difference test. Treatments followed by the same letter are not significantly different at P=0.05 significant level. Error bars represent the standard error of mean (SEM) of log CFU of bacteria / g of root. LSD: ■1.5101 □1.0964 ▨0.405.

4.4.2.3 Introduced Bacteria as a Percentage of Total Rhizosphere Bacteria

In all three sampling days, PA23 applied alone or in combination showed comparatively higher percentages. And in general PA23-314 also showed high percentages (Figure 4.6). In the 2nd week most of the strains showed their highest percentages. In the 2nd week the strains which represented above 25% of the total population were PA23 applied alone or in combination including PA23-314. Most of the PA23 containing groups and PA23-314 applied with PA23 showed percentages between 10-25 of total rhizosphere population. Contribution of other introduced strains for the rhizosphere community is very low, PA23-63-1 combined with PA23 having the lowest as 0.2%. Even though, the percentage of all strains considerably dropped by 4th week, the same trend was observed as in the 2nd week. By the 6th week, most of the strains that produce at least one antibiotic (Figure 4.6) had started to increase their numbers noticeably except the strains PA2-314 *gacS*, PA23-63 and PA23-314 *gacS* applied with PA23. However, PA23-1 (applied with PA23), which does not produce pyrrolnitrin also showed a notable increase in its percentage by the 6th week.

4.4.2.4 *Sclerotinia* Wilt Disease Assessment

The results of the three separate experiments were pooled and analyzed together. The percentage seedling emergence was calculated. Percentage seedling emergence is inversely proportional to early wilt disease incidence. Percentage seedling emergence shown by the treatments PA23 is significantly higher than all other bacterial treatments

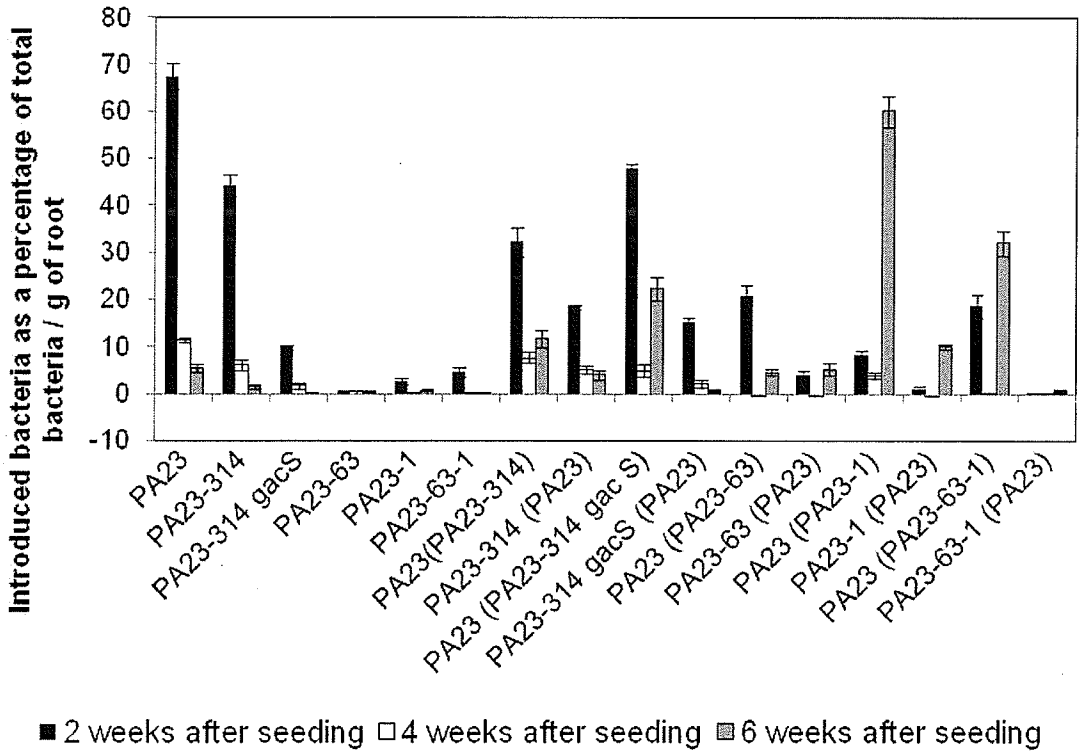


Figure 4.6. Introduced bacteria isolated from 1 g of roots treated with each strain as a percentage of the total bacteria isolated from 1 g of roots treated with each treatment. Appropriate dilutions were plated onto NA plates supplemented with particular antibiotics assigned for each strain to get the introduced bacterial density and onto NA plates without antibiotics to get the total bacterial count. LSD: ■ 35.754 □ 5.2791 ▩ 30.952.

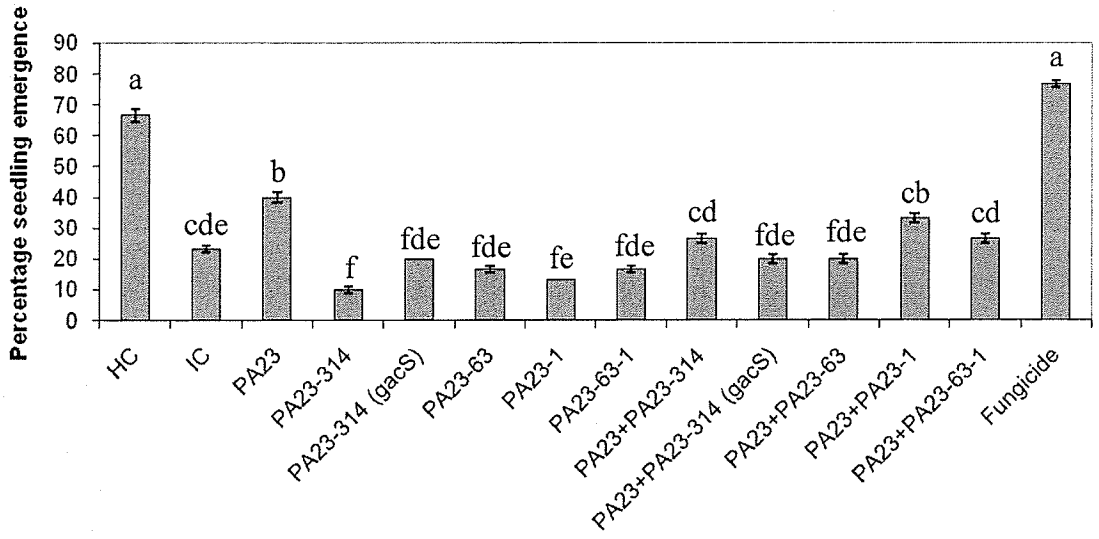


Figure 4.7. Percentage seedling emergence shown by sunflower seeds treated with different bacteria or bacterial combinations 2 weeks after seeding. Seeds were treated with 10^8 CFU/ml bacterial suspensions prepared in 1 % methycellulose in 0.1 M phosphate buffer (pH 7.0). Bars followed by the same letters are not significantly different, P=0.05, using the Fisher's least significant difference. LSD: 0.1194.

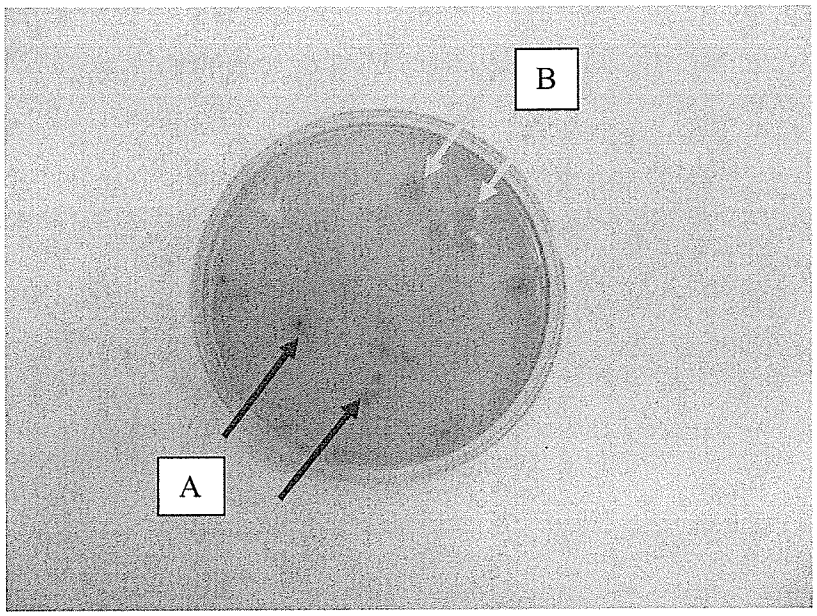


Figure 4.8. Colonies of PA23 isolated from 6-week old sunflower roots grown under growth room conditions. (A) Colonies with the typical bright orange color of PA23, however the color is restricted only to the middle area of the colony. (B) Colonies that do not show orange color typical to PA23.

including the inoculated control except that of PA23+PA23-1 (Figure 4.7). Fungicide (Ronilan) treatments showed the highest percentage emergence, which was significant in comparison to all other treatments except the healthy control. All other treatments exhibited a lower percentage seedling emergence than the inoculated control (Figure 4.7). The lowest seedling emergence was observed in the treatment PA23-314. The differences of percentage emergence between all other treatments in comparison to the inoculated control were not significant.

4.5 Discussion

In controlling soil-borne plant pathogens using biocontrol agents, their ability to establish in the rhizosphere at high levels for a long period of time is considered an important factor (Thomas et al., 2003). Successful root colonization acts as an efficient delivery system of antifungal metabolites, both non-volatile and volatile, along the root system (Thomas et al., 2003). Understanding of traits governing root colonization and antibiotic production by BCA and genes responsible for those traits is very important when studying the molecular mechanisms of biocontrol (Suslow, 1982; Lugtenberg et al., 1991; Bloemberg & Lugtenberg, 2001). Furthermore, volatile inhibitory compounds produced by a BCA are very important especially in controlling soilborne pathogens such as *S. sclerotiorum* that produce very resistant survival structures (sclerotia) in soil, since volatiles can diffuse towards pathogens and kill them.

In this study we tried to understand the involvement of the different genes in the inhibitory volatile production of the BCA *P. chlororaphis* PA23 and the contribution of

the different non-volatile antibiotics produced by PA23 to its root colonization ability and biocontrol ability in the rhizosphere.

The first experiment showed that the mutants that do not produce at least one of the antibiotics were not capable of inhibiting mycelial, sclerotial and ascospore germination significantly (Figure 4.1). This means that the particular mutated gene(s) might be responsible for this inhibition. The GC-MS analysis that was carried out to check this hypothesis showed that all mutants can produce the 3 volatiles; nonanal, benzothiazole and 2-ethyl–1-hexanol, like the wild type strain PA23. Therefore, there may be other organic or/and inorganic volatiles that were not detected from this method and most probably there may be concentration differences between the volatiles produced by the wild type and the mutants that might have resulted in the *in vitro* mycelial, sclerotial and ascospore inhibition observed. The method used in the current study may not have been the most appropriate one to compare the peak intensities due to their limitations associated with the sample collection method. Methyl chloride was used to extract the volatiles from the charcoal. Depending on the time period that each sample sat on the counter or in the fridge prior to injection into column, the concentration of the volatiles in samples can vary due to the highly volatile nature of methyl chloride even inside a tightly closed vial (Wayne Buchannon, personal communication). A method called Solid Phase Microextraction (SPME), a solvent free technology, is most appropriate in this regard, where it allows quantifying very small quantities of volatiles produced by organisms. In addition, the current study showed that the *S. sclerotiorum* inhibition shown by PA23-314 (*gacS* mutant) is significantly very lower than that was shown by PA23-443 (*ptrA* mutant). Based on this observation, it can also be suggested

that the production of organic volatiles in PA23 might be controlled somewhere in between GacS/GacA and ptrA gene in the secondary metabolite production regulatory pathway of PA23. However, apart from the above possibilities, HCN may have played an important role in the observations of the *in vitro* study. In a previous study, it has been shown that the HCN production is inhibited in PA23-314 and PA23-443 while in the others it remains or is restored (Poritsanos, 2005; Carrie Selin, personal communication). Therefore, the significant inhibition of *S. sclerotiorum* shown by other strains might be a result of accumulation of HCN in the headspace of the Petri plate. This is further supported by the fact that the strains or strain combinations where the above genes have been restored by complementation showed similar inhibition as the wild type. Castric (1983), Laville *et al (*1998) and Blumer & Hass (2000) found that the HCN production in *P. aeruginosa* and *P. fluorescens* cultures is optimally induced under oxygen limiting conditions. In the sealed Petri plate might have been the ideal condition since the growth of the bacteria reduce the oxygen level in the headspace enhancing the HCN production. However, GC-MS analysis did not show HCN. HCN has a very low molecular weight (50 Da) compared to other compounds. It might have eluted very early during the sampling period most probably with the methyl chloride and might have been masked (Wayne Buchannon, personal communication). To our knowledge this is the first time that a study has attempted to look at the genes involved in the production of the organic volatile organic antibiotics produced by antagonistic bacteria. However, there is research that has attempted to understand the regulation of HCN synthesis in *Pseudomonas* spp. HCN synthesis in *P. fluorescens* CHA0 was found to be encoded by the *hcnABC* gene cluster (Laville et al., 1998). Two regulatory proteins: the global activator GacA and the

anaerobic regulator ANR, have been demonstrated to control cyanogenesis in this bacterium (Laville et al.,1992, 1998) as well as in *P. aeruginosa* (Reimmann et al., 1997; Zimmermann et al., 1991). The "Gac" stands for the "global activation of cyanide synthesis". GacA, the response regulator of the two-component regulatory system GacS/GacA, has been found to be required for the synthesis of HCN in strain CHA0 (Laville et al., 1992; Sacherer et al., 1994). Blumer & Hass (2000) also support this idea that "cyanogenesis is sequentially activated by ANR at the level of transcription and by components of the GacA network at the level of translation".

These results suggest that none of the above genes tested is responsible for the production of three organic volatiles produced by PA23. The biochemical pathway of the production of organic volatiles, therefore, might be independent of that of organic non-volatile antibiotics. This is evident because HCN production is dependent on the biochemical pathway of non-volatile organic antibiotics since HCN is released as a product of secondary metabolism. Most of the antibiotics produced by bacterial antagonists are also secondary metabolites. That is why the observations of agar diffusible plate assay carried out to check the effect of different non-volatile antibiotics is similar to those of volatile plate assay. HCN affects sensitive organisms by inhibiting the synthesis of ATP mediated by cytochrome oxidase (Knowles, 1976). Among the factors that involve in the plant-microbe interactions and microbe-microbe interactions in the rhizosphere, HCN is thought to play an important role (de Bellis & Ercolani, 2001). HCN producing organisms are beneficial to plants where they suppress the harmful components of the microbial community in the rhizosphere (Lugtenberg, et al, 1991; Schippers et al., 1991) and they exert no harm to plant health and do not disturb the

elongation of plant rootlets (de Bellis & Ercolani, 2001). However, further research is needed to confirm this hypothesis.

In the root colonization study with different mutants of PA23, we observed that PA23 showed significantly higher bacterial density over the sampling period (Figure 4.4). Since PA23 was originally isolated from root tips, it is clear that PA23 showed successful colonization of sunflower roots. Furthermore, regardless of differences in antibiotic production, most of the strains showed an increase in the bacterial density by the 2nd week compared to the initial density on the seeds. This may be due to the short generation time in *Pseudomonas* spp. that has also been observed in many other studies (Thomas et al., 2003). For example, *P. fluorescence* WCS365 formed microcolonies on tomato root (Chin-A-Woeng et al., 1997; Bloemberg et al., 1997) one day after seed inoculation (de Weert et al., 2002). The reason for this increase might be the rich composition of root exudates produced by the newly emerging roots of sunflower seedlings. Sunflower seedlings take 10-13 days to emerge; therefore, 2 weeks after seeding is the exact time that new roots were forming. The strains that did not show an increase might have been influenced by the slightly lower initial bacterial density on treated seeds. After 2 weeks, in some strains bacterial numbers on roots increased up to week 4 while in other strains there was a continuous decrease leading to week 6 (Figure 4.4). Therefore, the strains differed in their rate of colonization or the time taken to attain their maximum population on roots. The rate of colonization of a BCA is assumed to be an important trait in biocontrol (Thomas et al., 2003). By the 6th week all strains showed a noticeable drop in population. This may be due to insufficient nutrient availability in the soil, since the pots were not fertilized after the initial fertilizer application. In

addition, by week 6, sunflower plants also had started to form the floral bud. This particular time period was critical for the plant itself. Therefore, bacteria might have had to compete with the roots for survival rather than increasing their number. Waisel et al. (1991) stated that the rhizosphere effect, which is the quantitative and qualitative variation of the soilborne microflora, varies according to the root exudate composition and the stage of the plant development is one of the factors affecting root exudate composition. Therefore the changes in root exudate composition together with soil nutrient availability might have been the reason for most of the variations in bacterial numbers on roots observed over the sampling period. The total bacterial count variation followed a similar pattern as introduced bacteria (Figure 4.5) and there was no prominent influence of the indigenous bacteria toward introduced bacteria or vise versa until week 4, further confirming this concept. This idea is supported by Botelho & Mendonça-Hagler (2006), where they state that "nutrient availability is the base for root colonization". Bloemberg & Lugtenberg (2001) state that the "colonization potential is related to the support of nutritional balance from roots to microbes, as well as, the genes related to rhizosphere colonization". Furthermore, Rainey (1999) identified 14 genes in *P. fluorescence*, which are involved in nutrient acquisition, stress response, or secretion. In addition, some antibiotic producing strains showed a sudden increase in their percentages (Figure 4.6) by week 6 whereas the antibiotic-deficient strains showed significantly similar densities as the PA23 single application early in the sampling period. It seems that the antibiotic producing strains started to take advantage by producing antibiotics in the late sampling period when the nutrients became limited. Mazzola et al., 1992 observed a similar trend where the density of introduced bacteria which could not

produce the antibiotic phenazine remained in bulk soil almost constant up to 40 days (approximately 5-6 weeks) of planting of wheat. Then they started to reduce considerably while that of antibiotic producing strain remained almost unchanged. Mazzola et al. (1992) also suggest that the nutrient availability can influence both antibiotic production and microbial competition. When resources are plentiful, phenazine production has little benefit to the producer and the antibiotic effect increases when the microbial population is high. This observation supports the theory of "quorum sensing" where increasing microbial density leads to increased production of antibiotics (Thomas et al., 2003); therefore, and bacterial number is the signal that induces the expression of antibiotic genes.

Throughout the sampling period most of the strains (except PA23-63) that can produce at least one antibiotic seemed to maintain relatively higher bacterial numbers than non producing strains and PA23-63-1 which produce neither phenazine nor pyrrolnitrin showed the lowest colonization (Figure 4.4). This means that the number of mutations is inversely proportional to the survival of bacteria on roots and mutations had reduced the ecological fitness of PA23 to various extents independent of the type of mutation. This is further supported by the observation that the strains having at least one antibiotic deficient mutation showed very low percentages (<5%) out of the total rhizosphere bacterial community in all sampling days (Figure 4.6). This finding partially agrees with that of Thomashow & Weller (1988) where there was no influence of different mutations on *P. fluorescence* 2-79RN_{10} on its rhizosphere colonization. However, there was no consistent pattern in the variation of densities of phenazine-deficient and pyrrolnitrin-deficient mutants and therefore the relative positive or negative

effect of these mutations on root colonization ability as well as the biocontrol ability of PA23 is unclear. This may be due to the fact that antibiotic production by a BCA can be affected by several biotic and abiotic factors such as oxygen, temperature, specific carbon and nitrogen sources (Duffy & Défago, 1997; Howie & Suslow, 1991; Ownley et al., 1992; Shanahan et al., 1992; Slininger & Jackson, 1992), plant host (Georgakopoulos et al., 1994; Maurhofer et al., 1995), pathogen (Duffy & Défago, 1997) and indigenous microflora (Wood & Pierson, 1994) when the experiment carried out in the unautoclaved soils. The natural ecological niche of PA23 was soybean root tips. With sunflower being the host plant in the current study, it is clear that the performance of PA23 could be reduced on a different host since the positive or negative inter-population signaling on the plant root therefore may influence the final efficacy of the biocontrol agent (Pierson et al., 2002). For example, Benizri et al., (1998) observed that exudates of maize rhizosphere can influence in the production of indol acetic acid by a strain of *P. fluorescens*. Furthermore, apart from phenazine and pyrrolnitrin PA23 produces an array of secondary metabolites including protease, lipase, hydrogen cyanide and siderophores (Poritsanos, 2005) that likely contribute to its biocontrol and colonization ability. There are situations where strains not producing antibiotics or siderophores suppressed diseases (B.X Zhang & D.M. Weller, unpublished) and also colonized roots successfully (Thomashow & Weller, 1988) and where strains producing antibiotics lost biocontrol ability (Chin-A-Woeng et al., 2000). Among the antibiotic producing strains, the densities of PA23-63 and PA23-314 *gacS* were considerably reduced overtime (Figure 4.4). Colonies of PA23-63 and PA23-314 *gacS* are white and bright orange in color respectively. In later sampling dates slightly orange colonies were observed in PA23-63

(typically white) agar plates during plate counts. Isolated colonies of wild type (Figure 4.8), PA23-314 *gacS*, PA23-1 were very pale rather than having the typical bright orange color. According to personal communication with Rahim Habibian (Research Associate in lab), mutations can easily be lost under more complex environments like soil especially with complemented strains like PA23-314 *gacS* (orange colored) where the inserted plasmid carrying the restored *gacS* gene can be lost making the strain PA23-314 white in color. This was further evidenced by Carrie Selin (Ph.D. student, University of Manitoba), who observed a reduction of typical orange color in cultures of PA23 upon slight variations in the temperatures in the lab and due to other unknown factors. Therefore when counting colonies sometimes colonies that did not produce the typical color were ignored. This might have caused a reduction in bacterial counts, especially in PA23-63 and PA23-314 *gacS* and provide proof of the effect of environmental factors on the production of antibiotics.

Another observation is that the PA23-314 showed a competitive colonization most of the time (Figure 4.4). From previous studies it has been shown that PA23-314 produced elevated amounts of siderophores than the wild type (Poritsanons, 2005) and this might have contributed to its successful colonization. Certain studies also demonstrated the importance of siderophores in the rhizosphere competition and thereby occurrence of large microbial populations on some plant roots (de Beliis & Ercolani, 2001).

There was no significant consistent additive or synergistic effect shown by combinations with antibiotic producing strains. However, generally slight reduction on the colonization or biocontrol ability of PA23 was observed upon combinations with any

strain (Figure 4.4 & 4.7). Single application of PA23 is showing the highest emergence than any other treatment and the addition of PA23-1 is not showing an additive or synergistic effect mean that combining antibiotic producing strain with PA23 can exert an additional competition, which can reduce the effectiveness of PA23. Percentage emergence of PA23+PA23-314 *gacS* and PA23+PA23-63-1 was not significantly different means that PA23-314 *gacS* and PA23-63-1 had not been able to give positive and negative effect on the performance of PA23 respectively. This supports the findings of Koumoutsi (2006) where he didn't observe a synergistic effect of two antifungal compounds due to the lower production of one antibiotic over the other.

In the sclerotinia wilt disease assessment, most of the treatments were not successful against the disease (Figure 4.7). In combined applications, depending on the bacterial densities the competition could have been between bacterial strains rather than between the pathogen and BCAs. Since the bacterial densities of inoculated strains on root were not monitored in the disease study, further explanations cannot be made. The bacterial density on the infected root especially at the point of infection is important (Wood et al., 1997), which was not determined in this study. Density of the BCA can vary along the roots and threshold value of antibiotics might therefore be lower at the sites of infection (Raaijmakers et al., 1999).

Antibiotic production, root colonization and biocontrol ability exhibited by a BCA are strongly interrelated to each other as well as with environmental factors and it is very difficult to determine the importance of one over the other in the control of soil borne plant pathogens. There may be undiscovered phenotypic characteristics of certain genotypes of BCA that positively contribute to disease suppression. With comparatively

low percentages of introduced bacteria observed in this study in comparison to total rhizosphere population (Figure 4.6), the capacity of introduced bacteria to withstand and perform successfully in the rhizosphere is questionable. Furthermore, results do not show any significant enhanced effect of phenazine over pyrrolnitrin or vise versa in comparison to rhizosphere colonization or wilt disease control. This might mostly be due to the enhanced expression of one antibiotic where the expression of the other antibiotic is low. In this way single mutants still retains a considerable inhibitory effect on pathogens and other indigenous microflora compared to the double mutant (Koumoutsi, 2006).

In summary, although the involvement of a particular trait in the biocontrol ability of a BCA has been proven under laboratory conditions, where interference of various environmental factors are minimal, the importance of the biocontrol under natural environment conditions can vary from plant to plant, pathogen to pathogen and be strongly affected by the prevailing environmental conditions. To better clarify these results, studies on spatial and temporal variations of colonization along the root are important (Weller, 1983; Raaijmakers et al., 1999). In addition, close examination of the influence of each environmental, host and pathogen factors is essential before applying a BCA in a particular host-pathogen system. Significant biocontrol attained by a BCA in a particular host-pathogen system under a given set of environmental conditions does not mean that it will work for every host-pathogen system under a range of environmental conditions. Each system should be studied separately.

CHAPTER 5

5.0 Identification of Antifungal Antibiotics of *Bacillus* Species Isolated from Different Microhabitats Using Polymerase Chain Reaction and MALDI-TOF Mass Spectrometry

5.1 Abstract

Although many *Bacillus* species are known to be good antibiotic producers capable of acting as biocontrol agents, the underlying antimicrobial mechanisms are often poorly understood. In this study, 21 *Bacillus* strains, demonstrating over 50% mycelial inhibition against *S. sclerotiorum* as well as significant control in plant assays, were examined for the presence of antibiotic biosynthetic genes. Primers specific for bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin and zwittermicin A were used to amplify biosynthetic genes from these bacteria using polymerase chain reaction (PCR). The majority of strains harbored surfactin (21/21) and iturin A (20/21) biosynthetic genes. Three strains (*B. subtilis* 3057, *B. amyloliquefaciens* BS6, *B. mycoides* 4079) were positive for bacillomycin D while four strains (*B. subtilis* H-08-02, *B. subtilis* 3057, *B. amyloliquefaciens* BS6, *B. mycoides* 4079) showed presence of the fengycin biosynthetic gene. The zwittermicin A gene was detected in *B. mycoides* S, *B. thuringiensis* BS8 and *B. amyloliquefaciens* BS6. Sequence analysis of purified PCR products revealed homology with corresponding genes from other *Bacillus* sp. in the GenBank database. Production of particular antibiotics in strains BS6, H-08-02, 3057 and 4079 was confirmed through matrix-assisted laser desorption ionization-time of flight-mass

spectroscopy (MALDI-TOF-MS). This study revealed the equivalent capability of different *Bacillus* strains from various microhabitats to produce the above mentioned antibiotics and highlights the possibility of using some strains as potential biocontrol agents under different microhabitats distant from their original habitat. Furthermore, it will enable research to develop rational strategies for the application of the antagonists and their metabolites within an agroecosystem.

5.2 Introduction

Many *Bacillus* species are capable of producing a wide variety of secondary metabolites that are diverse in structure and function. The production of metabolites with antimicrobial activity is one determinant of their ability to control plant diseases (McKeen et al., 1986; Silo-suh et al., 1994). These metabolites can be ribosomal compounds such as subtilin (Zuber et al., 1993), subtilosin A (Babasaki et al., 1985, TasA (Stover & Driks, 1999) and sublancin (Paik et al., 1998). A variety of non-ribosomally produced small lipopeptides belonging to surfactin family; surfactin, lichenysins (Kluge et a l., 1988), iturin family; iturin A, C, D and E bacillomycin D, F, L and mycosubtilin (Maget-Dana & Peypoux, 1994) and fengycin family; fengicins and plipastatins (Vanittanakom et al., 1986) as well as aminopolyols like zwittermycin A (Milner et al., 1996) are also common. The lipopeptides carry a hydrophilic peptide portion and a hydrophobic fatty acid portion (Roongsawang et al., 2002) and are produced via a multi-enzyme catalyzed pathway (Zuber et al., 1993). Iturin is a group of cyclic lipopeptides and shares a common sequence (β-hydroxy fatty acid-Asx-Tyr-Asx) with variation at the

other four positions (Maget-Dana & Peypoux, 1994). Iturin A, bacillimycin D and mycosubtilin are made of one β-amino fatty acid and 7 α-amino acids. Surfactin is also a cyclic lipopetide containing seven residues of D - and L- aminoacids and one residue of a β-hydroxy fatty acid (Kluge et al., 1988). The amino acid sequence in surfactin is completely different from the iturin group. Fengycin and plipastatin have 10 amino acids and a lipid attached to the N-terminal end of the molecule. The presence of unusual amino acids such as ornithine and allo-threonine make fengycin and plipastatin different from iturins and surfactins (Moyne et al., 2001). Antibiotics from the iturin family show strong antifungal and hemolytic activities with limited antibacterial activity (Maget-Dana & Peypoux, 1994) while the activity shown by fengycin is specific against filamentous fungi and inhibits phopholipase A_2 (Nishikori et al., 1986). Surfactin shows antiviral and antimycoplasma activities (Vollenbroich et al. 1997a, 1997b). Zwittermicin A is a linear aminopolyol (He et al., 1994) having a broad spectrum of activity against certain gram-positive, gram-negative, and eukaryotic microorganisms (Silo-Suh et al., 1998) and also has an insecticidal activity similar to the protein toxin produced by *Bacillus thuringiensis* (Broderick et al., 2000, 2003).

There are reports that *Bacillus* spp. especially *Bacillus subtilis*, *Bacillus cereus, B. licheniformis* and *Bacillus amyloliquefaciens* have been effective against plant and fruit diseases caused by soil-borne, aerial or post-harvest fungal diseases (Broggini et al. 2005; Szczech & Shoda, 2004; Yoshida et al., 2001; Havenga et al., 1999; Korsten & De Jager, 1995; Siddiqui & Mahmood, 1992). In most situations the involvement of antibiotics has been established. For example, zwittermicin A produced by *B. cereus* UW85 was found to be involved in the suppression of damping-off of alfalfa caused by *Phytophthora*

medicaginis (Stabb et al., 1994). Bacillomycin D showed strong antifungal activity against *Aspergillus flavus* (Moyne et al., 2001). Both surfactin and iturin A were involved in the control of *Rhizoctonia solani* damping-off by *B. subtilis* RB14 (Asaka & Shoda, 1996). Similarly, fengycin was found to be involved in the control of damping-off of bean seedling caused by *Pythium ultimum* and gray mold disease of apple caused by *Botrytis cineria*. Mycosubtilin overproducing strain *B. subtilis* BBG100 controlled *Pythium* damping-off of tomato seedlings (Leclère et al., 2005). Detection of antibiotic production by a particular bacterium is important in determining its capability to be a good biocontrol agent of plant diseases. Traditional method of selection and identification of potential biocontrol agents involving random isolation and screening procedure for antifungal activity is time consuming and laborious (de Souza & Raaijmakers, 2003). Therefore, screening candidate strains for particular antibiotic-encoding sequences followed by direct detection of antibiotic profile of a particular bacterium provides a rapid approach. PCR-based detection of antibiotic biosynthetic genes and MALDI-TOF-MS analysis are very appropriate approaches in this regard. MALDI-TOF-MS is an easy and less time consuming method for confirmation of antibiotic production when compared to biochemical extraction, purification and characterization of lipopeptides.

In the current study, twenty one *Bacillus* strains that have shown antifungal activity towards different economically important fungal pathogens of canola (*Brassica napus* L.), sunflower (*Helianthus annuus* L.) and wheat (*Triticum aestivum* L.) under laboratory, greenhouse and field conditions (Fernando et al., 2002; Zhang, 2004; Duncan et al., 2006) were selected and the antibiotic production was hypothesized to be one of the control mechanisms involved. Therefore, they were screened for the presence of

antibiotic biosynthetic genes for the antibiotics surfactin, iturin A, bacillomycin D, fengycin, mycosubtilin and zwittermicin A using specific polymerase chain reaction (PCR). Furthermore, the production of these antibiotics was confirmed through MALDI-TOF-MS analysis (Vater et al., 2002a).

5.3 Materials and Methods

5.3.1 Bacterial Isolates and Culture Conditions

Twenty-one strains of *Bacillus* species with different origins together with reference strains for each antibiotic tested (Table 5.1) were used in this study. Pure cultures of each bacterium were maintained in Luria-Bertani broth, amended with 20% glycerol (Fisher Scientific ^{TM}, Fair Lawn, New Jersey, USA) and stored at –80 °C. New cultures of each bacterium were started by streaking them onto Luria Bertani Agar (LBA; Difco Laboratories, Detroit, Michigan) and incubated at 28 °C for 24 h.

5.3.2 DNA Extraction and Quantification

Each strain was inoculated into 5 ml aliquots of Luria Bertani Broth (LBB) and incubated at 32 °C on a rotary shaker at 180 rpm for 16-18 h. DNA extraction was done using a cetyltrimethylammoniumbromide (CTAB)-based miniprep protocol (Ausubel et al., 1995) with slight modifications. One and a half milliliters of overnight cultures of bacteria were microcentrifuged at 7000 rpm for 10 min in a 1.5 ml microcentrifuge tube (Fisher Scientific^{TM}, Fair Lawn, New Jersey, USA). The pellet was resuspended in 567 µl of TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA pH 8.0). Thirty microliters of 10%

sodium dodecyl sulfate (SDS) and 3 μL of proteinase K (20 mg/mL; Sigma®, St. Louis, MO, USA) were added and mixed thoroughly followed by incubation for 1 h at 37 °C. The mixture was incubated at 65 °C for 10 min after addition of 100 μL of NaCl and 80 μL of CTAB/NaCl (CTAB: hexadecyltrimethlammonium bromide, Sigma®, St. Louis, MO, USA). After each addition the mixture was thoroughly mixed. Phenol was extracted with I volume of 24:1 chloroform/isoamyl alcohol (Fisher Scientific™, Fair Lawn, New Jersey, USA). It was microcentrifuged at 7000 rpm for 10 min after thorough mixing. The supernatant was transferred to a fresh tube and extracted with 1 volume of 25:24:1 phenol/chloroform/isoamyl alcohol (Phenol: Sigma®, St. Louis, MO, USA). After microcentrifugation for 10 min at 7000 rpm the supernatant was transferred to a fresh tube and DNA was precipitated with 0.6 volume of isopropanol (Sigma®, St. Louis, MO, USA) followed by gently mixing until a stringy white DNA precipitate formed. The DNA pellet obtained by centrifugation at 13000 rpm for 3 min was washed twice with 500 μl of chilled 70% ethanol and the final pellet was obtained after centrifugation at 13000 rpm for 3 min. The pellet was dried until no more ethanol droplets remained in the tube and it was resuspended in 100 μl of warm (65 °C) TE buffer. The DNA was stored at 4 °C overnight for further dissolving of the pellet. Then the DNA preparation was treated with 2 μl of ribonucleases (Sigma®, St. Louis, MO, USA) and incubated at 37 °C for 30 min to remove RNA. The DNA was precipitated, washed and resuspended in TE buffer as described previously and stored at –20 °C for further analysis. Genomic DNA was quantified in 1% agarose gel (Agarose DNA Grade, Electrophoresis Grade, Fisher Scientific™, Fair Lawn, New Jersey, USA) containing

ethidium bromide (0.5ug/ml) and 1x Tris-Acetate EDTA (TAE) buffer by comparing to the 1650 bp band in 1 kb-ladder (Invitrogen Life Technologies, CA, USA).

Table 5.1. List of bacterial isolates and the positive strains used in the current study.

Bacterium	Origin	Reference
Bacillus subtilis 240	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. subtilis 2031	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. subtilis 2090	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. subtilis 3057	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. subtilis H-08-02	Wheat head	Fernando et al., 2002
Bacillus amyloliquifaciens 226	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. amyloliquifaciens 248	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. amyloliquifaciens 265	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. amyloliquifaciens 268	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. amyloliquifaciens 2033	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. amyloliquifaciens BS6	Canola leaves, Saskatchewan	Zhang, 2004
Bacillus licheniformis 223	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. licheniformis 266	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. licheniformis 3039	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
Bacillus mycoides 4079	Sunflower sclerotia, Winnipeg, MB	Duncan et al., 2006
B. mycoides S-07-01	Wheat rhizosphere	Chen et al., 2001
Bacillus thuringiensis BS8	Canola leaves, Saskatchewan	Zhang, 2004
Bacillus cereus L-07-01	Wheat phyllosphere	Fernando et al., 2002
Strain G20	Alfalfa field soil, Carman, MB	Li Ru, unpublished
Strain LRC	Wheat field soil, Carman, MB	Li Ru, unpublished
Strain BLA	Wheat field soil, Carman, MB	Li Ru, unpublished

Reference strain	Antibiotic tested
Bacillus subtilis ATCC 13952	Iturin A and Fengycin
B. subtilis ATCC 6633	Surfactin
B. subtilis strain Bs49	Bacillomycin D
Bacillus cereus strain UW85	Zwittermicin A

5.3.3 PCR Analysis

Primer pairs used for each antibiotic are listed in Table 5.2. PCR reaction mixtures (25 µl) contained 20 ng of template DNA, 1X PCR buffer, 1.75 mM (for ITUD1F/1R, BACC1F/1R and SUR3F/3R), 1.5 mM (primers 677/678 and zwitF2/R1) and 2 mM (for FEND1F/FEND1R or FENA1F/FENA1R or FENB2F/FENB2R) of $MgCl_2$, 200 µM of each dATP, dCTP, dGTP and dTTP (Invitrogen Life Technologies, CA, USA), 20 pmol of each primer (Invitrogen Life Technologies, Carlsbad, CA, USA) and 0.1 U of Platinum® *Taq* polymerase (Invitrogen Life Technologies, Carlsbad, CA, USA). Amplifications were carried out with a PTC-100™ programmable thermal controller (MJ Research, Waltham, Massachusetts). For primers ITUD1F/1R, BACC1F/1R and SUR3F/3R the PCR program was as follows; initial denaturation at 94 °C for 3 min; 36 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 30 s, extension at 72 °C for 1 m 45 s; and final extension at 72 °C for 6 min. For primers FEND1F/FEND1R or FENA1F/FENA1R or FENB2F/FENB2R, initial denaturation at 94 °C for 3 min; 45 cycles of denaturation at 94 °C for 1 min, annealing at 62 °C for in min and extension at 72 °C for 1 min 45 s; and final extension at 72 °C for 6 min. For primers zwitF2/R1 the program was, initial denaturation at 94 °C for 5 min; 45 cycles of 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 2 min; and a final extension of 72 °C for 5 min. For primers 677/678, initial denaturation at 94 °C for 5 min with 45 cycles of 94 °C for 1 min; 56 °C for 1 min; extension at 72 °C for 2 min and a final extension at 72 °C at 5 min. *Bacillus subtilis* strain ATCC 6633 was used as the positive control for surfactin, and *B. subtilis* strain ATCC 13952 for iturin A and fengycin. *B. subtilis* strain Bs49 was the positive control for bacillomycin D and *B.*

cereus strain UW85 for the zwittermicin A positive control. An eight microlitre aliquot of PCR product was run on a 1% agarose gel, containing ethidium bromide, using 1X TAE buffer at 150-180 V for 45 min – 1h. The bands were visualized with a UV transilluminator.

Table 5.2. Specific primer sequences used for iturin A, bacillomycin D, surfactin, zwittermicin A (Ramarathnam, 2007) and fengycin (Ramarathnam et al., 2007) in this study.

Antibiotic	Primer Name	Primer Sequence
Zwittermicin A	ZWITF2	ttgggagaatatacagctct
	ZWITR1	gacctttgaaatgggcgta
Iturin A	ITUD1F	gatgcgatctccttggatgt
	ITUD1R	atcgtcatgtgctgcttgag
Bacillomycin D	BACC1F	gaaggacacggcagagagtc
	BACC1R	cgctgatgactgttcatgct
Surfactin	SUR3F	acagtatggaggcatggtc
	SUR3R	ttccgccacttttcagttt
Fengycin	FEND1F	tttggcagcaggagaagttt
	FEND1R	gctgtccgttctgctttttc
	FENA1F	gacagtgctgcctgatgaaa
	FENA1R	gtcggtgcatgaaatgtacg
	FENB2F	caagatatgctggacgctga
	FENB2R	acacgacattgcgattggta

5.3.4 Purification, Sequencing and n-Blast Search of PCR Products

The bands that showed positive results were reamplified with specific primers and purified with a High Pure PCR product purification kit (Roche Diagnostics Technologies, IN, USA) as follows. PCR products were verified for amplification by electrophoresis on

1% agarose gel and visualized under UV light. The procedure was started by adding binding buffer (500 µl, Roche Applied Science) to PCR product (100 µl). After a thorough mix the solution was transferred into a High Pure Filter Tube, which was placed on a collection tube. The solution in the filter tube was then centrifuged for 1 min at 13,000 rpm. Subsequently, the washing buffer (500 µl, Roche Applied Science) was added to the upper reservoir after discarding the flowthrough in the collecting tube. Flowthrough was discarded after centrifugation at 13,000 rpm for 1 min. Another 200 µl aliquot of washing buffer was added to the filter tube and centrifuged at 13,000 rpm for 1 min. Then the filter tube was placed on a fresh 1.5 ml microcentrifuge tube (Fisher Scientific™, Fair Lawn, New Jersey, USA) and 50 µl of eluting buffer was added directly onto the filter and centrifuged at 13,000 rpm for 1 min. The purified PCR product in the 1.5 ml microcentrifuge tube was transferred into another fresh 1.5 ml microcentrifuge tube (Fisher Scientific™, Fair Lawn, New Jersey, USA) and 4 µl of the product was run on 1 % agarose gel with a 1 kb-ladder for quantification. Products were sequenced at the MacrogenUSA (Rockville, MD). The sequences were searched for the specific homology in the GeneBank database through the NCBI n-blast search.

5.3.5 MALDI-TOF-MS Analysis of the Antibiotics Present in the Cell-surface Extracts of Bacterial Strains *B. amylolquifaciens* BS6, *B. subtilis* 3057 and *B. mycoides* 4079

Among the strains tested BS6, H-08-02, S-07-01, L-07-01, 3057 and 4079 showed the presence of multiple biosynthetic genes of antifungal lipopeptides surfactin,

iturin A, bacillomycin D and fengycin. Therefore, the surface extracts of these strains were subjected to MALDI-TOF-MS analysis for the detection of the production of the antifungal lipopeptides surfactin, iturin A, bacillomycin D and fengycin. *Bacillus subtilis* strain ATCC13952 was used as the reference control for iturin A and fengycin. *B. subtilis* strain ATCC6633 was used for surfactin while *B. subtilis* strain Bs49 was used for bacillomycin D. Preparation of bacterial surface extracts was done according to the methodology described by Vater et al. (2002a). Strains were grown on Landy agar (LA – 20 g glucose, 5 g L-glutamic acid, 0.5 g $MgSO_4$, 0.5 g KCl, 1 g KH_2PO_4, 0.15 g $Fe_2(SO_4)_3$.6H2O, 5 mg $MnSO_4.H_2O$, 0.16 mg $CuSO_4.5H_2O$, 15 g agar, 1000 mL distilled water, pH6) at 28 °C for 2 days. One to two loops of bacterial cells from the LA plates were suspended in 500 µL of 70% acetonitrile with 0.1% trifluoroacetic acid for 1-2 min. The suspensions were vortexed gently for homogenous suspensions. The bacterial cells were pelleted by centrifugation and the cell-free supernatant was transferred to a new mocrocentrifuge tube and stored in 4 °C for further analysis. One microliter of the surface extract was spotted onto the target of mass spectrometer with an equal volume of matrix solution (dihydroxy benzoic acid (DHB); 50 mg in 1 mL of 70% acetonitrile/0.1% trifluoroacetic acid. Samples were air dried and subjected to MALDI-TOF-MS analysis. The MALDI-TOF-MS analysis was performed at the Department of Physics and Astronomy, University of Manitoba. Analysis by MALDI-TOF-MS was performed in on the QqTOF prototype mass spectrometer which uses a combination of quadrupole-based RF-only collisional cooling, orthogonal ion injection and an electrostatic ion mirror to give mass resolution > 10,000 and stability on the order of 10 PPM. It is operated at 10kV of acceleration for positive ions. The working mass range is 500-5000 amu at an

orthogonal extraction frequency of 7500 Hz. MALDI ions are created using a 337 nm nitrogen laser coupled to a movable robotic target source using a 100 micron fused silica optical patchcord and custom aspheric optics. The laser repetition rate is typically 10-20 Hz; good statistics on mass spectra are obtained in less than 30 seconds. External calibration was done using a two-point quadratic of the singly-charged ions of dalargin, substance P and bee venom (monoisotopic masses 726.394, 1347.736 and 2845.762 respectively). Analysis of MS-MS was performed under very similar conditions as above except that the mass range was from 0 to parent ion amu.

5.4 Results

5.4.1 PCR Analysis and BLAST Search

Strains that were positive to each antibiotic and their GenBank homology are given in the Table 5.3. PCR products amplified from all strains using primer pair SUR3F/3R showed a 441 bp band corresponding to surfactin antibiotic biosynthetic gene (Figure 5.1-A). Sequenced products of BS6, S-07-01, H-08-02, 4079, 3057, 223, 266, 2031, 2033 showed high similarity to the GenBank# AF534916.1 (surfactin synthetase gene cluster of *Bacillus* sp. CY22), also confirming the presence of that gene in other strains positive for surfactin. All strains except 3039 showed a 647 bp band corresponding to iturin A antibiotic biosynthetic gene and the sequenced PCR products of strains BS6, BS8, H-08-02, L-07-01, 223, 248, 266, 3057 and 4079 (Figure 5.1-B) using primer pair ITUD1F/1R were highly homologous to iturin A synthetase gene cluster of *Bacillus* sp. with the GenBank# AF534617.1. Only strains BS6, 3057 and 4079 were

Table 5.3. Blast results of the sequenced products obtained from PCR amplification using gene-specific primers for biosynthetic genes of common bacterial antibiotics.

Antibiotic	Isolate	GenBank Accession #	GenBank Match	E value
Surfactin	*B. licheniformis* 223	AF534616.1	Surfactin synthetase gene cluster of *Bacillus sp* CY22	0.0
	B. licheniformis 266			0.0
	B. subtilis 2031			0.0
	B. amyloliquefaciens 2033			0.0
	B. subtilis 3057			0.0
	B. mycoides 4079			0.0
	B. amyloliquefaciens BS6			0.0
	B. mycoides S-07-01			0.0
	B. subtilis H-08-02			0.0
Iturin A	*B. licheniformis* 223	AF534617.1	Iturin synthetase gene cluster of *Bacillus sp* CY22	$3e^{-78}$
	B. amyloliquefaciens 248			0.0
	B. licheniformis 266			$8e^{-64}$
	B. subtilis 3057			0.0
	B. mycoides 4079			0.0
	B. amyloliquefaciens BS6			0.0
	B. thuringiensis BS8			$3e^{-161}$
	B. subtilis H-08-02			0.0
	B. cereus L-07-01			0.0
Bacillomycin D	*B. mycoides* 4079	AY137375.1	Bacillomycin D operaon of *B. subtilis*	0.0
	B. subtilis 3057	AJ576102.1		$2e^{-150}$
Fengycin	*B. subtilis* H-08-02	AJ011849.1	fenD gene of *B. subtilis*	0.0
Zwittermicin A	*B. amyloliquefaciens* BS6	AF235003.2	Zwittermicin D resistance protein (zmaR) of *B. thuringiensis*	0.0
	B. thuringiensis BS8	-do-		0.0
	B. mycoides S-07-01	-do-		0.0

positive for the presence of bacillomycin D biosynthetic genes showing the band at 875 bp (Figure 5.1-C) and their sequences were homologous to that of *B. subtilis* bacillomycin D operon. For zwittermicin A, two primer pairs were used, 677/678 for the zwittermicin A self-resistant protein, *ZmaR*, biosynthetic gene and ZWITF2/R1 for the zwittermicin A biosynthetic gene cluster. Strains BS6, BS8 and S-07-01 showed a 1 Kb band responsible for the *ZmaR* gene (Figure 5.1-D) while only strains BS6 and BS8 showed the presence of zwittermicin A biosynthetic gene cluster (Figure 5.1-E). Sequences of PCR products from strains BS6, BS8 and S-07-01 amplified using primers 677/678 showed high similarity to zwittermicin A resistance protein gene of *B. thuringiensis* (GenBank# AY083683). Only 3 strains; H-08-02, 3057, 4079 showed the 964 bp band corresponding to fengycin biosynthetic gene cluster (Figure 5.1-D). The particular band from H-08-02 was amplified using primers FEND1F/1R and that of strains 3057 and 4079 were amplified using the primers FENB2F/2R and FENA1F/1R respectively. Sequence of corresponding band amplified from H-08-02 was homologous to *fenD* gene of *B. subtilis* in the GenBank database. None of the 21 strains was positive for the presence of mycosubtlin biosynthetic gene.

5.4.2 MALDI-TOF MS Analysis

Strains showing the presence of multiple genes; BS6, BS8, H-08-02, S-07-01, 3057 and 4079 together with positive strains were subjected to MALDI-TOF MS analysis. Mass spectra obtained from strains BS6, H-08-02, 3057 and 4079 showed very clear peak clusters. The group of mass peaks at m/z = 1008.6, 1022.6, 1036.6 observed in

the mass spectra of strain BS6, 4079, 3057 and H-08-02 corresponded to the protonated forms of C13-C15 surfactins and the group of peaks at m/z = 1046.6, 1060.6 and 1074.6 in the mass spectra of above mentioned strains corresponded to the potassium adducts of C13-C15 surfactins (Figure 5.2 and 5.3). These peak groups were also detected in the mass spectrum of the positive strain ATCC 6633. The mass peaks corresponding to the antibiotic iturin A was detected in the mass range m/z =1070 – 1125. The peaks observed at m/z = 1071.6, 1085.6 in the mass spectra of 4079 and H-08-02 corresponded to protonated forms of C16-C17 iturin A. The peaks at m/z = 1093.6, 1096.6, 1098.6, 1107.6, 1109.6, 1123.6 and 1136.6 in the mass spectra of strains H-08-02, 4079, 3057 could be attributed to the sodium and potassium adducts of C16-C18 iturin A (Figure 5.2 and 5.3). These peaks were also observed in the mass spectrum of positive strain ATCC 13952. The group of peaks at m/z = 1031.5, 1045.6 and 1059.6 shown in the mass spectra of the strains 3057 as well as in the positive stain DFE16 was an indication of protonated forms of C14-C16 bacillomycin D. The peaks observed at m/z = 1053.6, 1067.6, 1081.6, 1069.5, 1083.5, 1097.5, 1095.5 and 1111.6 in the mass spectra of strains BS6, 3057, 4079 and the positive strain corresponded to sodium and potassium adducts of C14-C17 bacillomycin D (Figure 5.2 and 5.3). The group of peaks at m/z = 1449.7, 1463.8, 1477.8 and 1491.8 detected in the mass spectra of the strains BS6, H-08-02, 4079 and 3057 could be attributed to the protonated forms of C15-C17 fengycins and the peaks at m/z = 1501.7, 1515.7, 1529.7, 1539.7 and 1543. 8 in the mass spectra of the above strains corresponded to sodium and potassium adducts of C16-C17 fengycin (Figure 5.4). These peaks were also detected in the mass spectrum of the positive strain ATCC 13952. However, an interesting phenomenon could be observed in the mass spectra of 4 different

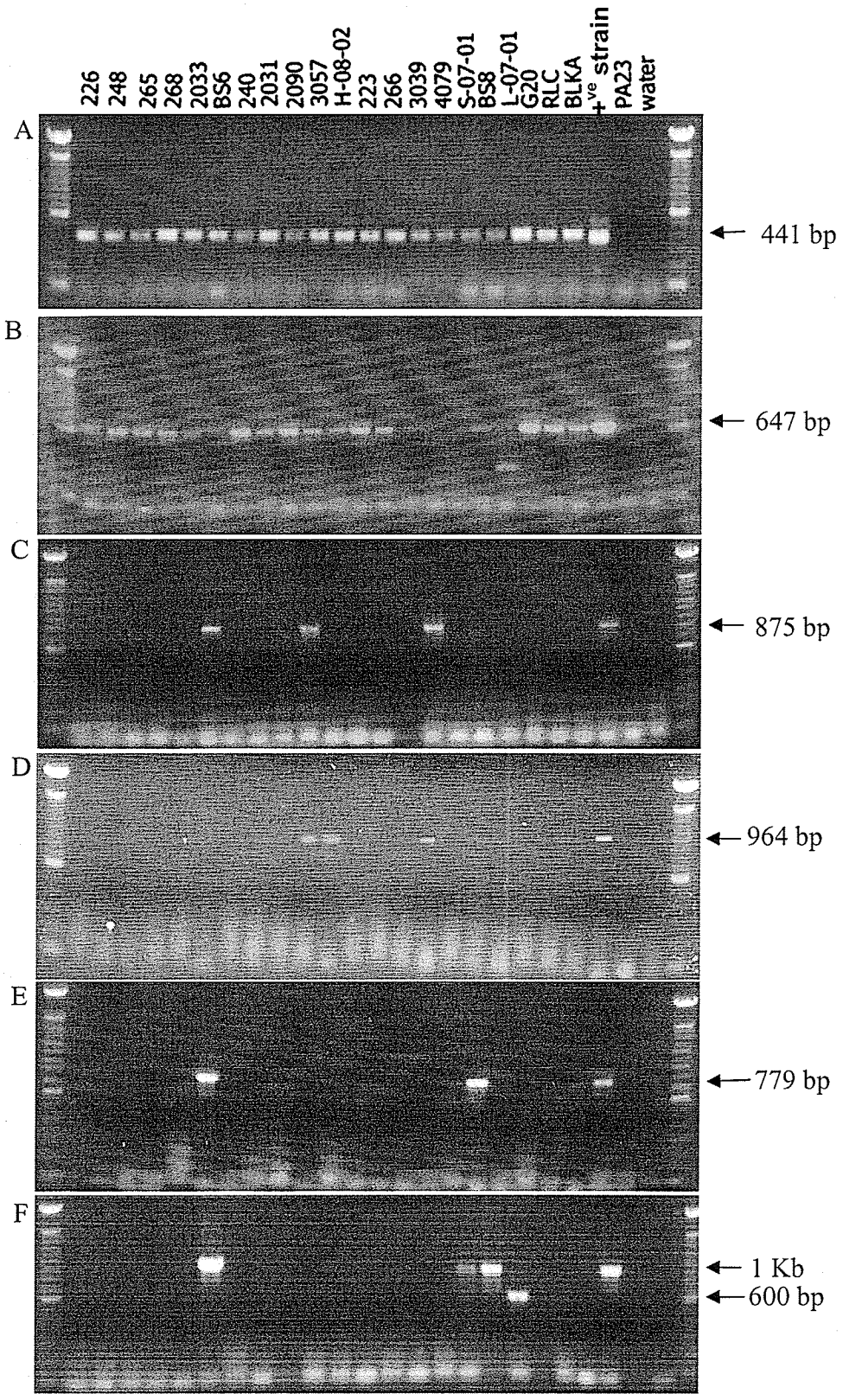


Figure 5.1. PCR amplification of biosynthetic genes corresponding to surfactin (A), iturin A (B), bacillomycin D (C), fengycin (D), zwittermicin A (E) and zwittermicin A self resistant protein, ZmaR (F). PCR products were separated on 1% agarose gel for 30 min and visualized by a transilluminator.

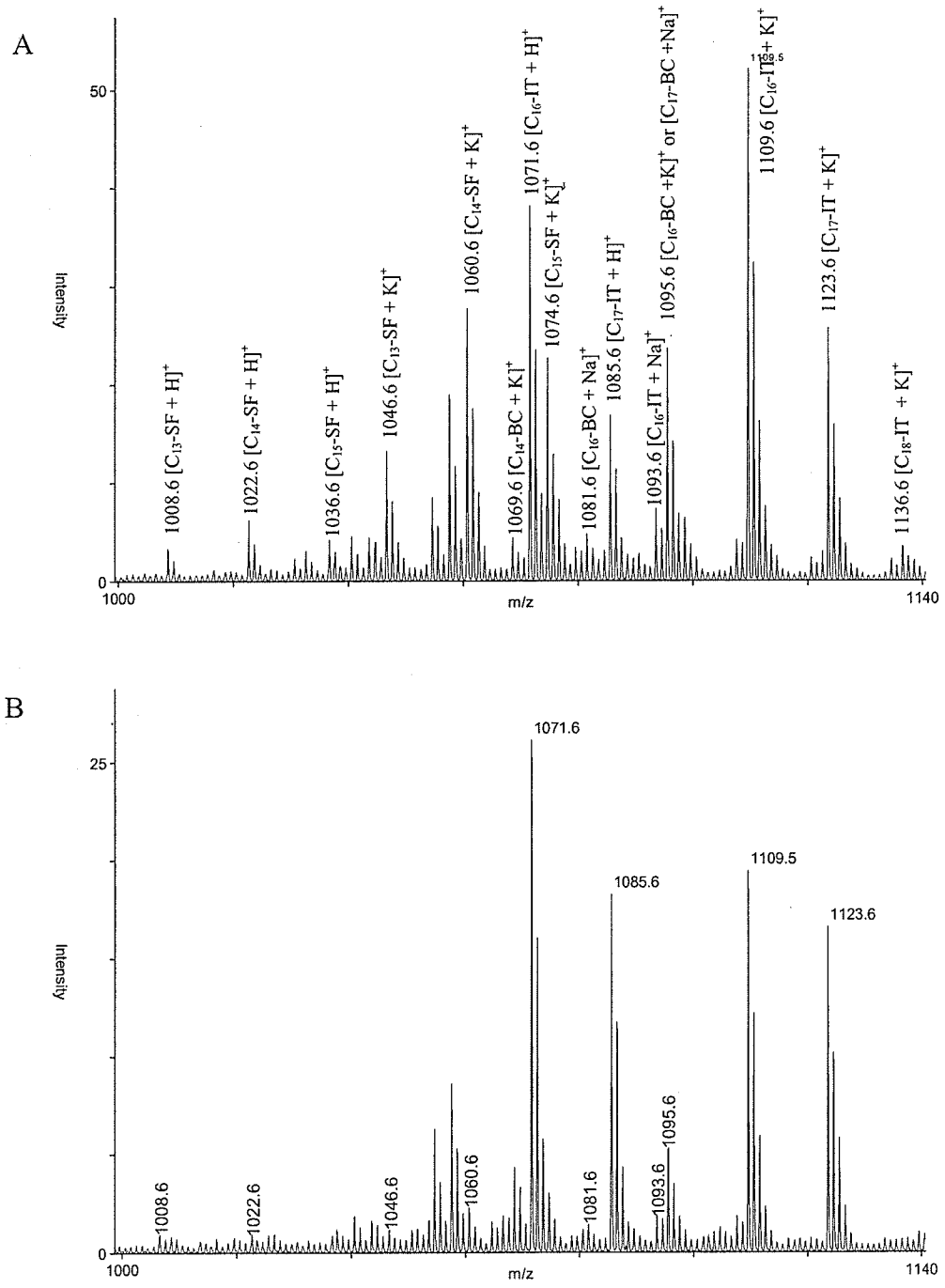


Figure 5.2. MALDI-TOF mass spectra of bacterial cell-surface extracts obtained from the strains BS6: *B. amylolquefaciens* (A) and 4079: *B. mycoides* (B), analysed for the presence of lipopeptide antibiotics surfactin (SF), iturin A (IT) and bacillomycin D (BC). Mass ranges were (m/z) 1008 – 1074, 1070 – 1150 and 1030 – 1111 for SF, IT and BC respectively.

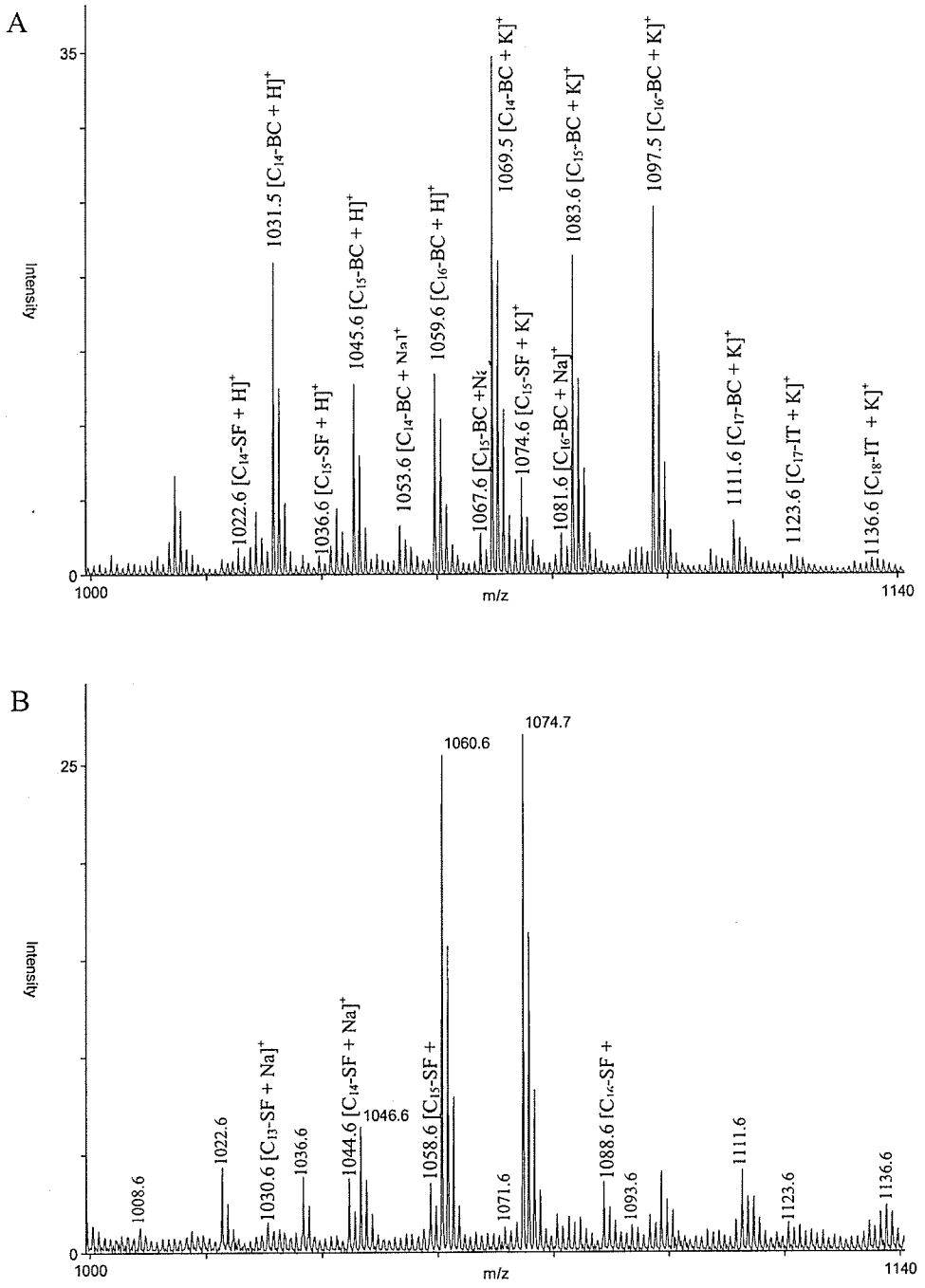


Figure 5.3. MALDI-TOF mass spectra of bacterial cell-surface extracts obtained from the strains 3057: *B. subtilis* (A) and H-08-02: *B. subtilis* (B), analysed for the presence of lipopeptide antibiotics surfactin (SF), iturin A (IT) and bacillomycin D (BC). Mass ranges were (m/z) 1008 – 1074, 1070 – 1150 and 1030 – 1111 for SF, IT and BC respectively.

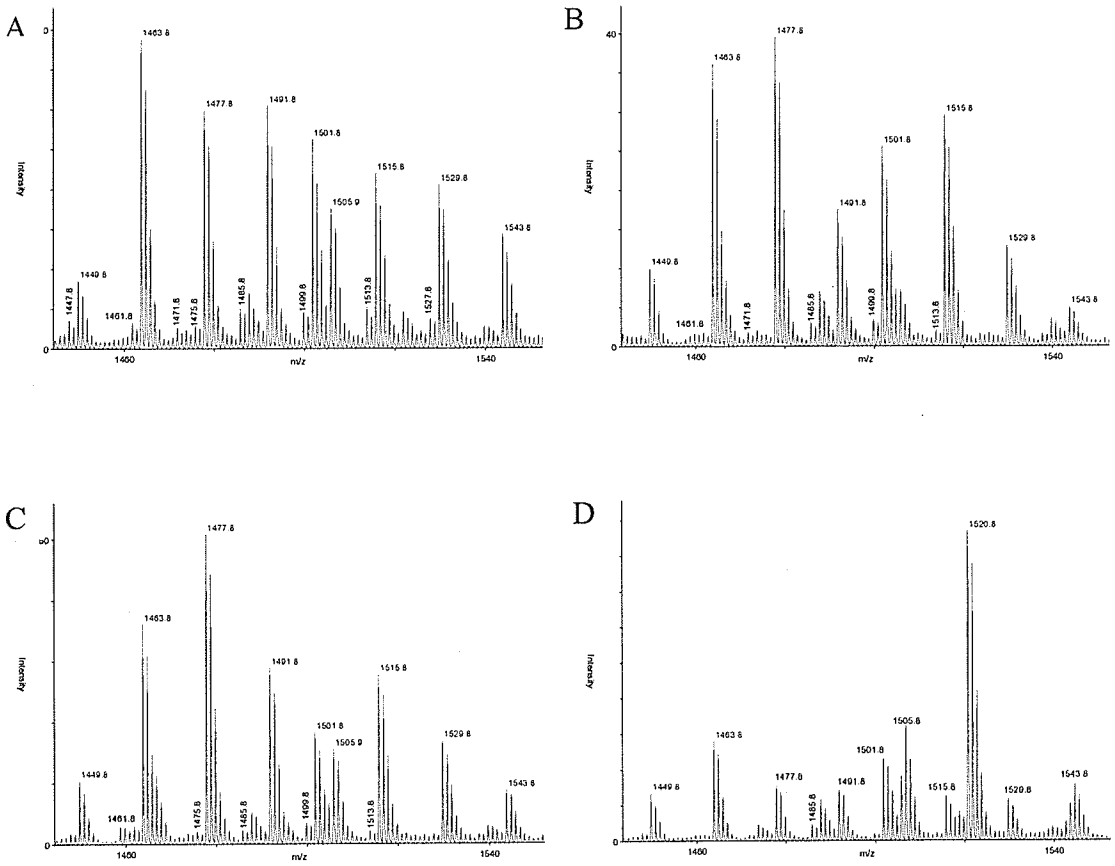


Figure 5.4. MALDI-TOF mass spectra of bacterial cell-surface extracts obtained from the strains H-08-02: *B. subtilis* (A), BS6: *B. amyloliquefaciens* (B), 4079: *B. mycoides* (C) and3057: *B. subtilis* (D) analyzed for the presence of lipopeptide antibiotic fengycin. Mass range was (m/z) 1047 – 1543. Mass peaks 1449.8, 1461.8, 1463.8, 1475.8, 1477.8, 1491.8, 1505.8 correspond to protonated forms of C15-C17 fengycin, other mass peaks correspond to the sodium and potassium adducts of C15-C17 fengycin.

strains, BS6, H-08-02, 4079 and 3057. In the spectrum of BS6, peaks corresponding to surfactin, iturin A and bacillomycin D could be detected in more or less similar intensities while in that of H-08-02 only peaks representing surfactin are prominent. In the spectra of 3057 and 4079 peaks representing bacillomycin D and iturin A are prominent respectively (Figure 5.2 & 5.3). This indicates that although these strains have biosynthetic genes for surfactin, iturin and bacillomycin D, under the given conditions the major antibiotics that are actually produced in elevated amounts will differ from strain to strain and might be dependent on environmental signals and the genetic make up of the bacterium.

The MS-MS analysis was not successful due to the complex cyclic structure of the fatty acid part of the antibiotic tested.

5.5 Discussion

All the strains used in the current study have shown >50% mycelia inhibition of *Sclerotinia sclerotiorum* or/and *Fusarium graminearum* under laboratory conditions and in addition some have shown significant green house and field control of stem rot disease in canola and fusarium head blight in wheat (Fernando et al., 2002; Zhang, 2004, Duncan et al., 2006). Furthermore, the buried sclerotia treated with strains 2031, 2033, 4079, 266 and 223 had shown significant reduction in their germination ability when they were recovered from soil after a period of time and cultured on PDA respectively (Paula Parks, personal communication). Therefore, one of the mechanisms of control was hypothesized to be antibiosis. This study was carried out to test this hypothesis where the presence of

biosynthetic genes encoding the antimicrobial antibiotics surfactin, iturin A, bacillomycin D, fengycin and zwittermicin A was investigated by PCR and their production was confirmed by MALDI-TOF mass spectrometry. PCR-based detection of specific antibiotic producing bacteria is favored over the time-consuming and laborious method of random isolation and screening procedures (de Souza & Raaijmakers, 2003). The detection of a particular antibiotic biosynthetic operon in a bacterial strain would signify the function of the operon and the production of the antibiotics. Any loss of function of the operon would be an exception to the rule (McSpadden Gardener et al., 2001). MALDI-TOF MS confirms the function of the operon detected by PCR. In the current study production of antibiotics by some of the strains was not observed through MALDI-TOF MS even though the specific genes were detected by PCR. A similar observation was made by Leenders et al (1999) where they detected conserved genes specifying surfactin and fengycin synthetase (Kunst et al., 1997)) in *B. subtilis* 168 but it did not produce corresponding antibiotics due to a mutation in the gene encoding 4'-phosphopantetheinyl transferase (Mootz et al., 2001). The same reason might be involved in the observation made in the current study where there could have been mutations occurring in any gene of the gene cluster. In the current PCR screening, almost all isolates including *B. subtilis, B. amylolquefaciens, B. licheniformis, B. mycoides,* and *B. cereus/thuringiensis* were positive for antibiotics surfactin and iturin A. This result is in support with earlier findings where these two antibiotics were detected from a wide variety of *Bacillus* sp. (Vater et al., 2002, Mikkola et al., 2004, Thaniyavarn, et al., 2003, Goursaud, 1989, McInerney et al., 1990, Ramarathnam, 2007) including all above mentioned species except from *B. mycoides*. This implies that surfactin and iturin A are

among the most common lipopeptide antibiotics produced by *Bacillus* sp. The specific surface- and membrane-active properties of the surfactins help bacteria to form biofilms, therefore, surfactin is thought to perform developmental function rather than defense functions in the environment (Hofemeister et al., 2004). This might be the reasons for most *Bacillus* strains to produce surfactins. To the best of our knowledge this study reports the production of surfactin, iturin A by a *B. mycoides* strain for the first time. Detection of *zmaR* genes in *B. thuringiensis* BS8, *B. cereus* L-07-01 and *B. mycoides* S-07-01 is in accordance with the findings of a previous study (Zhang et al., 2006). In addition, the present study detected the presence of *zmaR* and antibiotic biosynthetic genes for zwittermicin A and surfactin in *B. amylolquefaciens*, which has not been detected by Ramarathnam (2007). The presence of fengycin biosynthetic gene and the production of the antibiotic in the cell surface extract were observed only in a few strains. According to previous studies, fengycin was reported to be produced by *B. subtilis* (Vater et al., 2002; Ramarathnam et al., 2007) and *B. amylolquifaciens* (Koumoutsi et al. 2004). In the current study a *B. mycoides* strain (4079) was observed to contain the biosynthetic gene and produce fengycin, which has not been recorded yet in the literature. Although, the antibiotic biosynthetic gene for fengycin in the strain BS6 was not amplified using any of the 7 primer pairs used, MALDI-TOF MS analysis showed the group of mass peaks corresponding to isoforms of fengycin. As reported in other previously published research, bacillomycin D was detected in *B. subtilis* (Moyne et al., 2001; Ramarathnam et al., 2007) and *B. amyloliquefaciens* (Ramarathnam et al., 2007) strains in the current study. However, its production by a *B. mycoides* strain (4079), as observed in the current study, is not recorded in the literature. The detection of very low percentages of

bacillomycin D, fengycin and zwittermicin A producing bacteria among the studied isolates indicates that a comparatively low percentage of bacteria in the environment has the ability of producing these 3 antibiotics and within the same species the capability is rather variable. Not detecting mycosubtilin biosynthetic gene cluster in any of the strains tested might be an indication of the extreme rareness of mycosubtilin production among *Bacillus* antagonists.

The detection of antibiotic biosynthetic genes and their production by the *Bacillus* strains in the current study revealed that one of the mechanisms responsible for biocontrol exhibited by these strains could be antibiosis. Among the strains tested, the contribution of antibiotics in biocontrol could be very high for strains BS6, H-08-02, 3057 and 4079, in which several antibiotics were detected. However, there are strains that have shown >75% mycelia inhibition or control of *S. sclerotiorum* or/and *F. graminearum* (Fernando et al., 2002; Duncan et al., 2006) but detected to produce only surfactin and iturin A. There may be other types of antibiotics, which may be novel (not reported in literature) or different mechanisms might be involved in their biocontrol ability. In addition, the level of production of surfactin and iturin A by these strains could be higher than other strains. On the other hand, surfactin and members of iturin family (iturin A, bacillomycin D, etc.) are found to exhibit strong antifungal activity (Maget-Dana & Peypoux, 1994; Peypoux et al., 1999) and surfactin is considered as the most powerful biosurfactant known, which causes detergent-like action on biological membranes (Carrillo et al., 2003). These two themselves, therefore, might have the ability to exert significant pressure on pathogens. In several other studies the biocontrol ability of surfactin and iturin A were documented which supports this suggestion. For

example, Phae et al (1990) reported that more than 23 types of plant pathogens were suppressed *in vitro* by an iturin A and surfactin producing (Hiraoka et al., 1992; Asaka & Shoda, 1996) *B. subtilis* isolate. There are also instances in which some antibiotics showed stronger activity than others. Asaka & Shoda (1996) observed a significant stronger suppressive activity of iturin A against plant pathogens than surfactin. Kim et al. (2004) reported the stronger antifungal activity of fengycin producing strains in comparison to those producing surfactin and iturin A. Koumoutsi (2006) reported the comparatively higher effect of bacillomycin D than fengycin against various phytopathogenic fungi. The inconsistent performances exhibited by the same antibiotics in different studies suggest that their performance might be altered by various undetermined factors. Although the reasons have yet to be explored, differences in media used could be one possible explanation.

Another interesting phenomenon observed in this study was the types of antibiotics dominating in the mass spectra of different strains were different. For example, in the spectrum of BS6 peaks corresponding to surfactin, iturin A and bacillomycin D are prominent while in that of H-08-02 peaks representing surfactin are prominent. In the spectra of 3057 and 4079 peaks that represent bacillomycin D and iturin A are dominating reflecting higher intensity values of these antibiotics in the particular surface extracts. This indicates that although these strains have biosynthetic genes for surfactin, iturin A and bacillomycin D, at a certain time only one or two antibiotics are produced at high concentrations and the major antibiotic in action under a given set of conditions may vary from strain to strain. Since all the strains were subjected to similar conditions in the current study it can be inferred that the extents to which a

particular strain captures the environmental signals that induce the expression of different antibiotic genes differ among strains. Alternatively, the production of some antibiotics can be delayed relative to others (Hofemeister et al., 2004), which might have caused the differences in peak intensities. Although, a particular strain can produce more than one antibiotic a synergistic performance of them is occasional (Koumoutsi, 2006). This may be due to the lower production of some antibiotics over others under a given set of conditions. Strain 4079 is a good example of this where it was found to produce 4 of the antibiotics tested but the *S. sclerotiorum* mycelia inhibition shown was only 55%. The MALDI-TOF mass spectrum of strain 4079 showed high intensities for only iturin A and fengycin.

The results of this study together with those of other relevant studies emphasize the synergistic effect of several mechanisms in biocontrol besides antibiotics. However, at a certain time and under certain environmental conditions, antibiotic production may play the main role in its biocontrol of plant pathogens. This study revealed the equal capability of different *Bacillus* strains from various microhabitats to produce a variety of antibiotics and highlights the possibility of using some strains as potential biocontrol agents under diverse microhabitats distant from their origin. *Bacillus* sp. as BCA has several advantages over other organisms because of its resistance to heat and desiccation (Hou et al., 2006). Understanding the mechanisms of biocontrol at the molecular and biochemical level will enable us to develop rational strategies for the application of the antagonists and their metabolites within an agroecosystem.

6.0 GENERAL DISCUSSION AND CONCLUSIONS

The value of sunflower crop has been on the rise in Canada not only as an oil seed crop but as a confection crop, which has a wide value in snack food and baking industries. With the increase of the area of production of the crop, disease pressure has also increased remarkably. *S. sclerotiorum* is a pathogen as such, which causes three main diseases in sunflower. Due to the aggressiveness of the pathogen, lack of resistant germplasm of the plant together with various other environmental and economical issues, none of the available control methods are 100% successful. There is no registered fungicide to *Sclerotinia* diseases of sunflower in Canada. An integrated management system is, therefore, needed for the sunflower-sclerotinia host pathogen system and there is a need for continuous research on the management of *S. sclerotiorum* on sunflower in Canada.

The current study (chapter 3) investigated the efficacy of two control methods, BCAs and a fungicide, in controlling two sclerotinia diseases of sunflower under field conditions. In addition, the growth promotion ability of the BCAs was also investigated under field conditions. Conducting field trials is an important step towards the commercialization of the BCA since greenhouse trials under controlled environments do not demonstrate the real interactions of BCAs with the environmental factors. The results of the 2006 Morden trial further confirmed the fact that the excellent BCAs might not succeed against the pathogen when the disease pressure is high. The results also emphasized the importance of correct timing of application of the BCA and the pathogen. The method of pathogen application should also be appropriate so that it represents the

natural infection process. In the 2006 Morden trial, sclerotinia-infected ground millet seeds were used in addition to ascospores to induce head rot disease. Ground millet seed inoculum has mycelial fragments of *S. sclerotiorum*, which do not represent the natural infection process of head rot disease. Under appropriate environmental conditions PA23 and BS6 could be lethal to ascospore germination rather than to actively growing fragments of *S. sclerotiorum* resulting in a significant biocontrol. In addition, considerably higher bacterial densities maintained on petals 24 h after the application of PA23 and BS6 indicate the survival ability of these BCAs besides their natural ecological niche. However, whether BCAs can produce antibiotics and switch on other biocontrol mechanisms efficiently in environments outside their niches is a question and should be further studied for efficient use of BCA. According to the results of the 2007 Morden head rot trial, PA23 and BS6 have reduced DSI and AUDPC values compared to those of inoculated control although the reductions are not significant and no significant yield increase was documented. Therefore, biological and economical importance of the reductions is questioned. In the combined application of two BCAs, there was no consistent significant enhanced performance of one strain over the other which has also been observed by Zhang (2004) in the biocontrol study of stem rot disease in canola. However, PA23+BS6+Scl showed relatively lower (but not significant) DSI and AUDPC values than PA23+Scl and BS6+Scl. This supports the idea that to increase the efficacy of biocontrol, mixtures of BCAs should be used. This is mainly because the mixture provides the capability of using the product in a variety of environments, which at least one BCA in the mixture would perform well in different ecosystems. The natural habitat of PA23 is the rhizosphere and that of BS6 is the phyllosphere (Table 5.1). Therefore, by

adding BS6, the efficacy might have increased on the head. The fungicide tested, Ronilan, can be a fungicide against head rot but its use as a seed treatment against sclerotinia wilt would be more economical and advantageous since it has shown consistent increase in seedling emergence and yield over the two years. However, in the Morden head rot trial of 2007 some plants started to die and dry with thin stems and small heads. Collecting of data from those plants was not possible. This might have affected the results of the study and at least another field trial would be needed to further confirm these results. In addition, the significant reduction of DSI and AUDPC even under a low antagonist density on heads indicated that induced systemic resistance (ISR) could be one mechanism of biocontrol by PA23 and BS6.

None of the strains, either alone or in combination, controlled mycelial infections of *S. sclerotiorum* using both soil and seed treatments although the survival of bacteria on sunflower roots was considerable. Therefore, one of the reasons for the failure of these strains to control wilt could be the inappropriate timing of application of the BCA. If there was a way that BCA can be applied prior to the pathogen infection, then control might be achieved. Therefore, a greenhouse study which compares the efficacy of BCA against wilt disease when applied at certain time periods before the pathogen application should be studied prior to further field tests. This study highlighted the relative difficulty of using BCAs successfully in the rhizosphere compared to the phyllosphere.

Even with considerable bacterial numbers in the rhizosphere, a significant growth promotion could not be observed in both the 2007 Morden and Winnipeg trials. This indicates the inability of these strains to induce growth of sunflower or their growth promotion mechanisms might be hindered by the prevailing soil conditions. Since

bacterial volatiles found to induce growth of plants and both PA23 and BS6 are capable of producing volatiles, they may play a role in growth promotion. But the action of volatiles or their production might have been blocked in the wet soils that resulted from frequent rains that occured during the growing season.

Knowing the mechanisms of control and other factors that affect biocontrol of a BCA is important in order to interpret the field results and improve their efficacy. Therefore, the rest of the research focused on understanding the mechanisms of biocontrol of PA23 and BS6.

Studies in chapter 4 investigated genes involvedt in the production of organic antimicrobial volatiles by PA23. None of the genes tested was responsible antibioticproduction, which was not media dependent. This means that even under minimal nutrient conditions, PA23 can produce volatiles. Therefore, volatiles might help BCA to compete with other microorganisms when nutrients became limited in the environment. However, whether these particular genes can affect the amount of volatiles produced by PA23 is not clear and further studies with microextraction gas chromatography is suggested to test this hypothesis. Furthermore, the production of HCN by PA23 and some of its mutants can be a possible explanation for the *in vitro* inhibition of *S. sclerotiorum* shown by different mutants. This phenomenon indicates that the HCN might be more toxic to *S. sclerotiorum* than the organic volatiles since its absence caused a severe loss in its biocontrol ability. Furthermore, this study documented the limitations of using the particular volatile collection method for this type of investigation.

The root colonization ability of a BCA is important when controlling soil-borne plant pathogens and the role of antibiotics in the root colonization ability of a BCA is not

clear. Therefore, a greenhouse experiment was conducted to study this phenomenon (chapter 4). Unsterilized soil was used to mimic the real environment and to study the influence of indigenous microflora on the introduced BCA. The results showed that the rhizosphere microbe compositions, whether it is indigenous or introduced, seem to depend mainly on the nutrient availability. As long as there is no limitation of nutrients there might be no advantage of antibiotic production to a BCA. In the current study, the effect of antibiotic production by BCA on rhizosphere density seemed to appear after the 4^{th} week from seeding and until then no adverse effect of the number of introduced bacteria on indigenous microbes or vise versa was observed. Even though BCA was introduced at a high concentration, the percentage of sustainable population of the BCAs remained very low compared to the total bacterial population on the roots. Therefore, the amount of competition that the BCAs could exert on indigenous microflora and on a very aggressive pathogen such as *S. sclerotiorum* is questionable. Also, the rhizosphere colonization and antibiotic production are affected by a number of factors including temperature, moisture, etc. These may be the reasons for the failure of many BCAs in the field. Furthermore, siderophores seem to play a role in survival and rhizosphere colonization PA23. However, the results did not clarify the relative importance of one antibiotic (phenazine/pyrrolnitrin) over the other produced by PA23. This might be due to the fact that when the production of one antibiotic is blocked the production of the other antibiotic might have expressed more so that the BCA can equally perform.

Some *Bacillus* strains including BS6 isolated from our lab demonstrated control of *S. sclerotiorum* through agar diffusible plate assay. Therefore, one of the mechanisms of control was thought to be due to the production of agar diffusible antibiotics. To

investigate this hypothesis the presence of antibiotic related genes in 21 *Bacillus* strains were examined (chapter 5) using PCR and the production of particular antibiotics was confirmed by MALDI-TOF MS analysis. The results support the hypothesis that antibiosis could be one mechanism by which these strains inhibited *S. sclerotiorum*.

According to the results of this study, the potential use of *P. chlororaphis* PA23 and *B. amyloliquefaciens* BS6 in the control of head rot and wilt disease of sunflower is not well clarified and further research is needed specially with the formulation and delivery method. The researches should focus on the formulation and effective delivery systems early in the process of the development of a BCA rather than in the very late stage of the development. However, the possibility of developing the fungicide Ronilan as a short-term solution for wilt is apparent. The presence of antibiotic biosynthetic genes of several antibiotics in BS6 and other potential *Bacillus* sp. strongly support the idea that antibiosis is involved in control of pathogens shown by these strains. Apart from antibiosis (volatile and non-volatile), other mechanisms such as induced systemic resistanceand siderophore production, seem to be involved in the biocontrol of sclerotinia diseases by PA23 and BS6. However, the effect of each environmental factor on the biocontrol efficacy of these two strains should be studied separately before they are formulated for the commercial use. Furthermore, the failure of these strains in controlling diseases in sunflower-*Sclerotinia* host pathogen system demonstrates the fact that a particular BCA is not universally successful. Because a particular BCA is successful against a certain pathogen, on a certain crop under given set of conditions, does not mean that it would perform equally against the same pathogen in a different crop or different pathogen on the same crop. Each BCA/host-pathogen system should be studied

separately. Greenhouse studies, which involve continuous inoculation of the BCA to a particular host system and reisolation, are suggested before testing a BCA in a field study. This processs will allow the BCA to adapt to the particular host system and develop the BCA with it, gaining better survival. However, a aggressive pathogen such as *S. sclrotiorum* may develop resistance to a very successful BCA with repeated application for a period of time. And the future research should also been designed to address these possibilities.

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