

**MANAGEMENT OF VERTICILLIUM WILT OF POTATO
THROUGH THE USE OF BIOLOGICAL ALTERNATIVES
TO CHEMICAL PESTICIDES**

BY

AMARBEER K. UPPAL

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

Department of Plant Science
University of Manitoba
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**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of
Manitoba in partial fulfillment of the requirement of the degree**

Of

Master of Science

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ABSTRACT

Uppal, Amarbeer Kaur. M.Sc., The University of Manitoba, October, 2005. Management of Verticillium Wilt of Potato through the use of biological alternatives to chemical pesticides. Advisor; F. Daayf.

Verticillium dahliae Klebahn is an economically important pathogen in potato (*Solanum tuberosum* L.)-producing areas around the world. No single management practice provides consistent control of Verticillium Wilt of Potato. In this study, 18 bacteria and 4 plant extracts were tested for their potential biological control activity against Verticillium Wilt. Prior to biocontrol studies, two highly virulent *V. dahliae* (Vd1398-21 and Vd1396-9) out of 43 local isolates were first selected based on their higher pathogenicity. *In vitro* studies were then conducted to determine the efficacy of different bacteria in inhibiting the growth of the two *V. dahliae* isolates on two types of culture media. Three bacteria, S#1, W#2 (*Rahnella aquatilis*), and C#1 (*Bacillus amyloliquefaciens*) showed a high level of inhibition on the *in vitro* growth of the fungus. *Pseudomonas fluorescens* (DF#37) and *B. pumilus* (M#1) were included because of their previously reported ability to enhance plant defenses. These bacteria and four plant extracts (NH, RE, CE, SE) were then tested on two cultivars, Russet Burbank (moderately susceptible) and Kennebec (highly susceptible) under growthroom and field conditions during two growing seasons. Based on the first year results from growthroom and field trials, bacteria DF#37 and plant extract NH were found to be effective on Russet Burbank and Kennebec, respectively. During the second year's experiments, treatments were tested at two locations. Three treatments DF#37, M#1 and NH were significantly effective in reducing the disease and increasing the yield for cultivar Kennebec whereas for Russet Burbank, only DF#37 was effective.

FOREWARD

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

1.0 INTRODUCTION

Potato, a member of the *Solanaceae*, is the fourth most important food crop in the world (Desjardins et al., 1995). Manitoba is the second largest producer in Canada after Prince Edward Island (Field crop Report Series, Statistics Canada, 2004). It is primarily grown for processing (83%), seed (10%) and table-stock (7%) (Manitoba Agriculture and Food, 2003).

Verticillium Wilt is an economically important vascular disease of potato. It is one of the major components of Potato Early Dying Syndrome and is caused by *Verticillium dahliae*, a seed- and soil-borne fungus that is able to survive in the soil for several years as small, black, resting structures known as microsclerotia. Hence, microsclerotia are an important source of primary inoculum. This disease is characterized by premature senescence of affected plants and wilting of their foliage. In tubers, vascular browning is prominent (Rowe and Powelson, 2002).

Verticillium dahliae is known to have a wide host range, including vegetable and field crops, herbaceous ornamentals, shrubs, vines and trees (Bhat and Subbarao, 1999). *Verticillium dahliae* isolates vary in their pathogenic capabilities (Bao et al., 1998; Elena, 1999; Gennari et al., 2000).

Management strategies to control Verticillium Wilt aims at developing resistant cultivars, altering the efficacy of inocula or reducing the inoculum levels in the soil. Developing resistant cultivars would be an effective strategy. At present, there is no cultivar that is highly resistant to this disease (Rowe and Powelson, 2002). Due to the persistence nature and longevity of the pathogen in the soil, cultural practices such as crop rotation, vine removal are often not economically feasible (Subbarao and Hubbard, 1996;

Alstorm, 2001). Soil fumigation using chemicals such as methyl bromide or chloropicrin is an effective practice in reducing the inoculum levels in the soil (Fravel and Larkin, 2000). However, it is too expensive and incompatible with the cultural practices. Besides, concerns have been continuously raised regarding the use of chemicals in agriculture (Ioannou, 2000). There arose a need for more reliable alternative that can provide safer plant protection. Biological control seems to be the possible alternative to control this disease (Bloemberg and Lugtenberg, 2001) and can be incorporated into an integrated management strategy. Several biocontrol agents have been identified in the different plant-pathosystems (Keinath et al., 1991; Tjamos, 1993; Berg et al., 1994) but few have been tested against *V. dahliae*.

The objectives of the present study are as follows:

- (a) To select virulent isolates of *V. dahliae* from the local population of the pathogen;
- (b) To investigate *in vitro* inhibition of virulent isolates of *V. dahliae* by selected bacteria;
- (c) To explore the ability of (i) selected bacteria, previously shown to have either antibiotic production and/or defense reactions induction capacity and of (ii) plant extracts, to protect potato against the soil-borne fungus *V. dahliae* in growthroom and field trials, and
- (d) To investigate the responses of cultivars with different degrees of susceptibility to this disease under both growthroom and field conditions.

2.0 LITERATURE REVIEW

2.1 Potato (*Solanum tuberosum* L.)

2.1.1 Family and species:

Potato belongs to the family *Solanaceae*; the genus *Solanum*; and the species *tuberosum*. Many species from this family are very important to mankind. For example: potatoes, tomato, pepper as food crop; tobacco, deadly nightshade, thorn-apple, henbane, mandrake because of their alkaloid properties and as garden plants. There are more than 230 wild potato species (Hawkes, 1992).

2.1.2 Potato History:

Potato (*Solanum tuberosum* L.) originated in the Andes Mountains of South America (Salaman, 1949). The natives of Peru found potato as a valuable source of food and were the first to cultivate the earliest forms of potatoes approximately 4000 years ago (Ugent et al., 1982). In 1536, the Spanish invaded Peru and carried potatoes to their homeland. Further, potato was introduced into England in the 1590s (Hawkes, 1967). In 1565, potato was brought to Ireland and became very popular in the Irish diet. In Europe, a grain famine (1770), caused potato to become an important crop (Jordan, 1997).

In the 18th century, potatoes were introduced to North America when the British gained control of the French North American colonies (Hawkes, 1978). At present, potatoes are grown in all provinces and territories across Canada (Manitoba Agriculture and Food, 2003).

2.1.3 Acreage and Production:

Potato is the fourth most important food crop in the world after rice, wheat and corn (Desjardins et al., 1995). Potato production of major potato producing countries is

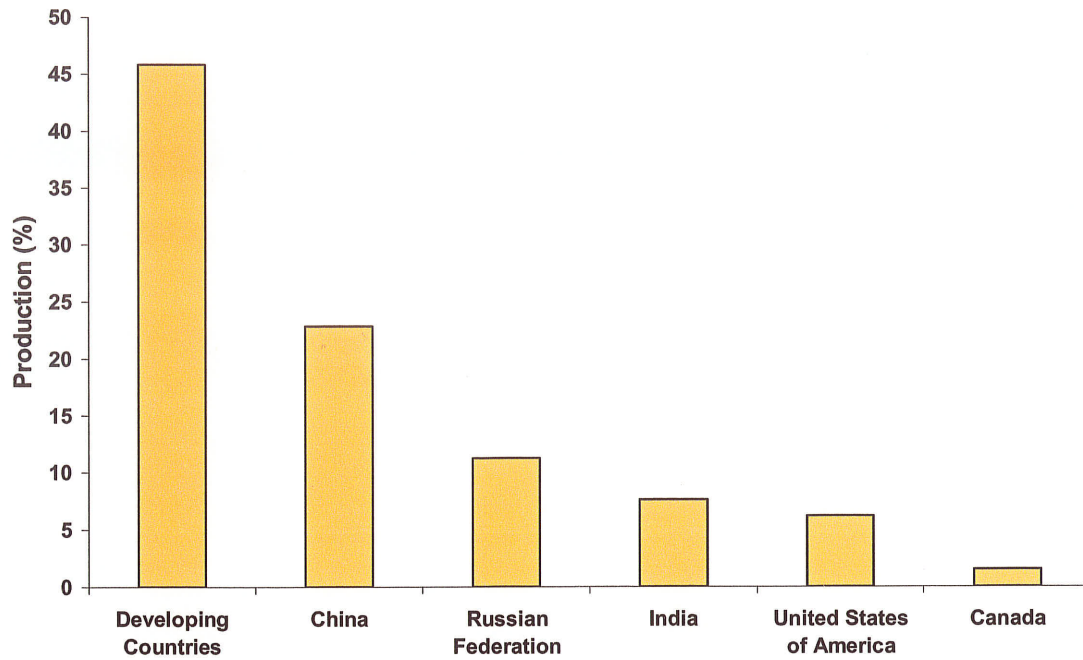


Figure 2.1 Potato production as a percentage of global production, 2004 (Data from World Food and Agriculture Organization Statistics, 2004)

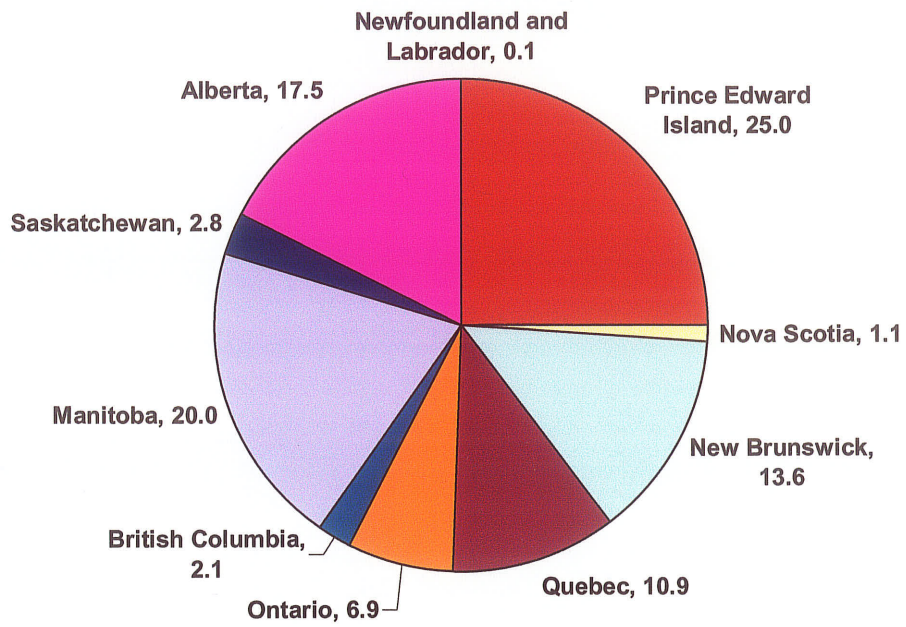


Figure 2.2 Potato production as a percentage of Canadian Production, 2004 (Data from Statistics Canada, 2004)

shown in Figure 2.1. More than 30 % of the global production now comes from developing countries (compared to 11 % in 1960s) (FAO Statistics, 2004). China is the largest producer (22% of the world's total production) followed by the Russian Federation (11%). Potato production of different provinces in Canada is shown in Figure 2.2. With 20% of Canada's potato production, Manitoba is the second largest producer after Prince Edward Island (25%) (Field Crop Report Series, Statistics Canada, 2004).

2.2 *Verticillium dahliae*

2.2.1 Introduction

Soil borne fungi of the genus *Verticillium* are the primary causes of PED in the different potato growing areas of North America. Two *Verticillium* species, *V. dahliae* Klebahn and *V. albo-atrum* Reinke and Berthier, have been identified in northern production areas, such as Maine and Red River Valley (Rowe and Powelson, 2002). *Verticillium dahliae* is the primary causal agent of Verticillium Wilt (Rowe et al., 1987; Rotenberg et al., 2004) but sometimes the fungus in conjunction with the root lesion nematode, *Pratylenchus penetrans* and other micro-organisms such as *Erwinia caratovora* and *Colletotrichum coccodes* causes a disease called Potato Early Dying (PED). This is also known as Early Die, Early Maturity Wilt, and Verticillium Wilt (Rowe and Powelson, 2002). *Verticillium dahliae* is more common in the north central states and Pacific Northwest where summer air temperatures frequently exceed 25°C.

2.2.2 Nomenclature and taxonomy

Verticillium dahliae belongs to the fungal class *Deuteromycetes* (Fungi Imperfecti), a group of fungi for which no sexual state is known (Hastie and Heale, 1984). In the genera *Verticillium*, parasexuality is the only known means of genetic exchange

among individuals (Heale, 1988). A heterokaryon is formed after hyphal anastomosis. Thus, vegetative incompatible isolates are genetically separated from each other. Isolates belonging to different vegetative compatibility groups (VCGs) may also vary in many characteristics, related to pathogenicity and aggressiveness (Korolev et al., 2000a; 2000b).

Verticillium is characterized by whorls of phialides produced along the length of undifferentiated filaments of conidiophores. Single-celled conidia are either ovoid or ellipsoid ($2.5 - 8.0 \times 1.4 - 3.2 \mu\text{m}$) in shape and borne on these phialides. The vegetative mycelium with 2-4 μm diameters is multinucleate and septate. *Verticillium dahliae* also forms dark brown coloured overwintering structure, also known as microsclerotia (30-60 μm in diameter) (Hawksworth and Talboys, 1970).

2.2.3 Host range

Verticillium dahliae is of major economic importance as it causes vascular wilt on a broad range of host plants (Agrios, 1997). This soil-borne plant pathogen is widely distributed throughout the world (Bhat and Subbarao, 1999). In North America, *V. dahliae* causes wilt on economically important crops such as vegetables, fruit and nut trees, legumes, forest trees, woody and herbaceous ornamentals (Goud and Termorshuizen, 2002). Distinct populations occur within the species and vary in their pathogenic capabilities (Bao et al., 1998; Elena, 1999; Gennari et al., 2000). *Verticillium dahliae* isolates exhibit different host specificity and differential pathogenicity on other hosts (Bhat and Subbarao, 1999). Isolates of *Verticillium* obtained from a given host can cause wilt symptoms in other hosts, but generally, the symptoms are more severe on the host from which they are obtained (Douhan and Johnson, 2001; Resende et al., 1994). Host specificity of *V. dahliae* is more pronounced on tomato, eggplant, bell pepper and cruciferous plants (Korolev et al., 2000a; 2000b). *Verticillium dahliae* isolates from

artichoke, eggplant, lettuce, tomato, watermelon can cause severe infection; isolates from bell pepper, cabbage, cauliflower were less aggressive and cause mild symptoms; and isolates from mint and cotton were non pathogenic on potato (Bhat and Subbarao, 1999).

2.2.4 Disease spread in the world

Verticillium Wilt is responsible for dramatic yield losses in many crops (Rotenberg et al., 2004). This disease is prevalent in the potato growing areas all over the world (Bhat and Subbarao, 1999). Verticillium Wilt is an important disease of potato in the irrigated areas of Central, Southern and western states of the United States (Rowe and Powelson, 2002). Potato has been a major crop every year in the areas of North Central States, the Red River Valley of North Dakota, Minnesota, and Idaho, where Potato Early Dying has developed slowly with time (Davis, 1981). Isolates of *V. dahliae* have been reported in Australia (O'Brien and Hutton, 1981), Morocco (Besri et al., 1984), South Africa (Ferreira et al., 1990), Negev desert in Israel (Nachmias et al., 1994), Northern Europe (Zielenski and Sadowski, 1995), Spain (Bejarano-Alcazar et al., 1996), China (Zhengjun et al., 1998), Cukurova region of Turkey (Kurt and Bicic, 1998), Greece (Elena, 2000), Japan (Kemmochi et al., 2000), Russia (Portenko, 2000). Verticillium Wilt also results in considerable losses in tree nurseries (Pierce and Gibbs, 1981; Sinclair et al., 1989).

2.2.5 Economic importance

Verticillium Wilt affects both the tuber size and the total marketable yield of the crop. The economic impact of Verticillium Wilt is significant as it lowers the yield by 30 to 50% and 10 to 15% in severely and moderately infected fields, respectively (Omer et al., 2000). Expensive management practices to control disease such as pre-plant soil

fumigation and low yields due to PED affects the income of the potato farmers in the irrigated production areas (Rowe and Powelson, 2002).

2.2.6 Sources of inoculum and pathogen spread

The fungus generally forms a large number of microsclerotia in infected potato vines (Schnathorst, 1981). Contamination of clean fields with *V. dahliae* can occur by wind, irrigation water or contaminated farm equipments (Huang, 2003). The primary means of infection is introduction of the fungus to the surface or within tissues of seed tubers. Certified seeds may be infected with *Verticillium* even though the tolerance level in certified seed is 0 % (Omer et al., 2000). Tuber samples collected from 224 certified seed lots in 14 U.S. states and four Canadian provinces were found to be infected with *V. dahliae* (Rowe and Powelson, 2002).

2.2.7 Disease cycle and Symptomology

Moist soils and a temperature range of 21-27°C are favorable for the fungus, but its growth is inhibited at temperatures above 30°C (Francl et al., 1990). Verticillium Wilt is a monocyclic disease (Figure 2.4). The fungus can survive for more than 10 years as microsclerotia in the soil, plant debris, or as mycelium in the vascular ring of the tuber (Schnathorst, 1981; Heale and Karapapa, 1999). The likelihood of plant infection depends on the inoculum density in the soil (Cappaert et al., 1992a; 1992b; Mol et al., 1996; Nagtzaam et al., 1997). The root exudates from the growing plants stimulate microsclerotial germination (Mol, 1995b). Nagtzaam et al. (1997) found that the microsclerotia must be present within a distance of 0.1 - 0.3mm from root surface. The germ tube of microsclerotia infect at or just behind the root tips (Bowers et al., 1996). After penetration, hyphae first colonize the cortical tissues and then, enter into vascular tissue (Bowers et al., 1996). In xylem vessels, the mycelium grows and conidia are

formed. Conidia move freely within xylem elements and through cell walls readily via pit apertures (Rowe and Powelson, 2002). The fungal mass and host reaction products in the vascular tissues block the upward movement of solutes and solvents in the plant. Systemic colonization of the plant soon results in chlorosis, necrosis and wilting of foliage. Further, the fungus begins to colonize the senescing tissues and the dark resting structures are formed in the dying leaves and stems (Mol, 1995a; Mol and Scholte, 1995a; 1995b). Subsequent cultivations incorporate the dead tissues into soil and release microsclerotia as the plant tissues decay. The largest contribution to soil inoculum is the incorporation of the dead infected tissues into the soil (Mol et al., 1996). Microsclerotia can remain viable for longer period of time but generally lose their viability when exposed to wet and warm soil as they are colonized and degraded by various bacteria and fungi (Tjamos et al., 2000).

Reduction in net photosynthesis and transpiration and increase in leaf surface temperature can occur several weeks before the appearance of visual symptoms (Saeed et al., 1997a, b; Bowden and Rouse, 1991). The most common symptom is the acropetal progression of chlorosis and necrosis of leaves followed by premature defoliation as shown in Figure 2.5 (Schnathorst, 1981). Sometimes, the symptoms can be visualized on only one side of the plant or on individual leaflet resulting in the death of symptomatic vines as shown in Figure 2.6 (Issac and Harrison, 1968). Verticillium Wilt is characterized by a premature defoliation 4 to 6 weeks earlier than natural senescence. A light brown vascular discoloration is often prominent in the lower stem of the infected plants and also in infected tubers (Figure 2.7).

2.2.8 Environmental influences on disease development

The development and progression of Verticillium Wilt is affected by abiotic

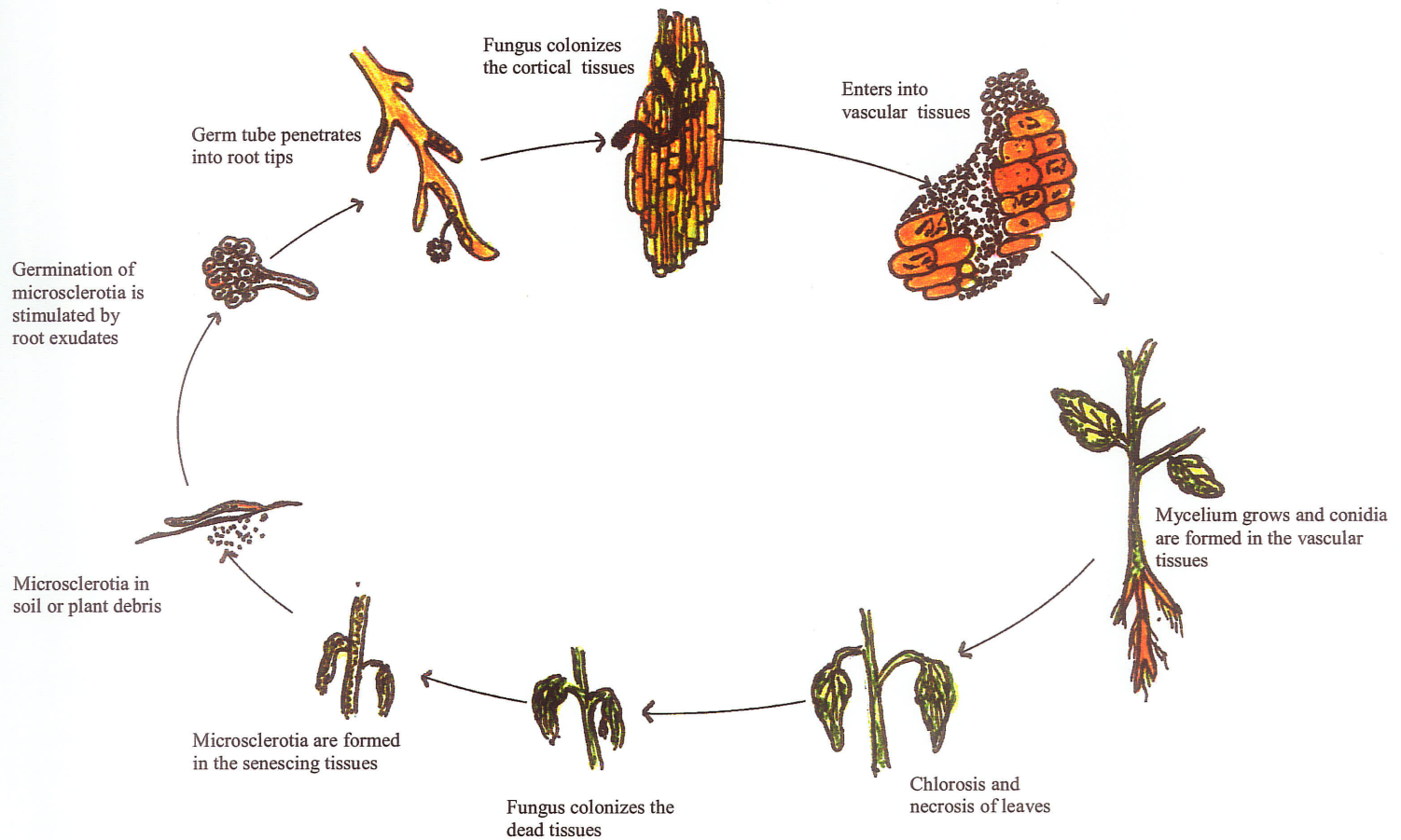


Figure 2.3 Life cycle of *Verticillium dahliae* in potato production system (Drawing and coloured by Amarbeer Uppal).



Figure 2.4 Acropetal progression of chlorosis and necrosis in potato plant (Photograph by: Amarbeer Uppal).



Figure 2.5 Chlorosis on one side of the individual leaflet (Photograph by: Amarbeer Uppal).

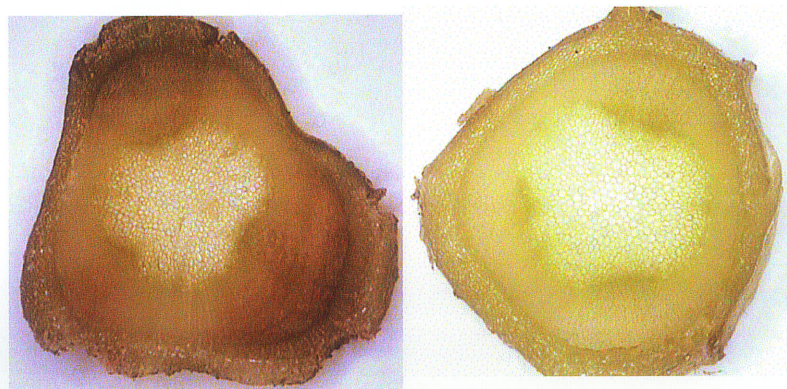


Figure 2.6 Cross-section of a potato stem with vascular discoloration (left) compared with a non-affected stem (right) (Photograph by: Amarbeer Uppal).

factors during the growing season. Temperature and moisture appear to be the most important factors (Francl et al., 1990).

Temperature varies with the geographic area. Development and severity of Verticillium Wilt symptoms and effects on yield in a geographical area depend upon temperature range during the potato-growing season of the region (Johnson, 1988). Potatoes grow optimally within a temperature range of 18 - 20°C. The optimum range for the growth of *V. dahliae* is 21 - 27°C (Powelson and Rowe, 1993). When the mean air temperature rises from 20 to 28°C during the potato-growing season, disease severity of the infected potato plant with *V. dahliae* tends to increase. In a field study, temperature effects on the symptom development and yield were analyzed by comparing results over different seasons or different locations. Francl and co-workers (1990) showed that periods of high temperature (>24°C) during early emergence and early tuber bulking reduced the yield from infected plants as compared to non-infected controls.

Soil moisture is another abiotic factor to affect the development of Verticillium Wilt in potatoes (Xiao and Subbarao, 2000). Cappaert et al. (1994) reported that Verticillium Wilt symptoms developed earlier and were more severe in the moderately resistant variety of potato during wet growing season than in a dry season. In field microplot study, irrigation treatments were applied either early or midseason to determine the effects of soil moisture status on the severity of Verticillium Wilt. Disease severity was greater in treatments with excessive as compared to optimal or deficit early season moisture treatments. Mid-season soil moisture status had no effect on the expression of wilt symptoms (Cappaert et al., 1992a; 1992b). Vascular diseases are favoured by soil moisture and evapotranspiration conditions that ease the flow of water, nutrients and conidia of the pathogen into the stem of the plant (Powelson and Rowe, 1993).

2.2.9 Role of root lesion nematodes and other micro-organisms

Verticillium dahliae is believed to be the primary pathogen in Potato Early Dying Syndrome but it has been found that root lesion nematodes and other micro-organisms such as *Erwinia carotovora*, *Colletotrichum coccodes* are associated with *V. dahliae* in many irrigated potato production areas (Tsror and Hazanovsky, 2001).

Root lesion nematodes are migratory endoparasites, i.e. they enter root tissues and can also move freely through soil from root to root. All stages persist in soil in the absence of host plants, but fourth-stage juveniles and adults are the primary overwintering forms. Nematodes hatch as second-stage juveniles and molt three times before becoming adults and reproducing. Juveniles and adult nematodes feed on root surfaces and burrow into the root cortex (Saeed et al., 1999). Many species of *Pratylenchus* have been described, but four are commonly found on potato in North America (*P. crenatus*, *P. neglectus*, *P. penetrans*, *P. scribneri*) (Brown et al., 1980; Wheeler et al., 1992). These nematodes have wide host ranges, including vegetable, forage and fruit crops, and many weed species. Some host favours the increase in nematode populations whereas others support only limited reproduction. Higher nematode populations, particularly of *P. penetrans* in soil can directly affect the growth and development of potato (Florini and Loria, 1990). Synergistic interaction between *Verticillium dahliae* and *P. penetrans* has been observed on potato (Rotenberg et al., 2004). The mechanism of the synergism with root lesion nematodes is not clear. *V. dahliae* enters into root tips, the region which is least favoured by *P. penetrans* (Zunke, 1990). Previous studies have shown similar phenomenon i.e., nematode feeding sites on potato roots were not spatially related to entry sites of *V. dahliae* on the same roots (Bowers et al., 1996). *Pratylenchus penetrans* feeds and remain outside the vascular system throughout its life cycle (Zunke 1990), where as

the fungus grows and reproduces within the root and stem (Rotenberg et al., 2004). Higher infections of root tip were seen in the presence of several species of *Pratylenchus* as compared to the plants infected with *V. dahliae* alone, which suggest that the nematode affects host physiology in a way that promotes vascular colonization (Bowers et al., 1996). It has been suggested that *P. penetrans* changes host nutrition and hormonal balances and also suppresses phytoalexin production (Saeed et al., 1999).

In potato, *E. carotovora* causes blackleg and aerial stem rot, results in premature death of foliage. The disease can be distinguished from Potato Early Dying by the completely hollowed pith of the dead vines (Tsrer et al., 1990). In some areas, *E. carotovora* systemically infects the vascular tissues, which may result in foliar symptoms similar to Potato Early Dying (Powelson and Rowe, 1993).

Colletotrichum coccodes causes Black dot of potatoes and is known to form abundant, small, black sclerotia on senescent and dead potato roots, stolons, and stems (Tsrer et al., 1999). The common symptoms are chlorosis and necrosis of leaves and rot of the below-ground stem and roots (Lees and Hilton, 2003).

2.3 Management of Verticillium Wilt:

2.3.1 Introduction

Potato Early Dying is a complex disease as its development is favoured by a number of factors such as populations of *V. dahliae* and other associated micro-organisms (*P. penetrans*, *C. coccodes*, *E. carotovora*); lack of host resistance; and conducive environmental conditions for disease development (Sedegui et al., 2000). Soil-borne diseases of potatoes can cause significant yield losses to this crop at all stages of growth: seedling, maturing plants and tubers (Xiao and Subbarao, 2000). Diseases can be

prevented by those management practices that enrich the native microflora, thereby keeping the pathogens from becoming established either directly or indirectly.

Management strategies for the control of this disease aimed at reducing the populations of these pathogens in soil, altering the efficacy of the inocula, or developing resistance in the host (Subbarao and Hubbard, 1996). Presently, Verticillium Wilt is controlled by application of chemicals (pre-plant soil fumigation with metham sodium, methyl bromide), cultural methods (crop rotations, organic amendments, irrigation, fertilization), host resistance, and biological methods.

2.3.2 Chemical control

For several years, soil fumigants such as chloropicrin, metham sodium, methyl bromide has been used effectively to control Verticillium Wilt of potatoes (Ben-Yephet et al., 1989; Fravel and Larkin, 2000). Soil fumigation can be easily accomplished either by applying in water directly through sprinkler irrigation systems or by incorporating fumigants into the soil through tillage followed by irrigation. For the soil fumigants, the recommended application rate varies from 140 to 180 kg a.i. /ha, resulting in an average cost of more than \$250 per hectare. In the soil, the soil fumigant (Metam sodium) is converted into methyl isothiocyanate (MITC). Soil fumigation is highly effective in reducing the populations of soil-borne fungal pathogens, nematodes, weeds, soil-borne insects. It has been reported that pre-plant fumigation of potato fields increases yield by 16 to 32% in Israel (Ben-Yephet et al., 1983) and of 5 to 10 tons/ha in the Pacific Northwest (Davis and Sorensen, 1986). In many potato production areas of North America, soil fumigation became a common practice during the last decade (Omer et al., 2000).

Despite of all these advantages, soil fumigation is expensive and incompatible with cultural and biocontrol practices. Concerns have been raised regarding environmental protection. Soil fumigation results in the enhanced destruction of the beneficial soil micro-organisms, potential contamination of the ground water, drift of sprinkler-applied products, depletion of atmospheric ozone (Noling and Becker, 1994). As this practice is detrimental to the environment, there is an urgent need for alternative solutions (Ioannou, 2000).

2.3.3 Cultural control

2.3.3.1 Crop rotation

Crop rotations have been used for many years to reduce the levels of soil inoculum as it deprives the pathogen of its host plant (Cook and Baker, 1983). Sometimes, the inoculum loses viability when it is lysed by other natural micro-organisms in the soil.

In many potato production areas, crop rotation is practiced to improve the soil structure and fertility. Long rotations that include non-host crops of *V. dahliae* can reduce levels of pathogens and pests in the soil but may not be economically feasible for growers (Xiao et al., 1998). Cereals (barley, buckwheat, field corn, oats, Sudan grass and wheat), legumes (Austrian winter pea, alfalfa, clover, and milkvetch), and *Brassica* crops (canola, radish, turnip) are grown in rotation with potatoes (Davis et al., 2000). As the root-lesion nematodes have a wide host range, which includes alfalfa, corn, soybeans, and wheat, different from that of *Verticillium* (Florini and Loria, 1990), crop rotation may not be appropriate alternative to manage Verticillium Wilt. The effectiveness of crop rotation in potato production as a cultural practice to control Verticillium Wilt is limited by the fact that microsclerotia can survive in the soil for several years even in the absence of

susceptible hosts (Harrington and Dobinson, 2000). So, crop rotation is not regarded as a practical approach for the management of Verticillium Wilt (Rowe and Powelson, 2002).

2.3.3.2 Soil Solarization

Solarization is a non-chemical approach to disinfest soil, first developed to control soil-borne pathogens (Katan, 1981). Pathogen control is accomplished by covering the soil surface with a clear plastic film to trap solar radiation and accumulate heat (Ioannou, 2000). Soil temperature can be raised to levels that are lethal to many plant pathogens. Soil solarization is usually conducted for at least 4 weeks, to achieve pathogen control to a depth of 45-60 cm (Gamliel et al., 2000). Soil solarization has been used to reduce the number of propagules but it is restricted to warm and sunny climates (Subbarao and Hubbard, 1996). This method basically utilizes solar radiation and has been proven to be effective against soil-borne diseases of open-field tomato, cucumber and eggplant (Ioannou, 1999). Because of cold weather conditions, Solarization is not feasible in Manitoba.

2.3.3.3 Organic amendments

Organic matter plays an important role in altering soil properties, which include aeration, structure, drainage, moisture holding capacity, nutrient availability, and microbial ecology (Davey, 1996). Incorporating organic amendments and managing the crop residues in the soil help to improve the plant health and crop productivity (Bailey and Lazarovits, 2003). Farmyard manures and by-products of seafood and livestock industries have been used by farmers to maintain fertility of agricultural soils for millennia (Davey, 1996; Barker et al., 2000; Lazarovits, 2001). The intensive production

system has abandoned the use of organic amendments and shifted the trend towards monoculture cropping, intensive tillage, use of chemical pesticides, fungicides and synthetic fertilizers (Katan, 1996). This change in production system has adverse effects on the soil health and quality of water, resulting in an increase in plant diseases and other pest problems (Pimental et al., 1991; Hoitink and Boehm, 1999).

Nitrogen availability has been associated with Potato Early Dying and poor N-nutrition can increase disease (Chen et al., 1995b). Davis et al. (2001) conducted a study on 100 commercial potato fields for soil characteristics, disease and yield and found that organic matter, organic nitrogen and nutrient availability are the important factors associated with reduced disease and higher yield.

It has been found that incorporation of certain organic amendments such as meat and bone meal, soymeal and poultry manure at rates of 37 T/Ha and at a 15 cm depth, significantly reduces the incidence of Verticillium Wilt, Common Scab of potato and plant parasitic nematode populations (Conn and Lazarovits, 1999). High-nitrogen amendments help to control soil-borne pathogens (Stirling, 1991; Shiao et al., 1999). Degradation of high-nitrogen amendments by the soil micro-organisms into ammonia (NH_3) and nitrous acid (HNO_2) kills microsclerotia in the soil (Tenuta, 2001).

Crucifer crops have been shown to possess disease suppressive characteristics in a number of host pathosystems (Olivier et al., 1999). In general, these crops contain glucosinolates. During the decomposition of crucifers, the glucosinolates break down to produce sulfides, isothiocyanates, thiocyanates and nitriles that have fungicidal properties (Subbarao and Hubbard, 1996). The types and amounts of glucosinolates vary with crucifer species (Mayton et al., 1996) and also determine the level of pathogen growth reduction. At the time of application of a soil amendment, the degree of dryness of

crucifer residues and the amount of glucosinolates content in the crop is related to disease suppressive ability of the residue. The mechanism by which crucifer residues acts on plant pathogen is assumed to be fungitoxic nature of volatile chemicals released after glucosinolate breakdown (Fenwick and Heaney, 1983).

Blok and his coworkers (2000) reported that incorporation of organic amendments (Broccoli and ryegrass) followed by tarping helps to reduce the inoculum density of *V. dahliae* in the soil. Application of plastic cover or incorporation of organic amendments alone did not result in significant reductions of viable propagules in the soil compared with the control. Factors such as soil temperature, anaerobiosis and crop-specific compounds were most closely related to inactivate the pathogen propagules.

The addition of compost to the soil can reduce diseases caused by soil-borne fungi (Hoitink et al., 1997; Zhang et al., 1996) and increase water holding and ion-exchange capacity of that soil (Maynard, 1994). LaMondia and his coworkers (1999) found that soil amendment with spent mushroom compost reduced Potato Early Dying disease severity and dramatically increased yields.

Liquid swine manure, another promising amendment (LSM, 57 hL/Ha) was shown to provide an effective control against Verticillium Wilt and Potato Scab for three consecutive years with only one application (Conn and Lazarovits, 1999). Tenuta et al. (2002) found that the volatile toxic component(s) of LSM kills microsclerotia in the headspace within one day after application to the soil. Soil pH is the most critical factor in regulating the activity of LSM. LSM kills microsclerotia only if the soil pH is below 6 (Conn and Lazarovits, 2000) or above 8 (Tenuta et al., 2002). Addition of liquid swine manure to soil increases the microbial population and stimulates the biological control agents (Lazarovits, 2001).

2.3.3.4 Irrigation management

Verticillium Wilt is a threat to potato production in many areas of North America. The severity of this disease is influenced by soil moisture content. Higher soil moisture was related to an increase in the severity of Verticillium Wilt on potatoes (Cappaert et al., 1992a; 1992b). Under irrigation conditions, microsclerotia of *V. dahliae* may sporulate several times, causing an increase in the soil inoculum density. So, the amount and time of irrigation can be manipulated to suppress the disease (Powelson and Rowe, 1993).

In potatoes, modifications in irrigation practices for disease suppression must occur prior to tuber initiation stage to reduce the chance of malformed tubers. The period between plant emergence and tuber initiation is about 3-5 weeks. Soil moisture must be maintained at 80 to 90% available soil water following tuber initiation, to optimize growth and prevent tuber disorders. In Oregon State, irrigation management not only helped to suppress this disease but also increased tuber yield (Cappaert et al., 1992a; 1992b). Irrigation treatment after tuber initiation had no effects on rates of foliar senescence (Cappaert et al., 1994) whereas the amount of water available to the plant during the vegetative growth stage has direct effect on plant development. In the arid regions, method of irrigation, frequency of irrigation, and soil water content can be controlled. High soil water content early in the season may be critical to the onset of infection of potato plants, whereas water stress on plants after infection may enhance symptom expression. Soil beneath dying plants remains wet for long periods of time and wet soil may indirectly lead to tuber disease problems. An increase in tuber water potential from -0.8 to -0.67 MPa dramatically increased lenticel swelling and the susceptibility of tubers to infection by soft-rot erwinias (Cappaert et al., 1992a). Under irrigation conditions, microsclerotia of *V. dahliae* may sporulate several times, causing an increase in the

population density of the pathogen. The movement of conidia in the vascular tissues of plants and subsequent wilt development increase with excessive irrigation (Xiao and Subbarao, 2000). Salinity of water also affects the incidence of wilt disease. Nachmias et al., 1993 showed an interaction between salinity and incidence of Verticillium Wilt in potatoes. Increased salinity was positively correlated with increase disease incidence and related traits such as potato stem colonization with the pathogen and plant height reduction. Katan (2000a) reported that rotation with paddy rice, where flooding of the crop is an important agronomic practice, helps in eliminating *V. dahliae* from soil.

2.3.3.5 Vine Removal

Microsclerotia are formed within dying infected plant tissues (Mol et al., 1996). When the dying plant tissues are ploughed back into the soil, the microsclerotia are slowly released into the soil as the crop residues decay. Flaming kills the microsclerotia in infected vines. Over 95% of *V. dahliae* propagules were killed in mint and potato stems treated with propane flaming (Mpfung and Hall, 2002) and those that did survive did not persist long in the soil. Mpfung and Hall (2002) reported that flaming alone can reduce populations of *V. dahliae* in soil, severity of wilt symptoms and increase tuber yield by 20-42%. Flamed vines produced ashes rich in phosphorous (P) and nitrogen (N) that can increase tuber yield (Erich, 1991).

Removing vines was ineffective as compared to propane flaming because it has no effect on pre-existing populations of microsclerotia in the soil. Propane flaming can raise temperature at the soil surface, thus reducing viability of microsclerotia in the soil. Microsclerotia that survive Propane flaming of vines lose its ability to germinate in soil.

2.3.4 Host resistance

The development of resistant cultivars would be the most efficient, effective, economical and eco-friendly strategy to manage Potato Early Dying Syndrome. In North America, mainly seven cultivars are grown. All except Ranger Russet are moderately to highly susceptible to Potato Early Dying. In 2000, percent of potato acreage under Russet Burbank, Russet Norkotah, Superior, Shepody, Atlantic, Norland, Ranger Russet and others was 30, 11, 11, 7, 5, 5, 4, and 27, respectively (Rowe and Powelson, 2002).

In *Solanum* species, resistance to *Verticillium* is polygenic and complex (Concibido et al., 1994; Corsini and Pavek, 1996; Secor and Gudmestad, 1999; Jansky and Rouse, 2000). In the past 20 years, potato breeders have devoted considerable effort toward developing two resistant cultivars, Russet Nugget and Bannock Russet. The first single dominant gene, V_c , which confers resistance towards *Verticillium* in *Solanum chacoense* has been reported (Lynch et al., 1997). The transfer of this gene to tetraploid germplasm and screening of germplasm using several isolates of the pathogen can provide resistance for short term (Rowe and Powelson, 2002).

2.3.5 Biological control

The development of biological approaches for disease control as a substitute to pre-plant soil fumigation in the intensive agricultural system presents numerous challenges. Fumigation is being used in the first place as it effectively controls the yield limiting soil pathogens. Due to high efficacy of fumigation for disease control, the production systems have been continuously using methyl bromide.

With the increasing public concern over chemical residues in food, restrictions could be imposed on the use of some chemicals in agriculture (Larkin et al., 1998). An environmentally friendly alternative to protect the roots against fungal and nematode

pathogens is bacteria-mediated biological control (Hasky-Günter et al., 1998; Bloemberg and Lugtenberg, 2001). Biological control involves the use of naturally occurring micro-organisms to suppress the growth of pathogens (Martin and Bull, 2002; Chernin and Chet, 2002).

Mechanisms of bacterial antagonists towards plant-pathogenic fungi include the competition for nutrients and space, the production of antibiotics and toxins, or the production of fungal cell wall-degrading enzymes (Lugtenberg et al., 2001; Whipps, 2001; Chernin and Chet, 2002; DeSouza et al., 2003). The goal of a biological-based strategy is to manage the rhizosphere populations to optimize the plant growth. This activity may be accomplished by enhancing the beneficial micro-organisms, thereby increasing competition for space and nutrients. Vascular wilt fungi occupy a selective niche: the xylem vessels of their hosts (Pennypacker et al., 1990). Thus, biological control of these pathogens might be more effective if bacterial antagonists also colonize the vascular system (Sharma and Nowak, 1997). Bacteria, especially the *Pseudomonads*, produce secondary metabolites like antibiotics or pyoverdine siderophores (Loper and Buyer, 1991) or, compete for nutrients (Andrews, 1992), thus suppressing the pathogen. Strains of *Bacillus* sp. isolated from root tips were able to occupy the rhizosphere, grow endophytically in *Verticillium*-susceptible hosts, and significantly reduce symptom development caused by *Verticillium dahliae* (Tjamos et al., 1998a; 1998b).

Rhizosphere competence is an important pre-requisite for obtaining a successful biocontrol agent. It is well established that root-infecting pathogens are often highly dependent on exudates to initiate the plant infections (Nelson, 1991). The ability of the biological antagonist to metabolize these exudate molecules (Green and Jensen, 1995) and

colonize the root elongation zone may be an important step in the biological processes (Tjamos et al., 2004).

Antagonistic root-associated bacteria are an important functional group of beneficial bacteria responsible for the control of soil-borne pathogens (Sorensen, 1997). Holflich et al. (1994) found that many root-associated bacteria have a direct positive influence on plant growth and can stimulate plant health indirectly. Numerous studies have shown the ability of several rhizobacteria to suppress diseases caused by fungal pathogens and plant parasitic nematodes (Whipps, 1997; Emmert and Handelsman, 1999; Kruze et al., 2000).

The ability of bacterial strains to induce resistance (Kloepper and Beauchamp, 1992; Maurhofer et al., 1994; Chen et al., 1995a; Pieterse et al., 1996; Raupach et al., 1996), cross protect the plant against diseases (Reddy et al., 1993; Cartwright and Bensen, 1995; Mari et al., 1996; Paulitz, 1997) and other environmental stresses (Nowak et al., 1995; Lazarovits and Nowak, 1997) have been well documented. It has been reported that rhizobacteria producing antifungal compounds were used to control *Verticillium* Wilt (Berg et al., 1994, 1999; Kruze et al., 2000). Some bacteria have been found with potential to confer beneficial effects on plants both in terms of growth promotion and protection against fungal and bacterial diseases (Sayonara et al., 1996; Hallmann et al., 1997; VonBerg and Bahl, 1997; Sharma and Nowak, 1997). Recently, it has been demonstrated that bacterial endophytes have beneficial effects on host plants, such as growth promotion and biological control of pathogens (Frommel et al., 1991a; 1991b; Chen et al., 1994; Struz et al., 1997; Downing and Thomson, 2000; Ashikari et al., 2001). Endophytic bacteria have been shown to promote host metabolic changes, including the

formation of physical and chemical barriers at the sites of potential fungal entry for conferring protection against fungal infection (Benhamou et al., 2000).

Bacillus species especially *Bacillus subtilis* are known for their ability to produce a variety of antimicrobial substances and these may be involved in disease suppression (Buchenauer, 1998; Schmidt et al., 2001; Wulff et al., 2002). Wulff et al. (2002) showed that *Bacillus* strain BB was able to colonize roots of several *Brassica* crops and provide long-lasting protection against *Xanthomonas campestris* pv. *campestris*. Xylem-colonizing *Bacillus* spp. reduced the percentage of silver maple stems colonized by *Verticillium dahliae* and endogenous bacterial isolates have been used in the control of wilt on oaks (Brooks et al., 1994).

Laboratory studies done by Nowak et al. (1995) have shown that bacterization of potato plantlets with growth promoting rhizobacteria *Pseudomonas* sp. strain PsJN made them resistant to low inoculum levels of *Verticillium albo-atrum* and *Rhizoctonia solani*. When PsJN were applied in combination with *Serratia plymuthica*, they increased resistance of tomato transplants to *Fusarium oxysporum* f. sp. *lycopersici* (Frommel et al., 1991a; 1991b). The bacteria form epiphytic and endophytic populations on both *in vitro* (Frommel et al., 1991a; 1991b) and *in vivo* (Frommel et al., 1993) grown potato and *in vitro* grown tomato (Pillay and Nowak, 1997). As an endophyte, PsJN has the advantage of escaping microbial competition and also influencing host's response to pathogen attack. Mercado-Blanco and his co-workers (2004) have reported the suppression of Verticillium Wilt in olive stockings by root-associated fluorescent *Pseudomonas* spp.

Two bacteria, *Bacillus subtilis* and *Pseudomonas fluorescens* offered a significant control of Verticillium Wilt of tomato and enhanced plant growth (Tu and Zheng, 1996). A few studies have reported induced resistance in plants with the use of biocontrol agents

(VanPeer et al., 1991; Wei et al., 1991; Bargmann and Schönbeck, 1992; Schippers et al., 1993; Benhamou et al., 1997; Kim et al., 1997). Biological control agent, *Bacillus mycooides* isolate Bac J, is capable of inducing systemic acquired resistance in sugarbeet (Bargabus et al., 2002).

The performance of biological control agents is highly variable (Johri et al., 2003). The efficacy of the biocontrol agents is influenced by a myriad of environmental factors, both biotic and abiotic. The major factors that contribute to this inconsistency are the variable expression of genes responsible for disease inhibition and poor root colonization (Raajmakers and Wellers, 2001). Plant genotype may have a selective effect on the microorganisms that colonize the rhizosphere (Edel et al., 1997) and different cultivars may support different populations of organisms responsible for biological control (Smith and Goodman, 1999; Smith et al., 1999).

Burpee (1990) emphasized the impact of several abiotic factors (soil pH, temperature, moisture, soil type, components of soil atmosphere, inorganic and organic constituents of soil and quantity and type of pesticide applied to soil) on interactions among soil-borne plant pathogenic fungi and microbial antagonists. Specific environmental factors, which include soil composition and pH (Ownley, 2003), water availability (Hase et al., 2001) and mineral and carbon source availability (Shaukat and Siddiqui, 2003) affects the efficacy of biological antagonists.

2.3.6 Integrated disease management

Improved control of Verticillium Wilt requires the use of an integrated management in which more elements are implemented before the crop is planted (Mao et al., 1998). No single practice provides a complete control for the disease. With the use of chemical fungicides, the perceived health and environmental risks related to these

synthetic chemicals have made the trend to grow towards alternate diseases management strategies (Jacobson and Backman, 1993). Despite of the economic importance of the disease, little is known about the biological control of Verticillium Wilt (Powelson and Rowe, 1993).

Hence, there arises a need for more reliable and eco-friendly system, which would offer a safe and long-term crop protection. Biocontrol seems to be a promising alternative as it involves harnessing disease-suppressive micro-organisms to improve plant health (Handelsman and Stabb, 1996). A promising disease management strategy for the replacement of chemicals could be the implementation of biocontrol technology, used individually or as an Integrated Pest Management (IPM) component as suggested by Mao et al., 1998. A few bacterial strains with antagonistic potential against *V. dahliae* in different hosts have been identified (Pegg and Brady, 2002). Previous studies have shown that bacterial strains of *Bacillus*, *Erwinia*, *Flavobacterium*, *Pantoea*, *Pseudomonas*, *Serratia*, *Sphingomonas*, *Stenotrophomonas* and *Streptomyces* from *Brassica* species, *Capsella* spp. and *Fragaria* spp. showed control on the Wilt disease in oilseed rape and strawberry (Berg, 1996, Berg et al., 1994; 2000). There is need to find more efficient biocontrol agents against *V. dahliae* (Berg et al., 2005). Biocontrol agents includes some plant extracts and beneficial bacteria, that can be explored and manipulated to fine tune their efficacy in controlling Verticillium Wilt of potatoes.

3.0 Pathogenic variability of *Verticillium dahliae* Kleb. isolates collected from potato tissue in Manitoba.

3.1 Abstract

Verticillium dahliae, an economically important pathogen of potato, is found in cultivated soils of Manitoba. Presently, there is no management practice that provides complete control of this disease. The objective of this study was to assess the pathogenic diversity of *Verticillium dahliae* isolates collected in Manitoba, and to select the highest virulent isolates for further biocontrol studies. A collection of 43 *Verticillium* isolates from infected potato stems and tubers collected in Manitoba were included in this study. PCR assay with universal and specific primers showed that all the isolates belong to the genus *Verticillium* and 39 out of 43 isolates were *V. dahliae*, respectively. A pathogenicity study was performed on these 43 *Verticillium* isolates to test whether isolates from different fields and tissues differ in their ability to cause disease in potato. There was no pathogenic variability among isolates of different potato fields and storage units in Manitoba. Also, there was no effect of potato tissue (i.e. stem or tuber) for isolating the fungus. The pathogenicity test resulted in the selection of two *V. dahliae* isolates (Vd1398-21 and Vd1396-9) which had the highest pathogenicity levels on potato. These two *V. dahliae* isolates showed significantly faster radial growth rate in culture and higher percent infection on potato plants as compared to other isolates. Both *V. dahliae* isolates were obtained from infected tubers.

3.2 Introduction

Verticillium Wilt is a vascular disease, primarily caused by the root-infecting pathogen *Verticillium dahliae* Kleb. (Tjamos et al., 2000; Stevenson et al., 2001). The

fungus survives as microsclerotia either in the soil or within the plant debris (Schnathorst, 1981) and as mycelium in the vascular ring of potato tubers (Powelson and Rowe, 1993). Under favourable conditions, microsclerotia germinate and infect potato tissues leading to yellow-bronze leaf spots, vascular discoloration, premature vine death and yield losses (Karagiannidis et al., 2002).

Verticillium dahliae has a wide host range, including vegetable and field crops, herbaceous ornamentals, shrubs, vines and trees (Bhat and Subbarao, 1999). Pathogenic variability is generally observed among *V. dahliae* isolates collected from one or different plant species. This variation mainly depends on the plant species from which the pathogen is isolated and of the plant species onto which it was inoculated (Tjamos et al., 2000; Mercado-Blanco et al., 2004).

Pathogenic variability among *Verticillium* isolates has been the focus of many studies (Daayf et al., 1995; Mingochi and Clarkson, 1994; Tjamos, 1981; Skotland, 1971; Brown and Wiles, 1970; Boyle, 1963; Issac, 1957). The reactions, ranging from virulent to non-pathogenic, have been reported in several host plants (Chang and Eastburn, 1994; Ciccicarese et al., 1987; Horner, 1954).

The objectives of this study were:

- (i) to identify the genera and species of 43 *Verticillium* isolates collected in Manitoba,
- (ii) to determine the pathogenic variability of the *Verticillium dahliae* isolates obtained from different potato fields and storage units in Manitoba, and
- (iii) to select the virulent *Verticillium dahliae* isolates from the local population of the pathogen.

3.3 Materials and Methods

3.3.1 *Verticillium* isolates

Forty-three isolates were obtained either from diseased aerial plant tissues or infected potato tubers from different potato fields (F1, F2, F3, F4, F5, F6, F7, F8 and F9) and storage units (S1, S2 and S3) in 2002 during field surveys done by Blaine Sangster, Kurtis Ginter, Bill Moons, Susan Mohlberg, Tom Gonsalves, Tracy Shinnors-Carnelley and provided by Ms. Mardi Desjardins (Manitoba Agriculture and Food, Rural Initiatives). Single-spore isolates were cultured and maintained on PDA (Table 3.1).

3.3.2 Polymerase chain reaction using *V. dahliae*-specific primers

In order to identify the genera and species of 43 isolates, PCR assays with universal primers (forward: 5'-CTCATAACCCTTTGTGAACC-3' and reverse: 5'-CCGAGGTCAACCGTTGCCG3') and *V. dahliae*-specific primers from the ITS region (5'-CCGGTCCATCAGTCTCTCTG-3') and 5'-ACTCCGCATCAGTCTCTCTG-3') were performed, respectively (Nazar et al., 1991; Robb et al., 1994).

DNA was extracted directly from fungal mycelium and spores. 50 mg of fresh mycelium was taken in 1.5 mL of eppendorf tube containing 125 µL of 0.5 M sodium hydroxide solution. The mycelium was ground with sterilized plastic micro-pestle. After centrifugation for 6 min at 19600 × gravity (14,000 rpm), the supernatant was transferred to 500 µL of 100mM Tris-HCl and 4mM EDTA (pH 8.0) and stored at -20°C. One µL of DNA per reaction tube was taken and PCR amplification was conducted using 25 µL PCR reaction mixture containing 2.5 µL of 10X PCR buffer, 0.25 mM of each deoxyribosenucleotide triphosphate, 1.5 mM MgCl₂, 0.4 mM of each primer, 0.1 U of Taq DNA polymerase and water. The amplification was performed using Flexigene

Table 3.1 Source, identification and number of *Verticillium* isolates.

Source¹	Species²	Number of isolates³
Potato stem tissue	<i>V. dahliae</i>	21
	<i>Verticillium</i> species	3
Potato tuber	<i>V. dahliae</i>	18
	<i>Verticillium</i> species	1

¹Source includes infected potato stem and tuber tissue from which the pathogen was isolated.

²Species identified with PCR assay using *Verticillium dahliae* - specific primers.

³Number of isolates collected from different potato tissue.

thermocycler (Techne, Princeton, N.J.) with 5 min initial denaturation at 94°C and 35 reaction cycles consisting of 30 s denaturation at 94°C, 45 s annealing at 60°C, 2 min elongation at 72°C and 10 min for final extension at 72°C. DNA products were suspended in 6x loading buffer and analyzed on a 1.8% agarose gel (stained with ethidium bromide 1%) at 104-105V for 30 min.

3.3.3 Growth test:

Mycelial growth of 43 isolates was evaluated at 3, 6, 9, 12, 15 days after transferring plugs onto the potato dextrose agar media. A five mm plug of fungal isolate was taken from the edge of an actively growing colony, transferred to the centre of a Petri-plate, and incubated at 27°C. The diameters of all colonies were then measured. There were three replicate plates per isolate.

3.3.4 Pathogenicity assessment of *Verticillium dahliae* isolates

Pathogenicity tests were conducted on potato cv. Russet Burbank. The inoculum was prepared by incubating cultures of 43 isolates on PDA Petri-plates for 15 days at 20°C. The conidial suspension was prepared by pouring sterile distilled water and scratching conidia from fungal cultures on PDA. The final concentration of the conidial suspension obtained from each *Verticillium* isolate was 10^7 conidia/mL. Inoculation was performed by applying 1 mL drop of conidial suspension to the seed piece. Control seed pieces were treated in distilled water prior to sowing. There were three plants grown for each isolate. Seed pieces were sown in clay pots filled with autoclaved soil mix (sand, soil, peat and perlite; 4:4:4:1, v/v/v/v). Four-week old seedlings were re-inoculated by pouring 10 mL of conidial suspension (10^6 conidia/mL) in holes made in the soil with plastic spatula. Ten mL of distilled water were applied to control plants' soil. These

experiments were conducted in a controlled growth chamber (24±2°C, 80-90% RH, 12 hours photoperiod).

Four weeks after re-inoculation, all the plants were assessed and percent infection was calculated as follows: Infection (%) = $(C_L/T_L) \times 100$, where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants. To detect the inoculated pathogens in the plant, cross-section of basal stem tissue was collected from all the plants. PCR assays using *V. dahliae*-specific primers were also performed in order to confirm the presence of the pathogen in the potato stem tissue.

3.3.5 Data analysis

Differences in *Verticillium dahliae* isolates for radial hyphal growth and percent infection were determined by analysis of variance and means were compared by Tukey's test ($P \leq 0.05$). All the data was analyzed statistically using General Linear Models (GLM) of SAS Package (Statistical Analysis Systems Institute Inc., Cary, N.C., U.S.A.). Data on radial hyphal growth and percent infection were analyzed as completely randomized experiment using three replicates per treatment. Data recorded as percentages were arcsine-transformed before analysis in order to obtain the homogeneity of variance. The relationship between percent infection and PCR detection were determined by linear regression analysis using SAS Package and r value was statistical significant ($P \leq 0.05$).

3.4 Results

3.4.1 Identification of *V. dahliae* isolates by PCR analysis

The genomic DNA of all tested isolates was successfully amplified with universal primers. Electrophoresis patterns of PCR-amplified fragments are shown in (Figure 3.1). All tested isolates were found to belong to the genus *Verticillium*. PCR assay with *V.*

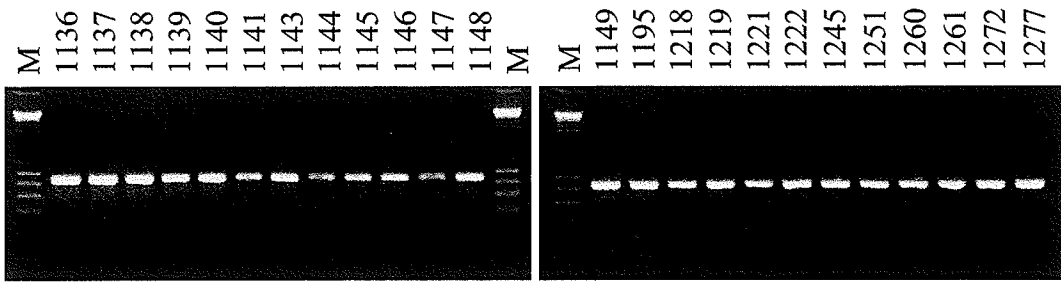
dahliae-specific primers amplified a 334bp DNA fragment (Figure 3.2) in 39 out of the 43 tested isolates. The 39 isolates were assigned to the species *V. dahliae* based on both PCR and morphological data, including the production of microsclerotia.

3.4.2 Radial hyphal growth of *Verticillium* isolates on PDA:

The isolates showed differences in the growth rate on PDA. Cultured colonies of two isolates (Vd1396-9 and Vd1398-21) achieved more than 75 mm of radial growth on PDA in 15 days. Only three isolates (Vd1219, Vd1221, and Vd1397-12) were found to be significantly different in their growth behaviour from the other isolates (Table 3.2).

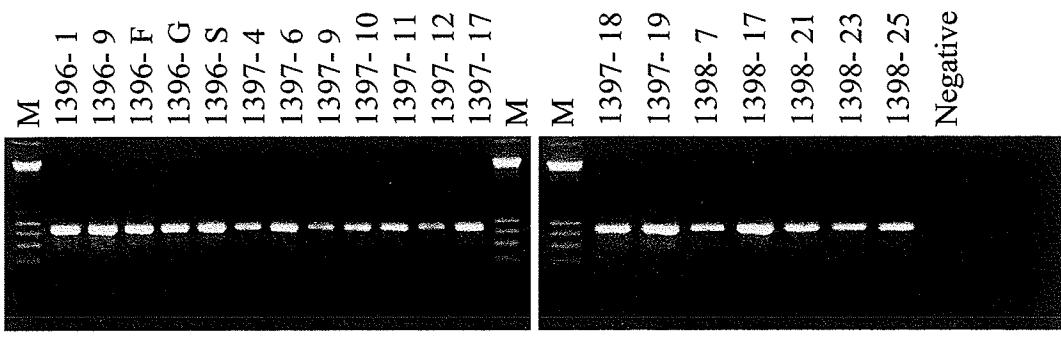
3.4.3 Pathogenicity of *Verticillium* isolates on potato plants

Potato plants infected with *Verticillium* isolates collected from infected potato stem and tuber tissues from different potato fields (F1, F2, F3, F4, F5, F6, F7, F8 and F9) and storage units (S1, S2 and S3) in Manitoba are shown in Table 3.2. All of the isolates were pathogenic to cultivar Russet Burbank although their pathogenicity varied widely. Isolates Vd1396-9 and Vd1398-21 showed the highest infection percentage. Vessel colonization of potato plants was confirmed by performing PCR analysis on basal stem sections. When basal stem sections were dissected and PCR-assayed for the presence of *Verticillium* species, most of them were found to be infected by *Verticillium* isolates. There was no significant effect of the source i.e. different potato fields and storage units on the pathogenic variability of the *Verticillium* isolate (Table 3.3). However, there exists a pathogenic variability among the isolates collected from the same potato field or storage unit. Differences in radial hyphal growth of the *Verticillium* isolates and percent infection caused by *Verticillium* isolates obtained from either infected stem tissue or tuber tissue on potato plant are shown in Table 3.4. There was no significant effect of the potato tissue i.e. stem and tuber on the pathogenic capability of the *Verticillium* isolates.



(A)

(B)



(C)

(D)

Figure 3.1 Agarose gel electrophoresis of PCR products (452 bp) using *Verticillium* species – universal primers. For (A), (B), (C) Lanes 1 – 12 and (D) Lanes 1 – 7 are *Verticillium* species. (D) Lane 8 Negative indicates the reaction mixture. The molecular size marker (M) is 100-bp DNA Ladder.

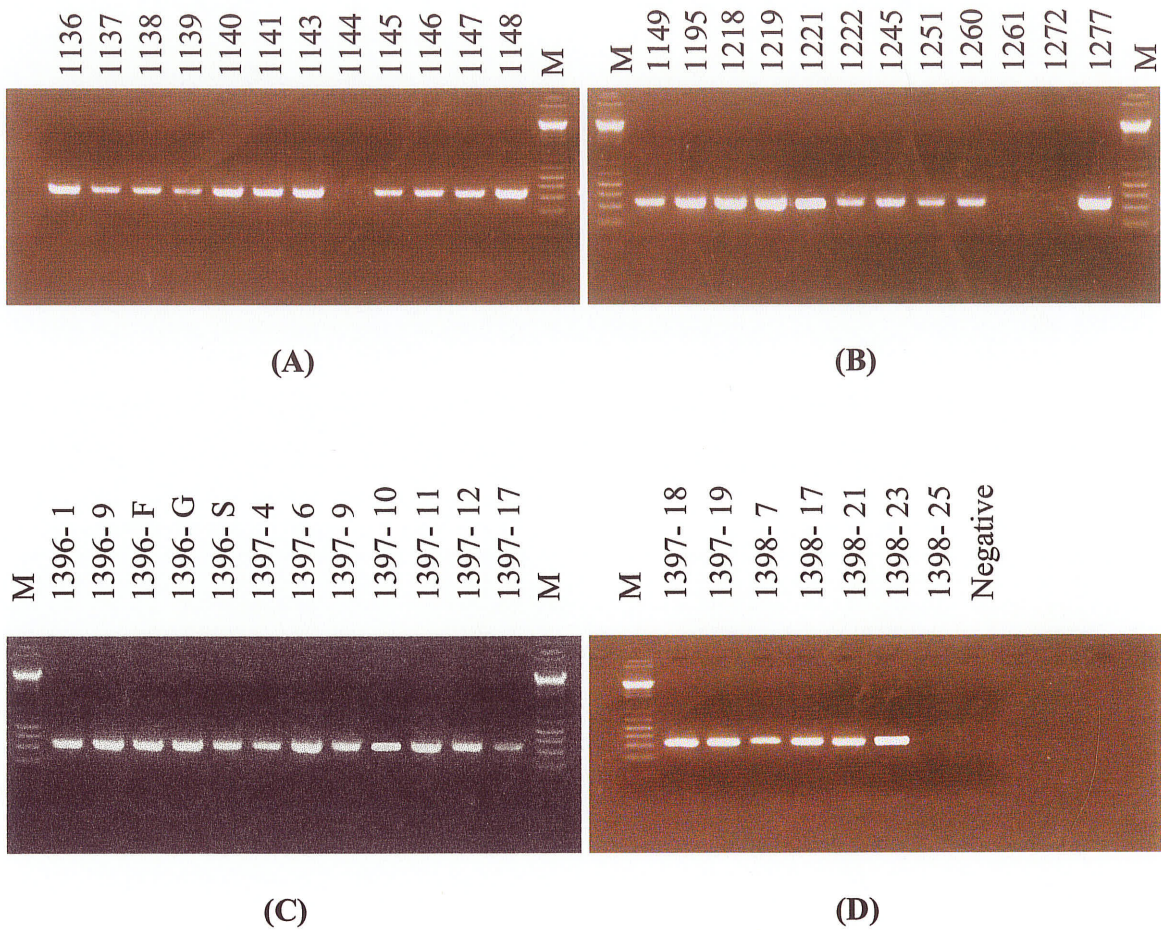


Figure 3.2 Agarose gel electrophoresis of PCR products (334 bp) using *Verticillium dahliae* – specific primers. For (A) Lanes 1 – 7 and 9 -12, (B) Lanes 1 – 9 and 12, (C) lanes 1 – 12 and (D) Lanes 1 – 6 are *Verticillium dahliae* isolates. For (A) Lane 8, (B) Lanes 10 – 11 and (D) Lane 7 are other *Verticillium* species. Negative indicates the reaction mixture. The molecular size marker (M) is 100-bp DNA Ladder.

The relation between radial hyphal growth in culture and percent infection caused by *Verticillium* isolates was determined. The linear regression showed that radial hyphal growth was proportional to percent infection ($P \leq 0.05$). The intercepts for percent infection and the slope of regression line differ significantly ($P \leq 0.05$) (Figure 3.3).

3.5 Discussion

In this study, the pathogenicity of 43 *Verticillium* isolates originating from infected potato stems and tubers was tested on cultivar Russet Burbank. The isolates were collected from different potato fields and storage units in Manitoba. This study showed a high pathogenic variability among these *Verticillium* isolates, which is consistent with previously reported findings (Strausbaugh, 1993; Bell, 1994; Resende et al., 1994; Ligoxigakis and Vakalounakis, 1994; Daayf et al., 1995; López-Escudero et al., 1996; Tsrer et al., 1998; Omer and Rowe, 1998; Katan, 2000b). However, such a pathogenic variability was found within each potato fields and storage units, but it was not observed among different potato fields and storage units. The lack of such pathogenic variability among fields and storages could be explained by the fact that both mildly and virulent isolates were found in both field and storage. PCR assay was performed to confirm the presence of the pathogen within the plant but the level of detection was not proportional to the level of symptoms observed in the plant. This discrepancy could be due to different spatial progression of each individual isolate within the plant. Isolates Vd1398-21 and Vd1396-9 were the most pathogenic whereas a few other isolates induced much less disease, as indicated by both foliar symptoms and vascular discoloration. These two most pathogenic isolates were isolated from tubers. The appearance of virulent isolates capable of causing severe wilt symptoms on potato could be due to the strong selection pressure

Table: 3.2 Radial hyphal growth of *Verticillium* isolates after 15 days of transferring plug on PDA media and Percent infection caused by *Verticillium* isolates on potato.

Source ¹	Designation ²	Radial hyphal growth ³	Percent Infection ⁴
F1	Vd1136	57.33a ⁵	12.74bc
	Vd1137	61.50a	13.47bc
	Vd1138	61.00a	16.84c
	Vd1139	59.33a	16.45c
	Vd1140	57.00a	8.44b
F2	Vd1141	60.67a	10.74b
	Vd1143	56.83a	11.45b
	V1144	68.33a	14.06bc
F3	Vd1145	68.17a	6.70ab
	Vd1146	67.00a	7.68b
	Vd1147	62.33a	15.58c
	Vd1148	68.00a	10.76b
	Vd1149	62.83a	9.81b
F4	Vd1195	63.67a	16.01c
	Vd1251	70.00a	18.98c
	Vd1260	64.67a	8.20b
F5	Vd1261	71.50a	16.48c
	Vd1219	49.67b	11.85bc
	Vd1221	52.00b	18.46c
F6	Vd1222	64.83a	16.07c
	Vd1218	54.33a	7.07ab
F7	Vd1245	66.67a	12.78bc
F8	V1272	62.33a	9.47b
F9	Vd1277	57.83a	18.27c
S1	Vd1396-1	58.33a	16.06c
	Vd1396-9	78.42a	26.39d
	Vd1396-F	55.17a	7.97b
	Vd1396-G	57.00a	15.16bc
S2	Vd1397-4	63.67a	13.03bc
	Vd1397-6	55.67a	11.29bc
	Vd1397-9	63.83a	8.33b
	Vd1397-10	67.33a	15.75bc
	Vd1397-11	59.00a	9.11b
	Vd1397-12	48.83b	11.80bc
	Vd1397-17	63.83a	8.96b
	Vd1397-18	62.17a	13.27bc
	Vd1397-19	61.00a	13.89bc
	Vd1398-21	78.67a	26.19d
S3	Vd1398-7	68.00a	8.38b
	Vd1398-17	60.17a	18.10c
	Vd1398-23	60.67a	18.61c
	V1398-25	63.17a	7.93b
-	Control	-	0.00a

¹Source of *Verticillium* isolates include different fields (F1, F2, F3, F4, F5, F6, F7, F8, F9) and storage units (S1, S2, S3) in Manitoba.

²Designation for *Verticillium* isolates to be addressed in the text.

³Radial hyphal growth of *Verticillium* isolates measured after 15 days of transferring the fungal plug on potato dextrose agar media. This study was conducted with 3 replicates per *Verticillium* isolates.

⁴Percent Infection was measured on all the plants (n = 3) per treatment after four weeks of re-inoculation. Percent Infection was measured as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

⁵Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

Table 3.3 Effects of *Verticillium* isolates collected from different sources (fields and storage units in Manitoba) on the radial hyphal growth and percent infection on potato plants (cultivar Russet Burbank).

Source ¹	Number of Isolates ²	Radial hyphal growth ³	Percent Infection ⁴
F1	5	59.23a ⁵	13.59a
F2	4	63.50a	10.74a
F3	4	65.04a	10.96a
F4	4	67.46a	14.92a
F5	3	55.50a	15.46a
S1	5	62.51a	16.30a
S2	10	61.65a	13.16a
S3	4	63.00a	13.26a

¹Source of *Verticillium* isolates include different fields (F1, F2, F3, F4, F5) and storage units (S1, S2, S3) in Manitoba.

²Number of isolates collected from each field and storage unit in Manitoba.

³Radial hyphal growth of *Verticillium* isolates measured after 15 days of transferring the fungal plug on potato dextrose agar media. This study was conducted with 3 replicates per *Verticillium* isolates.

⁴Percent Infection was measured on all the plants (n = 3) per treatment after four weeks of re-inoculation. Percent Infection was measured as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

⁵Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

Table: 3.4 Effects of *Verticillium* isolates obtained from different infected potato tissues (stem and tuber) on the radial hyphal growth and percent infection on potato plants (cultivar Russet Burbank).

Potato Tissue ¹	Number of isolates ²	Radial hyphal growth ³	Percent Infection ⁴
Stem	24	61.99a ⁵	12.48a
Tuber	19	62.16a	14.01a

¹Potato tissue includes stem and tuber tissue from which the pathogen was isolated.

²Number of isolates collected from different potato tissue.

³Radial hyphal growth of *Verticillium* isolates measured after 15 days of transferring the fungal plug on potato dextrose agar media. This study was conducted with 3 replicates per *Verticillium* isolates.

⁴Percent Infection was measured on all the plants (n = 3) per treatment after four weeks of re-inoculation. Percent Infection was measured as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

⁵Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

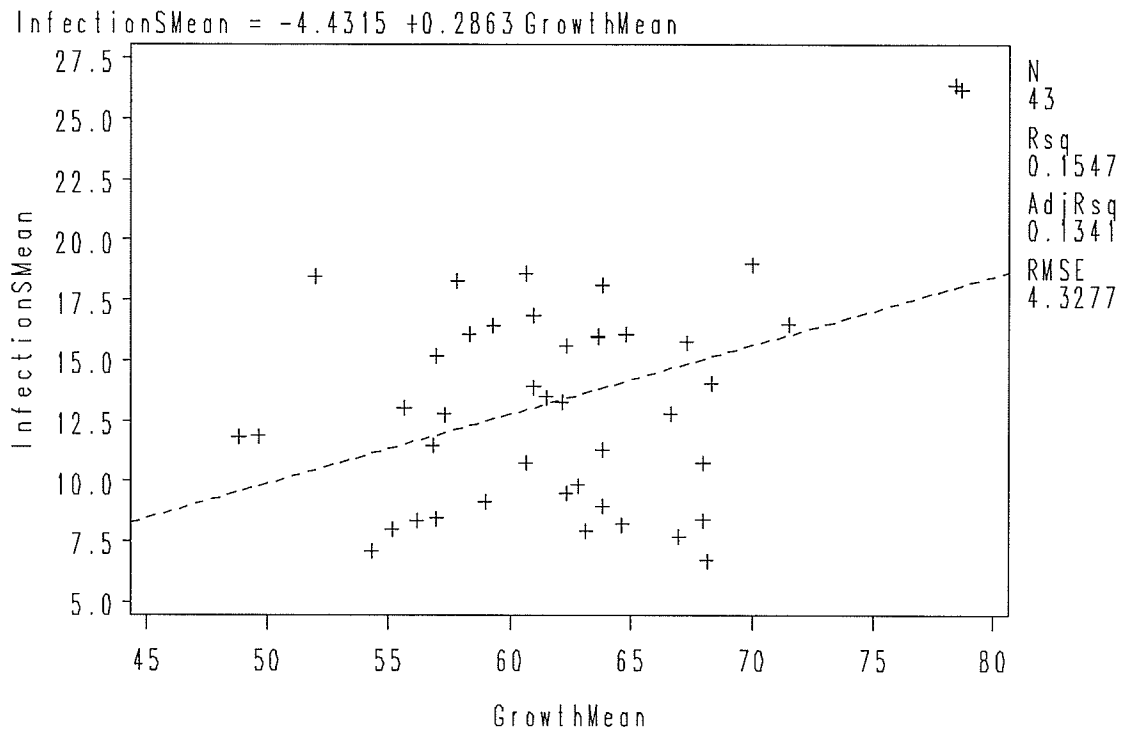


Figure 3.3 The regression of Percent infection with radial hyphal growth. Percent infection was measured on each plant after four weeks of re-inoculation with *Verticillium* isolates as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants. Radial hyphal growth of *Verticillium* isolates measured after 15 days of transferring the fungal plug on potato dextrose agar media. This study was conducted with 3 replicates per *Verticillium* isolates. Data were analyzed by linear regression, r value was statistically significant ($p = 0.05$)

exerted on the *Verticillium* isolates in the field (Bhat and Subbarao, 1999). The only mean of genetic exchange in genus *Verticillium* is asexual recombination, i.e. these isolates might be either clone of single isolate or they have evolved through asexual recombination.

Our results confirmed pathogenic variability among *Verticillium dahliae* isolates and the ability of most local isolates to cause the disease. The study also resulted in the selection of two virulent isolates that will be used later in biocontrol studies.

4.0 Comparative *in vitro*, growthroom and field screening of biological treatments for the control of Verticillium Wilt of Potato.

4.1 Abstract

Potato is an economically important vegetable crop worldwide. Verticillium Wilt disease, caused by *Verticillium dahliae* Kleb., is a constraint to potato production. Management of this disease has been pursued with a combination of chemical and cultural methods but none of them provide complete control. In this study, we aimed to explore biological treatments to control this disease. Eighteen bacteria were tested against two *V. dahliae* isolates previously selected for their highest pathogenicity levels. *In vitro* studies were initially conducted to determine the efficacy of these bacteria against the two *V. dahliae* isolates on two culture media (potato dextrose agar and nutrient agar). Based on the percentage of *Verticillium* mycelial growth inhibition, probably through antibiosis, *Bacillus amyloliquefaciens* 'C1', *Rahnella aquatilis* 'W2', and Unknown 'S1', were selected for further *in vivo* studies. Two bacteria, *Pseudomonas fluorescens* 'DF37' and *Bacillus pumilus* 'M1' were also selected for their previously demonstrated ability to enhance defense reactions in potato plants. In the *in vitro* tests, the inhibitory activity of the tested bacteria against the two *Verticillium* isolates was significantly higher on NA than on PDA media. Five pre-screened bacteria and four plant extracts were then tested on two cultivars Russet Burbank (moderately susceptible) and Kennebec (highly susceptible) under growthroom and field conditions during two growing seasons. First year results showed that one bacterium (DF37) and one plant extract (MV) were significantly effective in reducing Verticillium Wilt on Russet Burbank and Kennebec, respectively. Two application methods for plant extracts: seed treatment and incorporation into soil,

were tested. Disease expression was less when plant extracts were incorporated into soil as compared to seed treatment. During the second year, three bacterial treatments (DF37, M1 and MV) and one plant extract treatment (MV) were found to be effective in reducing the disease. Also, significant increases in yield, as compared to non-treated controls, were recorded for cv. Kennebec treated with DF37, M1 and MV, and for cv. Russet Burbank treated with DF37.

4.2 Introduction

Potato is an important crop in North America and the fourth most important food crop in the world (Desjardins et al., 1995). In recent years, the commonly called “Potato Early Dying Syndrome” has become one of the most important potato diseases in different areas of Canada and North America (Rowe and Powelson, 2002). This disease seems to be caused by a combination of pathogens, thereby the appellation “Early Dying Syndrome”. However, the main component to this disease seems to be a wilt caused by *Verticillium* sp. (Rowe & Powelson, 2002). This disease leads to premature senescence and wilting, leading to 30-50% yield losses (Omer et al., 2000). The second most known component of this complex is the root-lesion nematode, *Pratylenchus penetrans* (Rotenberg et al., 2004). This nematode interacts synergistically with *V. dahliae* to infect potato roots during the early stages of pathogenesis (Back et al., 2002).

Verticillium dahliae is a seed and soil-borne fungus that is able to survive in the soil for several years as small, black, resting microsclerotia (Schnathorst, 1981; Heale and Karapapa, 1999). The exudates from the plant’s growing root tips induce microsclerotial germination, and higher levels of inoculum in the soil favour plant infection (Nagtzaam et al., 1997). On potato plants, chlorosis of lower leaves followed by premature defoliation

is a common symptom (Rowe and Powelson, 2002). In the tuber, a brown ring is observed on cross-sections at the level of the vascular system (Ebihara et al., 2003).

Management of this disease is difficult not only due to the endophytic growth of the pathogen, but also because of its nature and longevity in the soil (Alstörn, 2001). The most common practice to control this disease is fumigation with broad-spectrum fumigants such as methyl bromide (Fravel and Larkin, 2000). The growing awareness about health and environmental risks related to the use of synthetic chemicals has made the trend grow towards alternate disease management strategies in many crops (Daayf et al., 2003, Ristaino and Thomas, 1997). Due to the economic importance of this disease, there is a need to find potential biological alternatives which can be incorporated with other management practices into more sustainable integrated management strategies.

Previous studies have reported that some bacteria and plant extracts can serve as excellent biological treatments against soil-borne pathogens (Pegg and Brady, 2002, Mercado-Blanco et al., 2004). Mechanisms of bacterial antagonism towards plant pathogens include (i) competition for nutrients and space, (ii) production of antibiotics, toxins, and host cell-wall-degrading enzymes, and (iii) elicitation of plant defense reactions known as “induced systemic resistance” (Ongena et al., 1999, 2000). Some bacteria, especially the *Pseudomonads*, produce secondary metabolites like antibiotics or pyoverdine siderophores (Thomashow and Weller, 1995), or compete for nutrients (Lugtenberg et al., 2003), thus suppressing the pathogen (Berg et al., 2000). *Bacillus* spp. are also well known as antibiotic producers with antagonistic activity against fungal and some bacterial pathogens (Krebs et al., 1998). Strains of *Pseudomonas* and *Bacillus* spp. isolated from root tips were able to occupy the rhizosphere, grow endophytically in *Verticillium*-susceptible hosts, and significantly reduce symptom development caused by

Verticillium dahliae (Lottmann et al., 2000; Tjamos et al., 2000). In addition to antibiotic production, the ability of these bacterial strains to grow endophytically has been considered as an important feature of the bio-control agents intended for use against vascular pathogens (Kloepper et al., 1999). However, only a few biological antagonists that may potentially develop into efficient and reliable biocontrol agents against *V. dahliae* have been identified (Berg et al., 2001). It has been also documented that plant extracts derived from seaweed, weed species, and *Brassica* species, may play a role in controlling different plant pathogens (Verkleij, 1992; Al-Abed et al., 1993; Olivier et al., 1999).

Currently, there seems to be many attempts around the world to initiate research about safer plant protection in general (Emmert and Handelsmann, 1999). Some biocontrol agents have been tested in different pathosystems (Whipps, 1997) but an effective biological treatment against Verticillium Wilt of potato is needed (Berg et al., 2001). Developing a more sustainable agriculture requires a re-thinking of plant protection strategies, which must integrate cultural practices, and both chemical and biological products against microbial pathogens.

One of the major initial problems in developing an effective biological control of *V. dahliae* is the screening method. Previous studies have reported inconsistency between the antagonistic effects under *in vitro* conditions and those on the whole plant system (Reddy et al., 1993). Weller (1988) suggested that biocontrol agents should be tested *in situ* rather than *in vitro*. However, such large-scale trials in the field are expensive and time consuming. In order to select potential biocontrol agents, *in vitro* screening is usually

primarily conducted. Then, field trials are conducted with those potential biocontrol candidates selected from the preliminary *in vitro* screening.

Three components seem to be essential for the choice of biocontrol agents (a) the choice of the pathogen's isolates and of the biological candidates to be tested, (b) the comprehensiveness of the testing system (*in vitro*, growthroom/greenhouse, and field trials), and (c) the ability to perform in specific environmental conditions.

In a previous study (Chapter 3), we assessed the pathogenic diversity of 43 *Verticillium* isolates collected from different potato fields and storage units in Manitoba. The two most virulent isolates were selected for the present study.

The objectives of this study were:

(i) to screen the bacterial strains for their ability to inhibit the growth of the two virulent *V. dahliae* isolates *in vitro*,

(ii) to compare the efficacy of selected bacteria and plant extracts to protect potato against *Verticillium dahliae* under different systems i.e. growthroom and field, and

(iii) to assess the efficacy of these treatments to two potato cultivars with different degree of susceptibility to Verticillium Wilt.

4.3 Materials and methods

4.3.1 *In vitro* studies

4.3.1.1 Bacteria

In the study, eighteen bacterial strains isolated from the phylloplane and rhizosphere of potato and canola were used (Daayf et al., 2003). These bacterial strains were identified with Biolog identification procedure. The identity and source of bacterial strains are shown in Table 4.1. The colonies of these bacterial strains were cultured on

Table 4.1 List of bacterial strains obtained from different sources.

Designation¹	Bacterial strains²	Source³
A1	Nd ⁴	Laboratory collection
A2	nd	Laboratory collection
B1	<i>Bacillus subtilis</i>	Laboratory collection
B2	<i>Bacillus pumilus</i>	Laboratory collection
B3	<i>Bacillus subtilis</i>	Laboratory collection
C1	<i>Bacillus amyloliquefaciens</i>	Laboratory collection
DF3	<i>Pseudomonas viridilivida</i>	Stem (cultivar Westar)
DF35	<i>Pseudomonas fluorescens</i>	Root tip (cultivar Cresor)
DF37	<i>Pseudomonas fluorescens</i>	Root tip (cultivar Cresor)
DF40	<i>Pseudomonas fluorescens</i>	Root tip (cultivar Cresor)
J1	<i>Bacillus subtilis</i>	Laboratory collection
M1	<i>Bacillus pumilus</i>	Laboratory collection
P1	<i>Pseudomonas putida</i>	Laboratory collection
R1	nd	Laboratory collection
S1	nd	Laboratory collection
W1	<i>Bacillus pumilus</i>	Laboratory collection
W2	<i>Rahnella aquatilis</i>	Laboratory collection
Y1	<i>Bacillus pumilus</i>	Laboratory collection

¹Designations used to address different bacterial strains in the text.

²Bacterial strains used in this study were identified with Biolog identification system.

³Source of the bacterial strains.

⁴nd stands for unknown bacterial strains.

nutrient agar media.

4.3.1.2 Pathogen cultures

Two *Verticillium dahliae* isolates (Vd1396-9 and Vd1398-21) previously screened for their high pathogenicity on potato were used to perform the *in vitro* studies. The isolates were maintained on potato dextrose agar (PDA) in Petri plates at 4°C. Five-mm plugs from *V. dahliae* cultures were transferred onto PDA Petri plates and then incubated at 25°C for 15 days.

4.3.1.3 Inhibition of pathogen growth *in vitro*

Eighteen bacterial strains recovered from different sources were cultured on nutrient agar media and incubated for two to three days at 20°C. Bacterial suspensions were prepared by adding 100 µL of sterile distilled water to the bacterial colony and mixing. Four replicates of 5 µL aliquots of the bacterial suspension were placed at four equally spaced spots on the circumference of each of the four Petri plates (9 cm in diameter). Two different media, potato dextrose agar (PDA) and nutrient agar (NA), were used in the study. A five-mm plug of *V. dahliae* isolate was transferred to the centre of the Petri plate. Four replicate Petri plates were prepared for each bacterial and fungal isolates. Petri plates were incubated at 20°C and the diameters of fungal colonies were measured over time using a ruler until the control colony reached 75 mm. This occurred 15 and 30 days after incubation on PDA and NA media, respectively. The percent mycelial growth inhibition provided by each bacterium was calculated as follows: (% inhibition) = 100 - [(B/V) × 100], where B is the radial growth of *V. dahliae* isolate when challenged with bacteria and V is the radial growth of the control *V. dahliae* isolate (Daayf et al., 2003).

4.3.2 Growthroom experiments

4.3.2.1 Plant material

Potato cultivars, Kennebec and Russet Burbank, were used in this study.

4.3.2.2 Bacterial treatments

Three bacteria were selected: *Bacillus amyloliquefaciens* (C1), *Rahnella aquatilis* (W2) and Unknown (S1) based on percent mycelial *in vitro* growth inhibition. Two other bacteria, *Bacillus pumilus* (M1) and *Pseudomonas fluorescens* (DF37) were also selected for their previously demonstrated ability to enhance resistance in potato plants against Late Blight. (Daayf et al., 2003). For bacterial inoculum preparation, colonies were each picked with autoclaved toothpicks and transferred into sterile 250 mL Nutrient broth in 500 mL Erlenmeyer flasks. The flasks were shaken for 2 days at 200 rpm and the suspensions centrifuged at $4080 \times$ gravity (5000 rpm) to settle down bacteria in the nutrient broth. Nutrient broth was drained from bottom of the plastic bottles. Bacterial inoculum consisted of the pellet transferred to sterile, distilled water, and adjusted to an absorbance of 0.14 at 640 nm (equivalent to 10^8 c.f.u./mL).

4.3.2.3 Plant treatments

Plant treatments were applied by two different ways: (i) incorporation of leaves in the soil, and (ii) application of plant extracts to potato seed piece. Different treatment application methods were first tested in the growthroom to assess the effects of treatments on Verticillium Wilt of potatoes.

4.3.2.3.1 Incorporation of leaves into soil:

The fresh leaves of Rapeseed from Winter-type *Brassica napus* cultivar Zhongyou 821 (RS) and Canada milkvetch "*Astragalus canadensis* L." (MV) were ground in liquid nitrogen. For Rapeseed and Canada milkvetch treatments, 20 g of powder was

mixed in 1 kg of an autoclaved soil-sand-peat-perlite mix (4:4:4:1, v/v) to fill each pot whereas for the Seaweed treatment *Ascophyllum nodosum* (SE), 20 ml of Seaweed extract (3 gm of Seaweed powder per litre of water) was added to each pot. The concentration of each treatment was 2 %.

4.3.2.3.2 Application of plant extracts to potato seed pieces:

Four plant extracts selected for growthroom and field experiments were Canola extract from cultivar Westar (CE), Rapeseed extract from Winter-type *Brassica napus* cultivar Zhong-you 821 (RS), Seaweed extract *Ascophyllum nodosum* (SE), and Canada milkvetch *Astragalus canadensis* L. (MV). Fresh leaves from each plant species were first washed in running tap water and distilled water. Thirty grams of air-dried leaves were ground into fine powder with liquid nitrogen. The extraction was done with hot water and 80 % ethanol. The flask was covered with aluminium foil and shaken overnight at room temperature. It was filtered through Whatman No. 1 filter paper. After filtration of the total extracts, the extracts were evaporated to dryness in vacuo and weighed. The extract was further diluted by 10^{-1} . Dilutions were prepared in sterile distilled water. The diluted liquid extract (1 mL/ seed piece) was used to treat each potato seed piece.

4.3.2.4 Fungal Cultures

Conidial suspensions of the two *V. dahliae* isolates (Vd1396-9 and Vd1398-21) were prepared by pouring sterile distilled water and gently dislodging conidia from mycelia cultures on PDA with a loop. 100 μ L of conidial suspension was transferred to Erlenmeyer flasks containing 100 mL of Sucrose Sodium Nitrate (SSN) medium. The flasks were shaken at 120 rpm for a week. The concentrations of the conidial suspensions of the two *V. dahliae* isolates were measured with haemocytometer. Suspensions from both isolates were equally mixed to prepare the inoculum with concentration 10^6

conidia/mL. Ten ml of conidial suspension (10^6 conidia/mL) was used to inoculate each plant in all growthroom experiments (GR1, GR2, GR3, and GR4).

4.3.2.5 Growthroom trials

Four experiments were carried out using potato cultivars Kennebec and Russet Burbank to study the efficacy of bacteria and of plant treatments against *V. dahliae* (Table 4.2). In experiment GR1, four bacterial treatments were included [*Bacillus amyloliquefaciens* (C1), *Bacillus pumilus* (M1), *Pseudomonas fluorescens* (DF37), and Unknown (S1)]. For experiment GR2, the same four bacteria and an additional bacterial treatment [*Rahnella aquatilis* (W2)] were tested. In experiment GR3, plant treatments [Rapeseed extract (RS), Canada milkvetch (MV) and Seaweed extract (SE)] were incorporated into autoclaved soil mix, whereas in experiment GR4, potato seed pieces were treated with plant extract solutions [Canola extract (CE), Rapeseed extract (RS), Seaweed extract (SE), and Canada milkvetch (MV)]. Potato seeds were sown in clay pots (10 cm in diameter) in an autoclaved soil-sand-peat-perlite mix (4:4:4:1, v/v). In experiments GR1 and GR2, treatments were as follows: (i) Water – Control, (ii) Pathogen alone, (iii) Bacteria alone, and (iv) Bacteria pre-treatment plus pathogen. In experiments GR3 and GR4, treatments included: (i) Water – Control, (ii) Pathogen alone, (iii) Plant treatment alone, and (iv) Plant treatment as pre-treatment plus pathogen. In the experiments GR1, GR2, GR4, pre-treatments were applied by dipping the seed piece in water, in plant extracts solutions, or in bacterial solutions whereas in GR3 experiment, pre-treatment was incorporated in soil. The potato seed pieces were treated 24 hrs prior to sowing and kept at seed storage conditions until further inoculation (4°C and 90-95% RH). This was repeated with five seeds. Inoculation with *V. dahliae* was performed 4

weeks after pre-treatments. Ten mL of fungal conidial suspension (10^6 conidia/mL) were poured in 6 cm-deep wells made around each plant. Control plants received sterile, distilled water instead of the fungal inoculum. Experiments were conducted in controlled environment chambers (22°C day and 17°C night, 16 h photoperiod, 80-90% RH). Six weeks after inoculation, plants were assessed for percent infection, wilt severity on individual plants and vascular discoloration of the lower section of the stem as follows:

Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

Rating scales were developed to assess the Wilt severity on potato (Figure 4.1) and discoloration of vascular tissues in the potato stem (Figure 4.2). Wilt symptoms were rated according to the following scale: 0 – no Wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves. The rating scale for evaluating the severity of vascular discoloration of potato stems caused by *V. dahliae* was as follows: 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

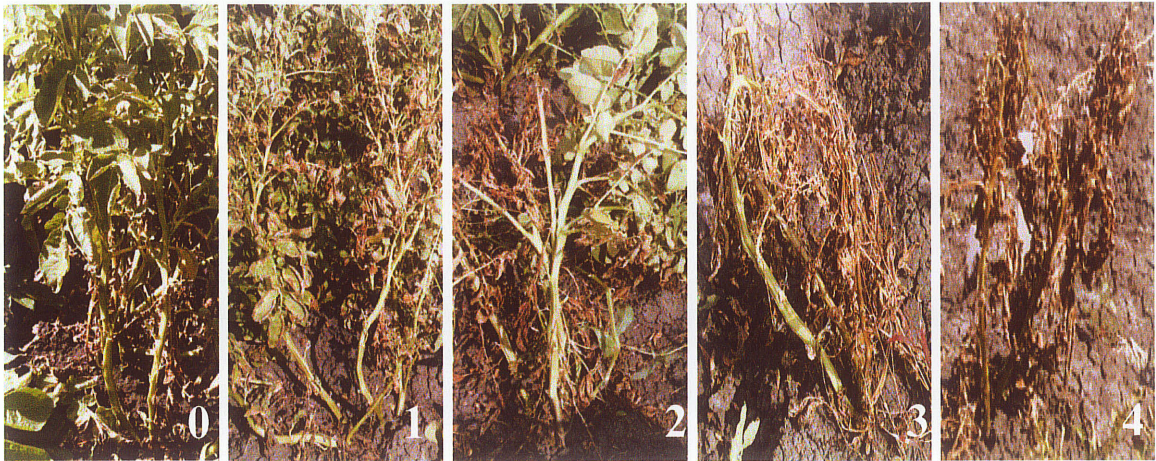


Figure 4.1 Rating scale for the Wilt severity on potato plants caused by *V. dahliae*: 0 – no Wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves.

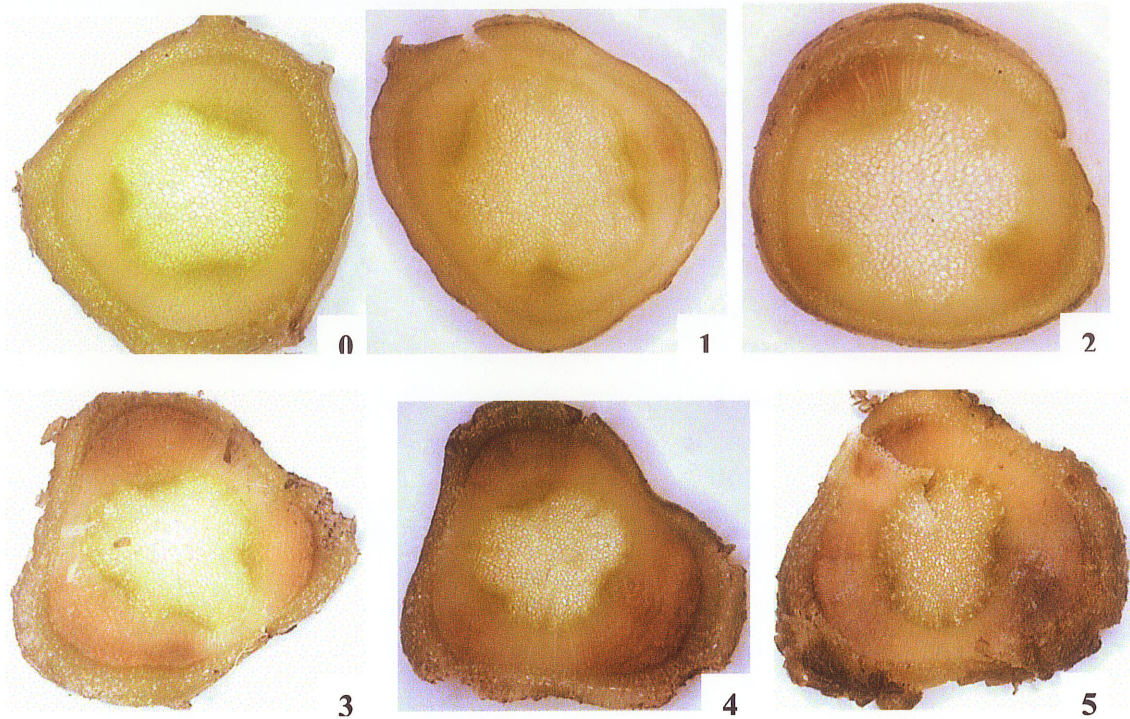


Figure 4.2 Rating scale for the severity of vascular discoloration of potato stems caused by *Verticillium dahliae*: 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

4.3.3 Field experiments

4.3.3.1 Pre-experiment soil analysis in the field plots

In all field experiments, there were three replicate blocks in each cultivar plot. In order to determine the initial inoculum present in each cultivar plot, nine cores of soil samples were randomly collected at two different depths 0-15 cm and 15-30 cm from each replicate block. Those nine cores of soil samples were then bulked for each depth.

4.3.3.1.1 Microsclerotia count:

In all field experiments, the inoculum level of *V. dahliae* was separately estimated at two different depths, 0-15 cm and 15-30 cm using the Sorensen's NP-10 medium method. The soil samples were air-dried for 2 weeks and then pulverised with a mortar and pestle. Five grams of powdered soil were placed in 100 mL of 1% agar solution and shaken for 2 minutes. One mL of soil water agar solution was plated on each Sorensen's NP-10 media Petri dishes. Plates were incubated at 24°C for 2 weeks and then the surfaces of the agar in the Petri dishes were gently washed under running tap water to dislodge and remove soil particles. Washed Petri-plates were examined for microsclerotia (Figure 4.3) using a stereomicroscope at 10x magnification. The number of propagules per gram of soil was expressed as follows:

Number of propagules per gram of soil = Total number of colonies on ten plates × 2

4.3.3.1.2 Nematode count:

Soil samples collected from all the field plots were used to determine the count of *Pratylenchus* species. For each soil sample, 100 grams of soil were weighed and poured in a beaker with 500 mL of water. The soil was mixed 20 times and sieved through a No. 10 mesh screen into a 2 L beaker. The solution was stirred in a circular fashion and when water almost comes to a rest, the contents were poured onto a No. 270 mesh screen. The



Figure 4.3 Colonies of germinating microsclerotia of *Verticillium dahliae* on Sorensen's NP-10 medium

sediments were rinsed on the No. 270 mesh screen and transferred with rubber spatula to round bottom centrifuge tube. The tube was centrifuged for 5 minutes at 2000 rpm. The liquid was then decanted and a sucrose solution (454 grams of sugar/ L) was added. The tubes were centrifuged for 1 minute at 1200 rpm. The solution was poured on No. 400 mesh screen and rinsed with tap water. Nematodes were then transferred to a conical centrifuge tube. The total number of nematodes was counted in a Petri-dish using a stereomicroscope at 10x magnification. The number of nematodes per gram of soil was expressed as follows:

Total nematodes per gram of soil = Total number of nematodes in a Petri-dish \times 2

To count the different species of nematodes present in the sample, the conical tubes containing nematodes were centrifuged for 2 minutes at 2000 rpm. The solution was siphoned off leaving 0.25 mL in the tube. Remaining solution was transferred onto the slide and for each sample, 100 nematodes were observed under a compound microscope.

4.3.3.2 Field trials

In all the field experiments, bacterial and plant extract solutions were applied to seed pieces before sowing. The concentration of the bacterial treatments used to treat potato seed pieces was 10^8 c.f.u./mL. Plant extract treatments were also applied to seed pieces in the same manner as in the growthroom trial. Treated seed pieces were kept overnight at seed storage conditions (4°C and 90-95% RH) prior to sowing. Control seed pieces were treated with sterile, distilled water.

Two field trials in 2003 and in 2004 were carried out in a field with clay loam soil located at University of Manitoba Campus farm, Winnipeg, Canada (Table 4.3). Two potato cultivars, Russet Burbank and Kennebec, used in the growthroom experiments

were grown in the cultivar plots P1 and P2, respectively. There were three replicate blocks per treatment per cultivar plot. The sizes of the replicate blocks were 10 × 14 m and 10 × 16 m for experiment 2003 and 2004, respectively. Twenty-five potato seed pieces were sown per treatment per cultivar (plant spaced 0.4 m within the rows and 1 m between rows). In the experiment FT1, treatments included four bacteria [*Bacillus amyloliquefaciens* (C1), *Bacillus pumilus* (M1), *Pseudomonas fluorescens* (DF37), and Unknown (S1)] and two plant extracts [Native herb (MV) and Canola Extract (CE)]. In field experiment FT2, previously tested treatments [three bacteria: *Bacillus pumilus* (M1), *Pseudomonas fluorescens* (DF37), and Unknown (S1), and one plant extract: Canada milkvetch (MV)] and few additional treatments [one bacterium: *Rahnella aquatilis* (W2), and two plant extracts: Rapeseed extract (RS), and Seaweed extract (SE)] were included.

Ammonium sulphate fertilizer (16:20:0:20) was applied at the rate of 300 kg/ha to the field in 2003 and 2004, respectively, using a manual feed Iron Age potato planter (Oliver corporation, York, Pennsylvania). Six-week-old plants were artificially inoculated with the two previously selected *V. dahliae* isolates in the field experiments 2003 and 2004 at the Point, University of Manitoba. A spade 15 cm-wide was placed 15 cm away from the base of the plant and sliced down to a depth of 15 cm. Then 100 mL of conidial solution (10^5 conidia/mL) were poured into the opening and then covered over. During the growing season, rainfall was the only water source (no irrigation). Potato plants received two foliar applications of systemic insecticide, Admire® 2F at 0.08 litre/acre at biweekly interval after 5 weeks of plant emergence to control Colorado potato beetle (*Leptinotarsa decemlineata*). Cultivation between rows was done to control weeds. It also involved hilling potatoes 8-13 cm below the hill top. When the plants were in their vegetative phase, weeds were pulled either manually or by shallow hoeing. Common

weeds noticed in the field were: Canada thistle (*Cirsium arvense*), Redroot pigweed (*Amaranthus retroflexus*), and wild buckwheat (*Polygonum convolvulus*).

Another field trial in 2004 was conducted in a commercial potato field with sandy loam soil located at Miami, Manitoba (Canada) where Verticillium Wilt disease had been prevalent in previous years (Table 4.3). The commercial potato cultivar Russet Burbank was used in this experiment. There were three replicates (plots) per treatment. Twenty-five potato seed pieces were sown per treatment in 10m × 10m plots (plants spaced 0.4m within the rows and 1m between rows). Treatments included four bacteria [*Rahnella aquatilis* (W2), *Bacillus pumilus* (M1), *Pseudomonas fluorescens* (DF37), and Unknown (S1)] and three plant extracts [Native herb (MV), Rapeseed extract (RS), and Seaweed extract (SE)]. No artificial inoculation of plants was done in this experiment, as the experimental plots were part of a commercial field. During the growing season, the plants were irrigated and fertilized by the potato producer, and plots were mechanically cultivated as needed to remove weeds.

4.3.3.3 Disease Assessment

Disease severity was recorded 2 weeks after artificial inoculation of potato plants in the field experiments at University of Manitoba Campus farm in the year 2003 and 2004. In the commercial potato field experiment in Miami (2004), eight weeks old plants were assessed as these plants were not artificially inoculated. All the plants were rated for Wilt symptoms according to wilt severity rating scale (Figure 4.1). Two and four visual assessments of plants were recorded at 15 days interval for the experiment in 2003 and 2004, respectively.

Ten stems were randomly collected from each treatment after last visual assessment of all the plants in all experiments and rated for the vascular discoloration.

The same rating scale for evaluating the severity of vascular discoloration of potato stems caused by *Verticillium dahliae* was used (Figure 4.2).

4.3.3.4 Yield analysis

Total weight of tubers from each treatment was measured and data was expressed as tonnes/ha. Total tuber yields from each treatment were compared in order to assess the effects of each treatment on the production.

4.3.3.5 Nematode count in potato plant roots

Ten potato plant roots were collected from each treatment after the last visual assessment to determine the presence of *Pratylenchus* species. The basic Baermann funnel technique, which utilizes a glass funnel with a wire mesh basket nested at top was used. A piece of rubber tubing is slipped over the stem and sealed with a clamp. For each treatment, the plant roots were cut in small pieces and weighed 5 grams. The roots were wrapped with a piece of Kimwipe tissue and placed in the funnel. The funnel was then filled with water to a level that will cover wrapped roots to be placed in the basket at the top of the funnel. A piece of Kimwipe tissue was used to wrap the roots and minimize the amount of soil that passes through. After 3 days, the water was collected from each funnel, passed through a No. 400 mesh screen and rinsed with tap water. Nematodes were trapped on the screen. The screen was rinsed so that the nematodes were washed to one spot and then collected in conical centrifuge tube. The number of nematodes on half of the area of a grided Petri dish was counted using a stereomicroscope at 10x magnification. The number of nematodes per gram of soil was expressed as follows:

Total nematodes per gram of soil = Total number of nematodes in a Petri-dish \times 2

To count the different species of nematodes present in the sample, the conical tube containing nematodes were centrifuged for 2 minutes at 2000 rpm. After centrifugation

the solution was removed by suction leaving 0.25 mL in the tube. The rest of the solution was transferred on the slide with glass Pasteur pipette and covered with cover slip. The edges of the slide were sealed with nail polish and for every sample, 100 nematodes were observed under microscope to determine the frequency of *Pratylenchus* species.

Total *Pratylenchus* counts were determined as total counts of nematodes \times frequency of *Pratylenchus*.

4.3.3.6 Data Analysis

Difference among the treatments for percent inhibition, percent infection, wilt severity, vascular discoloration and tuber yields were determined by analysis of variance and mean values were compared by Tukey's test ($P \leq 0.05$). ANOVA was conducted using General Linear Models (GLM) of the SAS Package (Statistical Analysis Systems Institute Inc., Cary, NC, USA). Data on percent inhibition against *V. dahliae* were analyzed as completely randomized experiment using two repetitions (four replicates per treatment). However, before analysis, the percent inhibition was arcsine square root transformed in order to normalize the data. Data on percent infection, wilt severity, vascular discoloration and tuber yields were analyzed using a statistical model with fixed effects: treatment, inoculation, treatment \times inoculation interactions, and blocks. Percent infection data was also arcsine square root transformed. Residuals of the transformed data were normally distributed.

4.4 Results

4.4.1 Comparative *in vitro* inhibition of *V. dahliae* by bacteria

Eighteen bacteria were screened for their ability to suppress *V. dahliae* using *in vitro* culture assays. The inhibitory effect of the bacteria against two *V. dahliae* (Vd1398-21

and Vd1396-9) isolates was higher on the nutrient agar (NA) than on PDA media (Table 4.2). The fungal growth was also slower on NA than on PDA media. Seven out of the 18 bacteria (S1, W2, C1, M1, Y1, J2, and B1) provided over 50% growth inhibition against *V. dahliae* on NA medium. Only bacteria S1, W2, and C1 showed an inhibitory effect on the growth of the two isolates on both media (Figure 4.4 and Figure 4.5). Only one bacterium (S1) strongly inhibited *V. dahliae* on both media. In this study, the screening resulted in the selection of three biological antagonists of *V. dahliae*, Unknown (S1), *Rahnella aquatilis* (W2), and *Bacillus amyloliquefaciens* (C1).

4.4.3 Growthroom experiments

In cultivar Kennebec (highly susceptible to *V. dahliae*), foliar symptoms were more pronounced compared to cultivar Russet Burbank (moderately susceptible to *V. dahliae*). There was a significant effect of inoculation with *V. dahliae* on potato plants in the growthroom (GR1, GR2, GR3, and GR4). Non-treated, inoculated control plants showed symptoms characteristics of those caused by *V. dahliae* in both cultivars Russet Burbank and Kennebec. No Wilt symptoms or vascular discoloration was observed in non-inoculated controls and in plants treated with plant extract, bacteria or when plant treatments were incorporated in autoclaved soil mix under growthroom conditions.

Similar results were observed in both experiments GR1 and GR2. Treatment S#1 significantly decreased the percent infection on Russet Burbank potato plants caused by *V. dahliae* and the browning of vascular tissues in lower stem sections as compared to non-treated controls. Treatment with bacteria DF37 and M1 significantly reduced the disease severity and vascular discoloration of cultivar Kennebec plants compared with the controls (Table 4.3 and 4.4).

Table 4.2 Effects of bacterial strains on the *in vitro* growth of *Verticillium dahliae* isolates (Vd1398-21 & Vd1396-9), expressed as percentage inhibition on nutrient agar and potato dextrose agar.

<i>Bacterial strains</i> ²	Designation ³	Media ¹			
		Potato Dextrose Agar		Nutrient Agar	
		Vd1398-21 ⁴	Vd1396-9 ⁴	Vd1398-21	Vd1396-9
<i>Rahnella aquatilis</i>	W2	52.77a ⁵	44.50a	60.14ab	68.28a
nd	S1	50.92a	55.48a	63.20a	68.93a
<i>Bacillus amyloliquefaciens</i>	C1	32.14b	31.60b	56.80ab	57.21b
<i>Bacillus subtilis</i>	B1	25.23b	23.91b	49.81ab	50.02b
<i>Bacillus subtilis</i>	B3	22.80b	15.31c	47.59ab	48.70b
<i>Bacillus pumilus</i>	M1	17.42b	14.82c	56.51ab	64.18ab
<i>Bacillus subtilis</i>	J1	17.00bc	12.94cd	49.84ab	51.00b
nd	A1	16.12bc	12.46cd	44.27bc	49.67b
<i>Bacillus pumilus</i>	Y1	15.17bc	7.67cd	45.65b	52.30b
<i>Pseudomonas fluorescens</i>	DF40	15.08bc	2.40d	38.99bc	49.33b
<i>Pseudomonas fluorescens</i>	DF35	13.78bc	10.04cd	10.03d	45.27bc
nd	R1	10.56bc	4.78d	23.58c	44.49bc
<i>Pseudomonas fluorescens</i>	DF37	10.08bc	10.51cd	24.85c	42.45bc
<i>Bacillus pumilus</i>	B2	10.03bc	2.40d	49.43ab	47.80bc
nd	A2	9.63bc	3.37d	49.01ab	39.68bc
<i>Bacillus pumilus</i>	W1	9.14bc	3.82d	33.07bc	47.68bc
<i>Pseudomonas putida</i>	PP1	7.34bc	7.67cd	27.76c	38.36cd
<i>Pseudomonas viridilivida</i>	DF3	7.30bc	7.16cd	29.30bc	28.42d

¹Media includes potato dextrose agar (PDA) and nutrient agar (NA).

²Bacterial strains (n= 18) tested for their ability to inhibit the growth of pathogen *in vitro*. nd stands for Unknown bacterial strains.

³Designations used to address different bacterial strains in the text.

⁴Vd1398-21 and Vd1396-9 were the virulent isolates of the pathogen.

⁵Within each column, percent inhibition values followed by same letters are not significantly different (Tukey's test; P > 0.05).

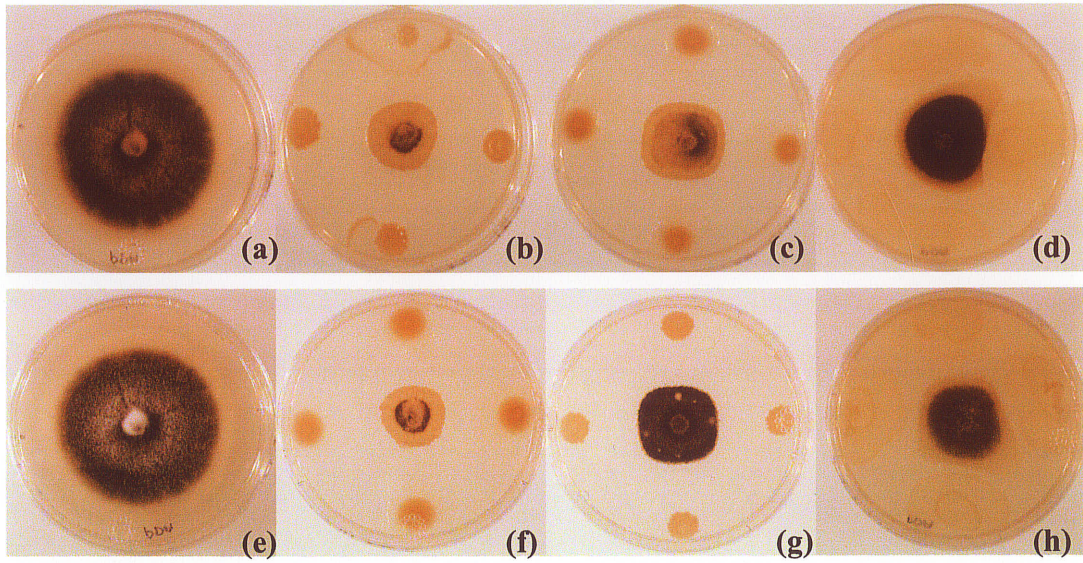


Figure 4.4 Inhibitory effects of bacteria on the *in vitro* growth of *V. dahliae* (Vd-1398-21 & Vd1396-9) on PDA media. (a) Control Vd1398-21, (b) S1 bacterium + Vd1398-21, (c) W2 bacterium + Vd1398-21, (d) C1 bacterium + Vd1398-21, (e) Control Vd1396-9, (f) S1 bacterium + Vd1396-9, (g) W2 bacterium + Vd1396-9, (h) C1 bacterium + Vd1396-9.

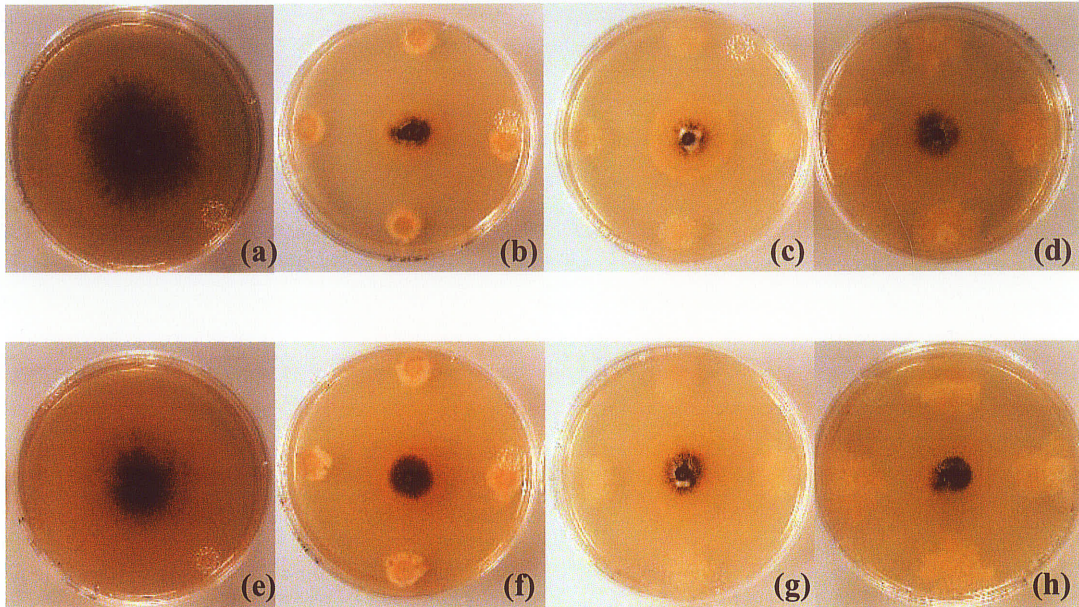


Figure 4.5 Inhibitory effects of bacteria on the *in vitro* growth of *V. dahliae* (Vd-1398-21 & Vd1396-9) on NA media. (a) Control Vd1398-21, (b) S1 bacterium + Vd1398-21, (c) W2 bacterium + Vd1398-21, (d) C1 bacterium + Vd1398-21, (e) Control Vd1396-9, (f) S1 bacterium + Vd1396-9, (g) W2 bacterium + Vd1396-9, (h) C1 bacterium + Vd1396-9.

In experiments GR3 and GR4, plant extract MV was effective in reducing wilt symptoms and the discoloration of vascular tissues in potato plants of both cultivars (Table 4.5). There was a difference in the degree of disease development in both experiments. The disease severity was lower when plant extracts were incorporated in the autoclaved soil mix. The effect of Native herb (MV) treatment was effective even when the methods of application of plant treatments were different.

4.4.4 Field experiments

4.4.4.1 *Verticillium* Inoculum level in the field experiments

In field experiment (2003), there was a significant difference in the level of inoculum (*Verticillium dahliae*) between the cultivar plots (Table 4.6). The distribution of inoculum at two depths was significantly different. The interaction effect between the cultivar plots and depths was also significant. The number of microsclerotia per gram of soil at 0-15 cm depth was significantly higher in cultivar plot P1 where Russet Burbank was grown (Table 4.7). There was no significant difference in the number of microsclerotia per gram of soil at two depths and across the cultivar plots in the field experiments at University of Manitoba Campus farm (2004) and commercial potato field in Miami (2004).

4.4.4.2 Nematode counts

In the field experiments, total nematodes were counted in the soil and in potato root samples (data not shown). Different species counts of nematodes showed plant parasitic nematodes belonging to genera *Xiphinema* and *Heterodera* were observed in only few soil samples (data not shown). The root lesion nematodes, *Pratylenchus* species were absent in the soil samples and in the potato plant roots collected from all the field experiments.

Table 4.3 Effects of bacterial treatments on Verticillium Wilt in cultivars Kennebec and Russet Burbank under growthroom conditions (2003).

<i>Treatments</i> ²	<i>Cultivars</i> ¹			
	Kennebec		Russet Burbank	
	Percent Infection ³	Vascular Discoloration ⁴	Percent Infection	Vascular Discoloration
Control	21.25a ⁵	1.88a	17.88a	1.50a
C1	19.63a	0.75b	14.00a	1.50a
S1	12.00b	0.50b	8.38b	0.25b
M1	8.13b	0.50b	10.75a	1.13a
DF37	4.25b	0.75b	9.13a	0.63b

¹Cultivars include Kennebec (Highly susceptible to Verticillium Wilt) and Russet Burbank (Moderately susceptible).

²Treatments applied to seed pieces includes; water (Control); Bacterial strains, C1 (*Bacillus amyloliquefaciens*), S1 (Unknown), M1 (*Bacillus pumilus*), DF37 (*Pseudomonas fluorescens*).

³Percent Infection was measured on all the plants (n = 5) per treatment after six weeks of inoculation. Percent Infection was measured as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

⁴Vascular discoloration was assessed on the lower stem sections of all the plants (n = 5) per treatment after six weeks of inoculation based on the 0-5 rating scale in which 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

⁵Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

Table 4.4 Effects of bacterial treatments on Verticillium Wilt in cultivars Kennebec and Russet Burbank under growthroom conditions (2004).

<i>Treatments</i> ²	<i>Cultivars</i> ¹					
	Kennebec			Russet Burbank		
	Percent Infection ³	Wilt Severity ⁴	Vascular Discoloration ⁵	Percent Infection	Wilt Severity	Vascular Discoloration
Control	17.30a ⁶	0.80a	1.10a	15.30a	0.65a	0.70a
C1	11.20b	0.55ab	0.50ab	10.20ab	0.35ab	0.40ab
S1	7.20bc	0.50ab	0.40ab	9.60ab	0.43ab	0.20ab
W2	4.20c	0.25b	0.80ab	7.40ab	0.43ab	0.30ab
DF37	3.60c	0.18b	0.10b	6.00b	0.25b	0.10b
M1	3.20c	0.25b	0.30b	8.50ab	0.35ab	0.10b

¹Cultivars include Kennebec (Highly susceptible to Verticillium Wilt) and Russet Burbank (Moderately susceptible).

²Treatments applied to seed pieces includes; water (Control); Bacterial strains, C1 (*Bacillus amyloliquefaciens*), S1 (Unknown), W2 (*Rahnella aquatilis*) M1 (*Bacillus pumilus*), DF37 (*Pseudomonas fluorescens*).

³Percent Infection was measured on all the plants (n = 5) per treatment after six weeks of inoculation. Percent Infection was measured as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

⁴Wilt severity rating was done all the plants (n = 5) per treatment after six weeks of inoculation. Rating is based on a 0-4 scale in which 0 – no wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves.

⁵Vascular discoloration was assessed on the lower stem sections of all the plants (n = 5) per treatment after six weeks of inoculation based on the 0-5 rating scale in which 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

⁶Within each column, mean values followed by same letters are not significantly different (Tukey's test; P > 0.05).

Table 4.5 Effects of plant treatments when applied to seed piece and incorporated into autoclaved soil mixture on *Verticillium* Wilt in cultivars Kennebec and Russet Burbank under growthroom conditions (2003 and 2004).

Treatment application method ²	<i>Cultivars</i> ¹						
	<i>Treatments</i> ³	Kennebec			Russet Burbank		
		Percent Infection ⁴	Wilt Severity ⁵	Vascular Discoloration ⁶	Percent Infection	Wilt Severity	Vascular Discoloration
<i>Incorporation in soil</i>	Control	10.75a ⁷	0.58a	0.83a	7.83a	0.40a	0.58a
	RS	6.91ab	0.50ab	0.50ab	6.25ab	0.33ab	0.50ab
	SE	3.00b	0.17b	0.41ab	5.17ab	0.21ab	0.25ab
	MV	1.67b	0.13b	0.25b	1.50b	0.08b	0.17b
<i>Seed piece treatment</i>	Control	17.90a	1.15a	1.80a	12.40a	0.75a	1.10a
	SE	10.00b	0.75ab	0.70ab	5.60b	0.60ab	0.60a
	CE	8.60bc	0.70b	1.30ab	7.40ab	0.50ab	0.80a
	RS	5.60bc	0.63b	0.80ab	6.40ab	0.56ab	0.80a
	MV	4.10c	0.42b	0.50b	3.40b	0.27b	0.50a

¹Cultivars include Kennebec (Highly susceptible to *Verticillium* Wilt) and Russet Burbank (Moderately susceptible).

²Treatment were applied by two different methods: 1) incorporation of leaves into soil; 2) application of plant extracts to seed pieces.

³Treatments include; water (Control); RS (Rapeseed), SE (Seaweed), CE (Canola), MV (Canada milkvetch).

⁴Percent Infection was measured on all the plants (n = 5) per treatment after six weeks of inoculation. Percent Infection was measured as follows: Infection (%) = $(C_L/T_L) \times 100$ where C_L is the number of chlorotic leaves and T_L is the total number of leaves on potato plants.

⁵Wilt severity rating was done all the plants (n = 5) per treatment after six weeks of inoculation. Rating is based on a 0-4 scale in which 0 – no wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves.

⁶Vascular discoloration was assessed on the lower stem sections of all the plants (n = 5) per treatment after six weeks of inoculation based on the 0-5 rating scale in which 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

⁷Within each column, mean values followed by same letters are not significantly different (Tukey's test; P > 0.05).

Table 4.6 Analysis of variance of the distribution of microsclerotia in soil at different depths in the three experiments.

Experiments¹	Source of Variation²	df³	Mean Square	Pr > F⁴
FT1(2003)	Cultivar plot	1	833.33	0.0130
	Depths	1	645.33	0.0232
	Cultivar plot*Depth	1	705.33	0.0191
FT2(2004)	Cultivar	1	1.33	0.8158
	Depths	1	48.00	0.1866
	Cultivar plot*Depth	1	85.33	0.0903
FT3(2004)	Depths	1	1734.00	0.2088

¹Experiments conducted in two years during the study.

²Source of variation: Two cultivar plots (Russet Burbank and Kennebec) in the field; Nine cores of soil samples were collected each replicate block (n = 3) of each cultivar plot at two depths (0 - 15 cm and 15 - 30 cm).

³df = degrees of freedom

⁴P = Probability (P > 0.05)

Table 4.7 Number of microsclerotia per gram of soil at different depths in two cultivars for the three experiments.

<i>Cultivar plot</i> ²	Depths ³	<i>Experiments</i> ¹		
		FT1 (2003)	FT2 (2004)	FT3(2004)
Russet Burbank	0 - 15 cm	40.0a ⁴	14.7b	87.33a
	15 - 30 cm	10.0b	13.3b	53.33a
Kennebec	0 - 15 cm	8.0b	8.7b	-
	15 - 30 cm	8.7b	18.0b	-

¹Experiments conducted in two years during the study.

²Cultivars include Russet Burbank and Kennebec.

³Depths includes 0 - 15 cm and 15 - 30 cm. Nine cores of soil samples were collected at two depths from each replicate block (n = 3) of each cultivar plot.

⁴Within each column, mean values followed by same letters are not significantly different (Tukey's test; P > 0.05).

4.4.4.3 Field trials

In field experiment at University of Manitoba Campus farm (2003), Native herb (MV) plant extract significantly reduced the wilt severity and vascular discoloration on cultivar Kennebec whereas on cultivar Russet Burbank, bacterium DF37 was the most effective as compared to non-treated/inoculated controls (Table 4.8). Bacterium S1 showed less vascular discoloration in Russet Burbank potato plants. There was no significant effect with different treatments on yields of both cultivars in the experiment FT1.

In field experiment at University of Manitoba Campus farm (2004), there was poor emergence of Russet Burbank potato plants. Therefore, the data could not be statistically analyzed for this cultivar. Results from cultivar Kennebec showed that three treatments (M1, DF37, and MV) were significantly effective in decreasing the foliar wilt symptoms and discoloration of vascular tissues, as compared to non-treated controls (Table 4.9). These treatments also showed a significant increase in the yield of cultivar Kennebec.

In commercial potato field experiment in Miami (2004), treatment DF37 lessened the severity of external and internal symptoms of wilt disease on cultivar Russet Burbank compared with the controls. Treatment DF37 was also found to enhance the tuber yield of cultivar Russet Burbank as compared to non-treated control plants (Table 4.10).

4.5 Discussion

The *in vitro* screening of 18 bacteria on two different media resulted in the selection of three bacteria which effectively inhibited the mycelial growth of *V. dahliae*. Many differences were observed on two types of media. The bacterial strains showed high

Table 4.8 Effects of different bacterial and plant extract treatments on Verticillium Wilt in cultivars Kennebec and Russet Burbank under field conditions at University of Manitoba Campus farm during the growing season 2003.

<i>Treatments</i> ²	<i>Cultivars</i> ¹					
	Kennebec			Russet Burbank		
	Wilt Severity ³	Vascular Discoloration ⁴	Yield ⁵	Wilt Severity	Vascular Discoloration	Yield
Control	2.18a ⁶	2.46a	27.91a	1.82a	2.23a	25.90a
DF37	1.76ab	1.90ab	28.09a	1.35b	0.73b	26.02a
M1	1.72ab	2.00ab	27.96a	1.52ab	1.80ab	26.04a
C1	1.62ab	1.77ab	27.40a	1.60ab	2.10ab	24.88a
S1	1.37ab	1.97ab	28.83a	1.78ab	0.97b	26.32a
CE	1.96ab	1.77ab	27.42a	1.79ab	1.67ab	26.41a
MV	1.20b	1.00b	29.28a	1.57ab	1.50ab	26.14a

¹Cultivars include Kennebec (Highly susceptible to Verticillium Wilt) and Russet Burbank (Moderately susceptible).

²Treatments applied to seed pieces includes; water (Control); Bacterial strains, S1 (Unknown), W2 (*Rahnella aquatilis*), M1 (*Bacillus pumilus*), DF37 (*Pseudomonas fluorescens*); Plant extracts, CE (Canola), MV (Canada Milkvech).

³Wilt Severity rating was done on all the plants before harvesting (25 plants per treatment and replicate blocks = 3). Rating is based on a 0-4 scale in which 0 – no wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves.

⁴Vascular discoloration rating was done before harvesting. Ten stem per treatment (Replicate blocks = 3) were randomly collected and lower stem section from each stem was assessed for the level of vascular discoloration based on the 0-5 scale in which 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

⁵Total yield for each treatment was measured in tonnes/hectares. 25 plants per treatment (Replicate blocks = 3).

⁶Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

Table 4.9 Effects of different bacterial and plant extract treatments on *Verticillium* Wilt in cultivar Kennebec under field conditions at University of Manitoba Campus farm during the growing season 2004.

<i>Treatments</i> ¹	<i>Wilt Severity</i> ²	<i>Vascular Discoloration</i> ³	<i>Yield</i> ⁴
Control	3.00a ⁵	3.73a	33.29b
S1	2.70a	3.70a	38.71ab
W2	2.67ab	3.37ab	37.96ab
M1	2.45b	2.70b	39.86a
DF37	2.33b	2.58b	39.34a
SE	2.55b	3.12ab	36.86ab
RS	2.54b	3.80a	33.88b
MV	2.43b	2.90b	39.12a

¹Treatments applied to seed pieces includes; water (Control); Bacterial strains, S1 (Unknown), W2 (*Rahnella aquatilis*), M1 (*Bacillus pumilus*), DF37 (*Pseudomonas fluorescens*); Plant extracts, SE (Seaweed), RE (Rapeseed), MV (Canada Milkvetch).

²Wilt Severity rating was done on all the plants before harvesting (25 plants per treatment and replicate blocks = 3). Rating is based on a 0-4 scale in which 0 – no wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves.

³Vascular discoloration rating scale was done before harvesting. Ten stem per treatment (Replicate blocks = 3) were randomly collected and lower stem section from each stem was assessed for the level of vascular discoloration based on the 0-5 scale in which 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

⁴Total yield for each treatment was measured in tonnes/hectares. 25 plants per treatment (Replicate blocks = 3).

⁵Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

Table 4.10 Effects of different bacterial and plant extract treatments on Verticillium Wilt in cultivar Russet Burbank under field conditions in commercial potato field in Miami, Manitoba during the growing season 2004.

<i>Treatments</i> ¹	Wilt Severity ²	Vascular Discoloration ³	Yield ⁴
Control	2.68a ⁵	4.37a	30.68b
S1	2.59a	3.80a	36.16ab
W2	2.65a	3.27ab	32.90ab
M1	2.40a	3.47ab	31.20b
DF37	1.90b	2.50b	38.12a
SE	2.68a	3.27ab	31.90ab
RE	2.58a	3.63a	31.11b
MV	2.42a	3.60ab	32.01ab

¹Treatments applied to seed pieces includes; water (Control); Bacterial strains, S1 (Unknown), W2 (*Rahnella aquatilis*), M1 (*Bacillus pumilus*), DF37 (*Pseudomonas fluorescens*); Plant extracts, SE (Seaweed), RE (Rapeseed), MV (Canada Milkvetch).

²Wilt Severity rating was done on all the plants before harvesting (25 plants per treatment and replicate blocks = 3). Rating is based on a 0-4 scale in which 0 – no wilt symptoms; 1 – interveinal chlorosis in the lower leaves; 2 – moderate necrosis and defoliation of the lower leaves; 3 – severe leaf necrosis and defoliation, stunted growth; and 4 – severe defoliation accompanied by pronounced stunting, chlorosis and necrosis of the remaining leaves.

³Vascular discoloration rating scale was done before harvesting. Ten stem per treatment (Replicate blocks = 3) were randomly collected and lower stem section from each stem was assessed for the level of vascular discoloration based on the 0-5 scale in which 0 – no vascular discoloration; 1 – trace to less than 9% of the stem cross-section show vascular discoloration; 2 – 10 to less than 24 % of the stem cross-section show vascular discoloration; 3 – 25 to 49 % of the stem cross-section show vascular discoloration; 4 – 50 to less than 74 % of the stem cross-section show vascular discoloration; and 5 – 74 to 100 % of the stem cross-section show vascular discoloration.

⁴Total yield for each treatment was measured in tonnes/hectares. 25 plants per treatment (Replicate blocks = 3).

⁵Within each column, mean values followed by same letters are not significantly different (Tukey's test; $P > 0.05$).

inhibition effects on the growth of the two *V. dahliae* isolates on NA medium. The two *V. dahliae* isolates achieved 75 mm of radial growth after 15 days on PDA as compared to 30 days on NA media. The higher inhibitory ability of the bacterial strains and the slower growth of fungus on NA than PDA could be explained by the fact that nutrient agar is more selective growth media for bacteria. The *in vitro* assay allows many repetitions, screening of a large number of bacterial strains and is generally designed to screen biocontrol agents with antibiotic activity (Daayf et al., 2003). In the present study, Bacteria Unknown S1, *Rahnella aquatilis* W2 and *Bacillus amyloliquefaciens* C1 showed growth inhibition effects on *V. dahliae*. The production of antibiotics, toxic metabolites or siderophores could be the possible mechanism of action of such bacteria (Swadling and Jefferies, 1996). However, the *in vitro* screening eliminates the candidates which might have the potential of inducing resistance in the host but don't have inhibitory activity against the fungus. Bacteria such as M1, DF37 provided lesser inhibition against the pathogen *in vitro*, but were selected for further studies in the growthroom and field trials. Daayf et al. (2003) previously found that these bacterial strains are capable of inducing defense mechanisms in potato.

Ideally, biological treatments should be screened on the plants rather *in vitro*, however, in many situations it is impractical to conduct large-scale screening trials on whole plants. In the present study, we developed a rational strategy to conduct preliminary screening *in vitro* and to move to secondary screening in planta which are more relevant to the natural situation as the numbers of potential antagonists have been reduced to appropriate numbers.

In vitro studies, only a small number of selective *Verticillium* antagonists (S1, W2, C1) were found. These bacteria strongly inhibited the growth of fungus on culture media,

which suggests antibiosis to be the possible mechanism of action involved in the protection provided by such bacteria. These bacteria along with the other two selected bacteria will be further tested in the growthroom and, later in the field. The successful performance of a biocontrol agent to control the disease under different systems confirms the efficacy of the antagonist.

This study has also confirmed that repetitive testing of different treatments including bacteria and plant extracts, using the whole plant systems under growthroom and field conditions was the most realistic rather than screening the treatments *in vitro*. The *in vitro* tests have been designed to select bacterial candidates producing antibiotics but the major drawback of this technique is the elimination of potential candidates with resistance-inducing ability. Growthroom experiments using different bacteria had shown that bacteria *Rahnella aquatilis* W2 and Unknown S1 strongly inhibited *V. dahliae* *in vitro* but provided only a moderate level of protection on whole plants. Strong inhibition *in vitro* can be explained by the fact that these bacteria can produce high levels of antibiotics. *Bacillus amyloliquefaciens* C1 provided a relatively moderate level of inhibition against *V. dahliae* *in vitro*, but was ineffective when tested on the whole plant system. The moderate level of protection also indicated that these bacteria possibly induce plant defense responses. The inefficacy of this bacterium might be due to lack of competitiveness, short-term survival or detoxification of antibiotic by the pathogen in soil. Another important reason for the moderate or low efficacy of these treatments in the field trials could be that only highly virulent isolates of *V. dahliae* were used in the screening process. *Pseudomonas fluorescens* DF37 and *B. pumilus* M1 were effective in reducing the disease expression on cultivar Kennebec and Russet Burbank although they provided little inhibition when challenged to *V. dahliae* *in vitro*. These bacteria were also

previously shown to enhance resistance in potato plant against highly aggressive *P. infestans* (Daayf et al., 2003). The highest level of protection achieved with these bacteria might be due to the induction of resistance mechanism in the plant despite of the little inhibition of the fungus *in vitro*, suggesting the poor antibiotic production ability of these bacteria.

As biological alternatives, bacteria have often been the focus of research in sustainable agriculture systems (Whipps, 1997) because of their ability to suppress diseases caused by soil-borne plant pathogens (Emmert and Handelsman, 1999). Previous studies have shown extracts from different plant species to act as a source of natural chemicals and play an important role in controlling several plant pathogens (Verkleij, 1992; Mojtahedi et al., 1993; Qasem and Abu-Blan, 1995; Subbarao and Hubbard, 1996). Plant treatments were applied differently in the growthroom experiments: Incorporation of leaves in autoclaved soil and application of plant extracts to seeds. One major disadvantage of leaves into the soil was that large amounts of fresh plant tissues are needed, which is not feasible for the farmers. Seed treatment was the preferred technique in order to make the treatments user-friendly. We observed a high efficacy of Canadian milkvetch plant extract (MV) regardless of the method of application. This might be due to the production of fungitoxic compounds or induction of defense response mechanisms in the plant. Canola, Rapeseed and Seaweed plant extracts showed little effect in reducing disease expression, which indicates that they probably have limited potential as biological alternatives. Contradictory and supportive documents on Seaweed extracts have been reported in the past (Verkleij, 1992). The magnitude of pathogen suppression by *Brassica* spp. is linked to the composition and the concentration of glucosinolates, which can vary greatly among plant species, developmental stage, and tissue type (Kjaer, 1973; Fenwick

and Heaney, 1983; Sang et al., 1984). The plant extracts, being a biological product, are prone to decay either by transformation of the fungitoxic compounds into non-toxic forms (Verkleij, 1992) or loss of volatile compounds with time (Qasem and Abu-Blan, 1995). The success of a biological control agent against the pathogen *V. dahliae* must be judged by its performance under field conditions. A single field experiment is not sufficient to draw final conclusions. Field trials in our study were conducted during two growing seasons and at two different locations. We found similar results in the greenhouse and in the field. The field trials over the two years demonstrated that bacterium DF37 and plant extract MV was effective on cultivar Russet Burbank and Kennebec, respectively. Consistency in the performance of these treatments indicates that they could be potential biological control agents towards *V. dahliae*. The MV extract treatment effective on the highly susceptible cultivar (Kennebec) was found to provide no control against the disease in the moderately susceptible cultivar (Russet Burbank). The resistance level of these cultivars to *V. dahliae* could explain the differential effect of plant extract treatments. Bacterium treatment M1 showed inconsistent effects on cultivar Kennebec under the field conditions although it was effective in the growthroom. The higher biocontrol efficacy of the treatments under growthroom conditions than under field conditions might be due to abiotic and biotic factors, which are rather difficult to control in the field. The use of highest virulent isolates for inoculating plants in the field trials at University of Manitoba Campus farm and the presence of high inoculum level in Miami soil may also explain the inefficacy of some treatments under field conditions. Treatments that were inconsistent could be considered for further research work in this regard. In field trials, expression of wilt symptoms in non-treated and treated control plants was due to presence of natural inoculum in the soil.

Bacteria DF37, and M1 and MV plant extract were effective in controlling Verticillium Wilt on cultivar Kennebec under growthroom and field conditions. Only one bacterial treatment DF37 significantly reduced the disease on cultivar Russet Burbank. Further research is needed to assess the role of the treatments and improvement in the formulations and methods of application may further stabilize the performance of these bacteria and plant extracts as biocontrol agent against Verticillium Wilt of potato. Systemic effects of the treatments could be investigated in order to check whether induced plant resistance is expressed only locally at the site of treatments, or also systemically. This would give way to new formulations, which would serve as a better alternative to chemical fungicides. The pathogen could be combated in a more sustainable manner in contrast to the chemical fungicides, which are extremely costly and pose the problem of environmental pollution. Implementing a long-term disease management strategy, which such biological treatments are integrated with cultural practices and minimal chemical treatments, would be beneficial both to the potato industry and the general public.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Verticillium dahliae Kleb. (Verticillium Wilt), is an economically important soil-borne pathogen of potato. Complete management of this disease with soil fumigation, cultural practices and low levels of host resistance are not feasible. The purpose of this research was to identify biological alternatives either bacteria or plant extracts, effective in reducing Verticillium Wilt disease. The use of biological means would be an appropriate strategy if it is combined with other management practices.

To confirm the efficacy of different bacterial strains against *V. dahliae*, *V. dahliae* isolates with the highest level of pathogenicity were used in the biocontrol studies. An assessment of pathogenic variability in the local population of the pathogen was first investigated. In order to select the most pathogenic *V. dahliae* isolate, a pathogenicity test was conducted on 43 *Verticillium* isolates collected from both infected potato stem tissues and infected potato tubers in Manitoba (Chapter 1). A pathogenic diversity was shown within the collected *Verticillium* isolates. Two of the most pathogenic isolates, Vd1398-21 and Vd1396-9, were identified and kept for further studies.

For selecting an effective biological treatment, a step wise screening procedure was followed. The treatments were primarily screened *in vitro*, then in growthroom and finally in the field.

The bacterial strains were initially screened *in vitro*, thereby reducing the potential candidates to an appropriate number. This step was useful in identifying the bacterial strains with antibiotic-producing ability but it's major drawback was that it eliminates those bacteria that have a different mechanism of action such as triggering plant's own defense reactions. For this reason, two other bacteria with plant resistance-inducing ability

(Daayf et al., 2003) were also selected. Preliminary screening *in vitro* resulted in the selection of three bacterial strains with high inhibitory effects on the mycelial growth of the fungus. These bacterial strains appeared as excellent biocontrol candidates for introduction in the field.

Extracts derived from different plant species were also used as biological alternatives in this work. Introduction of bacteria and plant extracts to the whole plant system to protect it against Verticillium Wilt was the second biological control strategy in this study (Chapter 2). Five pre-screened bacterial strains along with four plant extracts were further tested on two cultivars, Kennebec (highly susceptible) and Russet Burbank (moderately susceptible) under growthroom and field conditions. *Pseudomonas fluorescens* DF37 and *B. pumilus* M1 were the most effective bacterial treatments. Bacteria *Rahnella aquatilis* W2 and Unknown S1 showed an inconsistency in reducing the disease. For plant extracts, two different application methods were tested (Chapter 2). One of the plant extracts, Canada milkvetch (MV) was more effective when incorporated into the soil, in comparison to application to seed piece. Incorporation of plant extracts into soil would not be feasible for the producers as large quantity of fresh material is needed. However, this plant may have potential as a green manure treatment. Few treatments W2, M1, and SE were found to be effective on cultivar Kennebec but not on Russet Burbank. The differential effects of these treatments might be due to the different degrees of resistance of these cultivars. In the growthroom experiments, the number of treatments identified with potential bioactivity was higher, which in general could be explained by the fact that all the abiotic and biotic factors could be controlled in the growthroom. Results were also consistent with those from *in vitro* studies.

Responses to the biological control treatments under growthroom and field conditions were variable; only a few treatments were found to be effective in the field. Bacterium *Pseudomonas fluorescens* DF37 not only reduced wilt disease but also increased the yield of both cultivars. Two other treatments (*Bacillus pumilus* M1 and Canada milkvetch MV) showed similar results on cultivar Kennebec. Since no direct inhibiting effect of these treatments was detected against *Verticillium*, their mode of action would probably be through the induction of plant's own defense reactions. In field conditions, many abiotic factors such as temperature, moisture and biotic factors may be responsible for the variable performance of these treatments. The high pressure of inoculum in the field was another reason for low efficacy of some of the treatments. Treatments showing discrepancy in their performance under different screening systems could be considered for further biocontrol studies. With in depth formulation research prior to performance testing in the field, this variation in efficacy may be reduced.

Formulations will need to be assessed along with colonization potential and longevity of the bacteria. The type of formulation desired depends on the intended end-use. For large potato farms, a liquid formulation would be more appropriate. As *Pseudomonas* spp. have short-life, these bacteria could be formulated as frozen cell pellets and kept at low temperature before use. Dry powder formulations can be prepared from *Bacillus* spp. as these bacteria forms hardy structures and can be dried easily. The plant material, Canada milkvetch, could be prepared as a plant extract and should be stored at low temperature. The liquid formulation can be applied to seed pieces a day prior to sowing and the treated seed pieces can be stored at storage conditions. Future research should assess the potential of these bacteria under several different formulations and application procedures, determining the full extent of their applicability and potential

risks. This research is a long-term effort, but initial screening of potentially effective biological treatments has been accomplished.

Different screening procedures were investigated in this thesis to identify a potential biological treatment to control Verticillium Wilt of potato. *In vivo* testing was found to be more realistic than the *in vitro* one. *In vivo* studies also revealed the differential effects of treatments in controlling the disease on two cultivars. Some treatments found to be effective on highly susceptible cultivar Kennebec were ineffective on moderately susceptible cultivar Russet Burbank. Further investigation is needed to study the mechanism of action of these treatments involved in controlling the disease and confirm the potentials of these treatments. These studies on the mechanism of action may lead to the discovery of new bioactive molecules that can be used in the biological control of early dying syndrome.

Biological alternatives were researched in this thesis. The use of the biological alternative will aid in controlling Verticillium Wilt and also contributes to the reduction in the use of chemical fungicides. A good level of Verticillium Wilt in potatoes could be achieved by integrating the use of biological treatments with other management techniques. It would be also beneficial to growing potato industries in Manitoba that is perceived to be suffering from losses caused by this disease.

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