CROSS PATHOGENICITY OF *VERTICILLIUM DAHLIAE* BETWEEN POTATO AND SUNFLOWER

BY

HASSNA ALKHER

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

MASTER OF SCIENCE

Department of Plant Science
University of Manitoba
Winnipeg, Manitoba, Canada

THE UNIVERSITY OF MANITOBA

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GENERAL ABSTRACT

Alkher, Hassna. M.Sc., The University of Manitoba, May, 2009. Cross Pathogenicity of *Verticillium dahliae* between Potato and Sunflower. Advisors; F. Daayf and K. Y. Rashid.

Verticillium dahliae and *V. albo-atrum* are soil-borne pathogens, and the main causes of Verticillium wilt disease. They are able to attack more than 200 plant species, including economically important crops such as potato and sunflower. The cross infection of *V. dahliae* between potato and sunflower were not clear. In this study, 10 isolates of *V. dahliae* isolated from potato and 10 from sunflower infected tissues and two highly aggressive *V. dahliae* isolates (Vd1396-9, Vd1389-21) and one weakly aggressive *V. albo-atrum* isolate, were used to investigate the cross pathogenicity of *V. dahliae* on potato and sunflower. The isolates were tested on two potato cultivars (Kennebec, a susceptible cultivar, and Ranger Russet, a moderately resistant one), and two sunflower hybrids (IS8048, susceptible, and 6946, moderately resistant). Most *V. dahliae* isolated from potato were more aggressive on potato and sunflower compared with the ones isolated from sunflower. However, each group of isolates was more aggressive on their original host. Four weakly aggressive *V. dahliae* isolates (P47, P09 from potato; S07, S09 from sunflower) were chosen to study the effect of passing these isolates several generations through their alternative host and their host of origin on their pathogenicity. The isolates were passed through the susceptible potato and sunflower cultivars for four generations. The comparison was among the initial isolates and their 4^{th} passed counterparts. The results showed that potato isolates exhibit gain in their pathogenicity on either potato or sunflower when passed through susceptible potato cultivar Kennebec for four generations compared to the ones from sunflower.

FOREWORD

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. The First manuscript has been published in the European Journal of Plant Pathology, and the second manuscript will be submitted to the Canadian Journal of Plant Pathology.

1.0 GENERAL INTRODUCTION

Potato (*Solanum tuberosum* L.) is one of the major food crops in the world (Rowe, 1993). Manitoba is the second largest potato producer in Canada, after Prince Edward Island (Canadian Potato Production, statistics Canada, 2006). There are many potato varieties grown in Manitoba some of which are important for chip and French fry production (Manitoba Agriculture and Food, 2003).

Sunflower (*Helianthus annuus* L.) also is an important crop in Manitoba. Approximately 85 per cent of the Canadian sunflower crop is grown in this province. Both oil seeds and confection type are significant (Manitoba Agriculture, Food and Rural initiative, 2005).

Verticillium wilt is a serious disease that causes major yield losses in many plant species. The causal agent, *Verticillium* spp., attacks over 200 plant species (Agrios, 2005). The main pathogenic species are *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke, which are soilborne pathogens. They can survive in the soil for many years as resting structures called microsclerotia, in the case of *V. dahliae* (Rowe and Powelson. 2002), or as dark mycelium in the case of *V. albo-atrum* (Dez and Clewes, 2003; Agrios, 2005). *Verticillium dahliae* is the major cause of Verticillium wilt on many economically important vegetable, fiber, legumes, trees and flower crops (Bhat and Subbarao, 1999).

Verticillium dahliae is a pathogen of potato and sunflower and causes significant losses in these crops by reducing tuber quality and yield in potato (Rowe and Powelson, 2002), reducing yield, head size and oil concentration in sunflower (Gulya et al., 1997). Management practices for the control of Verticillium wilt, including cultural controls, use of resistant or tolerant varieties, soil fumigation, or biological control are not efficient because of the long-term survival of microsclerotia in the soil (Alstörm, 2001; Subbarao

and Hubbard, 1996). One of the first important steps in reducing the impact of Verticillium disease is understanding this pathogen's interactions with its hosts.

Verticillium species are known for their high pathogenic variability on different hosts. *Verticillium dahliae* isolates can cause a range of symptoms on different hosts, with the most severe on the host from which the isolates were obtained (Bhat and Subbarao, 1999). Such variability causes difficulty in understanding the behavior of this pathogen on different crops. Another difficulty is due to the fact that *V. dahliae* is soilborne. In order to understand the interactions of this pathogen with its hosts, the cross-pathogenicity of *Verticillium* spp. toward important crops need to be determined.

2.0 LITERATURE REVIEW

2.1 Potato (*Solanum tuberosum* L.)

2.1.1 Taxonomy

Potato belongs to the *Solanaceae*, a family of about 90 genera and 2,800 species, in the genus *Solanum*, and the species *S. tuberosum* (Correll, 1962; Hawkes, 1992). There are more than 225 wild potato species (Spooner et al., 1994).

2.1.2 Origin and movement of potato cultivation

Potato originated in the Andean highlands of South America. Potato was the main crop and a valuable source of food for the native people in Peru, where it has been cultivated for at least 8,000 years (Rowe, 1993). The plant was introduced to Europe in the 1590s by the Spanish (Rowe and Powelson, 2002). Over time, the crop became a staple in northern Europe, particularly in Ireland. From Europe, potatoes traveled to many other parts of the world including North America (Rowe, 1993).

2.1.3 Potato varieties

There are different varieties of potato based on their genetic and various traits. Specific varieties are required by processors for chip and French fry production. The major varieties used by French fry companies in the Canadian Prairie are Russet Burbank, Shepody and Ranger Russet, while Atlantic, Conestoga (Manitoba only), Snowden, Niska and Nor Valley are the main varieties used for chip production (Lynch et al., 2003).

2.1.4 Importance and production

Potatoes are one of the major nutritional crops for mankind, ranking fourth in world food production, after wheat, corn, and rice (Desjardins et al., 1995; Rowe, 1993). China

is the world's largest producer of potatoes with 70,048,000 metric tons, followed by the Russian federation with 35, 914, 240 metric tons (National potato council NPC Statistic, 2004).

Manitoba is the second largest potato producer in Canada, after Prince Edward Island. Manitoba showed a great increase in potato production in 2006, where the production reached 21,735,000 cwt compared to 15,960,000 cwt in 2005 (Canadian Potato Production, 2006).

2.2 Sunflower (*Helianthus annuus* L.)

2.2.1 Taxonomy

Sunflower belongs to the family *Asteraceae*, and the genus *Helianthus*, in which 67 species are known (Dedio et al., 1980).

2.2.2 Origin and movement of sunflower cultivation

Sunflower originated in North America and can be found growing wild from the northern region of the Canadian Prairie to Mexico and further south into Latin America (Dedio et al., 1980). The Spanish introduced sunflower to Europe, from which its cultivation spread through to Russia, where it was readily adapted (Putnam et al., 1990).

In 1875, sunflower was reintroduced to North America with the arrival of immigrants. Commercial production of sunflower in Canada started in Saskatchewan and Manitoba (Dedio et al., 1980).

2.2.3 Sunflower seeds

The sunflower is cultivated primarily for its seeds which yield an important source of edible oil. Mainly, there are two types of sunflower seeds: oilseed and confection type. The seed of oilseed cultivars is smaller in size and higher in test weight than that of

confection cultivars. Sunflower oil seed contains 44% oil on a dry weight basis. The seeds of confection cultivars (non-oilseed) are larger than that in oilseed type (Putnam et al., 1990). Confection Sunflower seed are used as roasted snack, food in the shell or as dehulled seeds for the baking industry, while oil-seed varieties are used in birdfeed and crushing industry for oil production (Manitoba Agriculture, Food and Rural Initiative, 2005).

2.2.4 Importance and production

The sunflower is important as a source of food for people around the world. Overall, about 95% of world sunflower production is the oilseed type and only 5% the confection type. Manitoba reported 86% of the Canadian production in 2002-2003, followed by Saskatchewan at 11%, Alberta at 2.5%, and Ontario accounting for most of the remaining 0.5%. South-central Manitoba is the main producing area, followed by south-western Manitoba and south-eastern Saskatchewan (National sunflower Association of Canada Inc, 2003).

2.3 Verticillium Wilt

Verticillium wilt is a serious disease that affects many plant species (Agrios, 2005). The disease is caused by the common soil-borne fungi *Verticillium albo-atrum* Reinke and *Verticillium dahliae* Kleb (Rowe and Powelson, 2002).

2.3.1 *Verticillium dahliae*

The soil-borne pathogen *Verticillium dahliae* is the major cause of Verticillium wilt (Rowe et at., 1987). Sometimes the fungus interacts with the root lesion nematode *Pratylenchus penetrans* and the root rot bacteria *Erwinia coccodes* to cause a complex vascular wilt disease known as potato early dying (Rotenberg et al., 2004; Rowe and

Powelson, 2002). *Verticillium dahliae* is widespread in the north central states and Pacific Northwest where the temperatures frequently exceed 25 C°.

Verticillium dahliae causes wilt in a wide range of plant species. It can affect more than 200 plant families (Agrios, 2005) including important vegetable crops, such as potato, tomato, artichoke, peppermint, and cauliflower (Bhat and Subbarao, 1999; Douhan and Johnson, 2001; Xiao et al., 1997), and fruits such as strawberry and olive (Levin et al., 2003; Harris and Yong, 1996)

2.3.2 Nomenclature and taxonomy

Verticillium dahliae belongs to the fungal class Deuteromycetes (fungi imperfecti), which is a group of fungi that have no known sexual state (Hastie and Heale, 1984). *Verticillium* is characterized by hyaline, septate, multinucleate and simple or branched mycelium. The conidia are ovoid or elongate, and are produced on the phialides. These phialides are carried in whorls around the conidiophores at different points (Fradin and Thomma, 2006). (Figure1: A and B) The Parasexual stage is the only stage which is clearly demonstrated in *Verticillium dahliae* (Typas, 1983; Typas and Heale, 1978). Independent *Verticillium* isolates can freely able to anastomose and form heterokaryons. This ability is termed vegetative compatibility (Puhalla, 1979).

2.3.3 Disease cycle

Verticillium dahliae and *V. albo-atrum* are soil-borne pathogens that invade the plant through the roots. Verticillium wilt is recorded as a monocyclic disease, which means it has only one cycle of disease and inoculum production for each season (Rowe and Powelson.2002). The microsclerotia of *V. dahliae* survive in the soil more than ten years and it can germinate in response to root exudates secreted from the root tip (Mol, 1995).

The hyphae grow out of germinating resting structures reach the plant host via nutrient gradients (Fradin and Thomma, 2006). *Verticillium dahliae* infection of a host takes place either by penetration or invasion of the cortex. From the cortex, the hyphae invade the xylem (vascular tissue) where it can form conidia. The conidia are discrete within the vascular tissue and move through cell walls via pit apertures (Rowe and Powelson. 2002). The accumulation of fungal material and plant reaction products can cause plugging in the vascular system, preventing the water and the nutrients from reaching the upper part of the plant. Chlorosis, necrosis and wilting appear as a result of the systemic colonization of the plant (Mol, 1995). At later stages of the disease, *V. dahliae* starts colonizing the non-vascular tissue and forms microsclerotia on the senescing and dead tissue and which are released to the soil. The fungus can also survive for many years either as mycelium or conidia in the vascular system of perennial plants (Mace et al., 1981; Mol, 1995).

2.3.4 Microsclerotia (resting structure)

The microsclerotia of *V. dahliae* are resting structures that maintain the fungus in the soil in the absence of a susceptible host (Schreiber and Green, 1993). Microsclerotia, are dense aggregate of darkly stained, thick-walled hyphal cells (Figure 1: C).

Griffiths (1970) described the fine-structure of developing microsclerotia of *V. dahliae* and summarized it in three stages. Swelling and septation of the neighboring hyphae occurs in the first stage. The swelling or the budding continues to form a mass of yellow or brown microsclerotial-initial cells. In the second stage, melanin leaves the living hyphae and deposits in the cell walls and internal space. In the mature microsclerotium, the melanin accumulates in the individual cells where it surrounds the cells to form a

thick-dark cell wall. Microsclerotia are 30-60 μm in diameter (Hawksworth and Talboys, 1970).

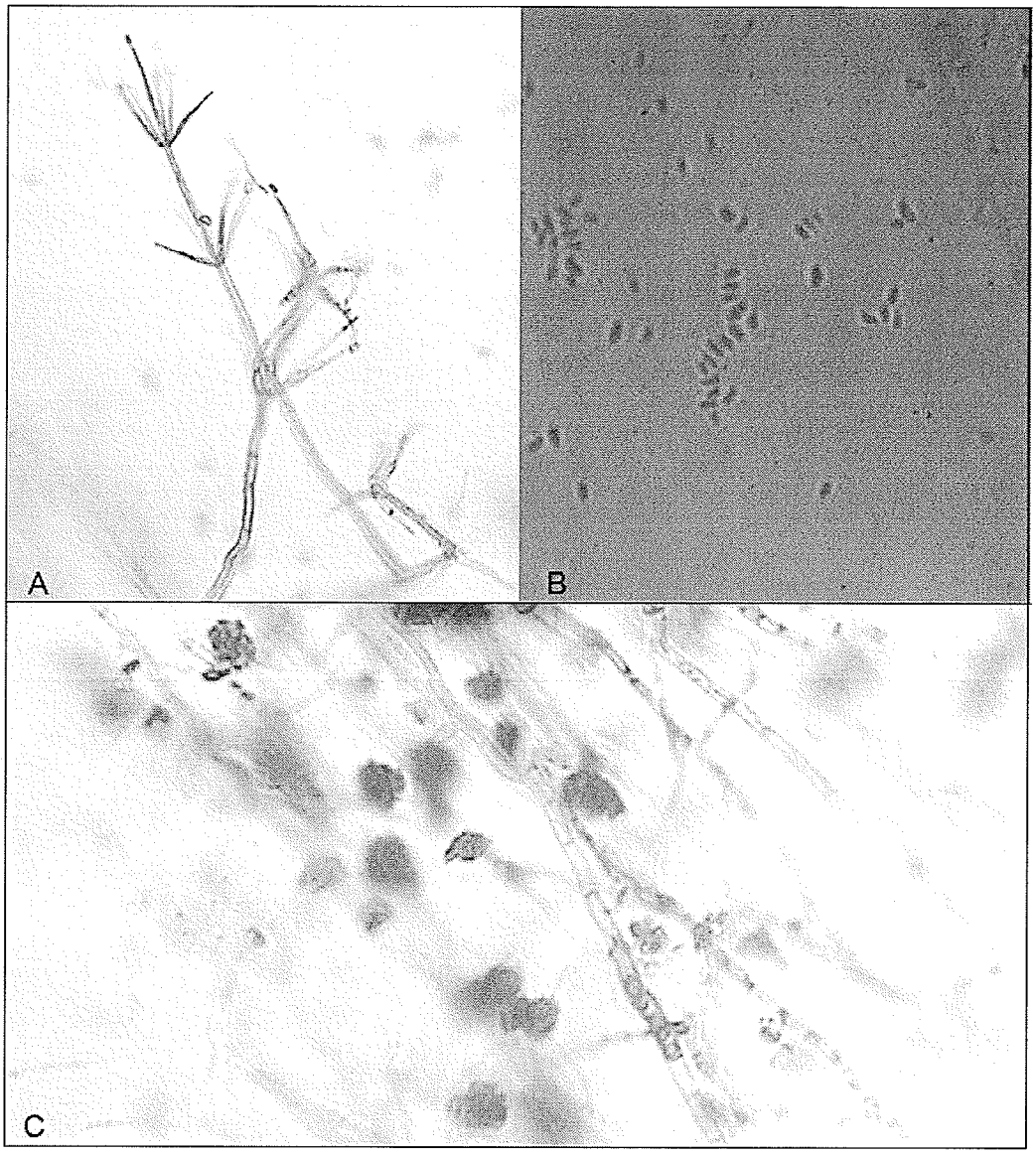

Figure 2.1. Morphological structure of *Verticillium dahliae.* A) microscopical observation of *V. dahliae* conidiophores in verticillate disposition with released conidia. B) *V. dahliae* ovoid conidia. C) microsclerotia produced by *V. dahliae* on PDA media.

2.3.5 Disease symptoms

Many *Verticillium* isolates cause different symptoms on several hosts. Symptom severity from an isolate is more pronounced on the original host of this isolate (Bhat and Subbarao, 1999). There are no exclusive symptoms that fit all hosts because of the variation among the symptoms of *Verticillium* on hosts. Even though this disease is known as Verticillium wilt, wilt does not occur in every infection. The typical wilt usually starts at one side of the lower leaf of the infected plant (Figure 2 A). The lower leaves turn yellow and the leaf edges die, causing the typical V-shaped lesion (Figure 2 B). Eventually, the chlorosis turns to necrosis (Fradin and Thomma, 2006; Mace et al., 1981). Internal symptoms arise from the plugging of the vascular system which shows brown rings in the vascular tissue (Figure 2 C). If an infected stem or potato tuber is cut laterally, a brown dark circle can be observed in some plant species (Beckman and Talboys, 1981). The disease is also known as leaf mottle in sunflower (the symptoms appear as a mottle affected by the contrast between the yellow and the persisting green areas), first described by Sackston in 1957 (Figure 2 D). The symptoms may appear early in the season but generally are close to the time of flowering (Sackston, 1957). Including the typical symptoms of Verticillium wilt, stunting with head diameter reduction 16-42% can occur (Hoes, 1972). Later, black, streaky pieces appear at the base of the stem in severely infected plants (Gulya et al., 1997).

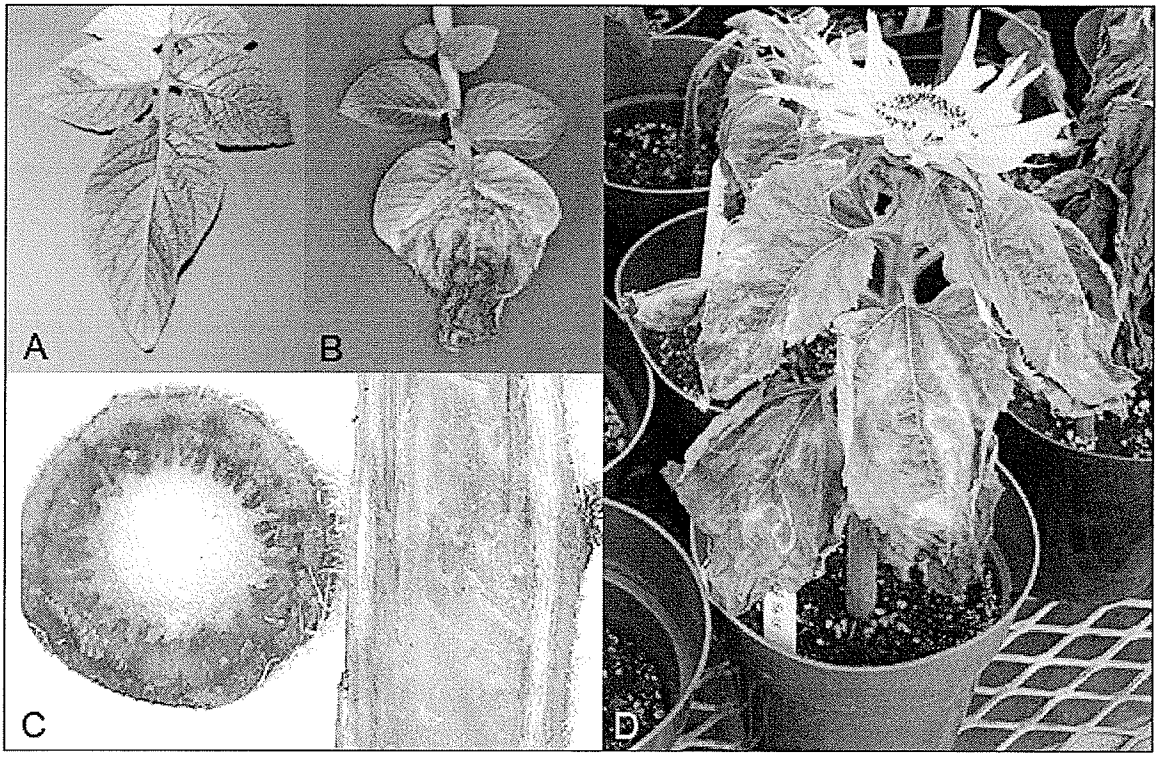

Figure 2.2. Verticillium wilt symptoms on diseased potato and sunflower. A) Chlorosis on one side of the lower leaf of potato plant. B) Necrosis with **V** shape on potato leaf. C) Elongate stem section (right) and cross section (left) show the vascular discoloration on sunflower stem that caused by *V. dahliae*. D). symptoms of Verticillium wilt/leaf mottle on sunflower.

2.3.6 Environmental influence

Temperature appears to be the most influential factor in the development of *Verticillium* species (Rowe and Powelson. 2002). *Verticillium dahliae* favors moderate to high soil temperature (21-27 C°) (Francl et al., 1990). However, it is inhibited at temperatures over 30 C° (Mace et al., 1981). In contrast, *V. albo-atrum* prefers cooler conditions (21-24 C°) (Francl et al., 1990), such as those found where most potato production is located in the northern U.S.A and southern Canada. In these regions, the average soil summer temperature is not more than 25 C° (Rowe et al., 1987; Platt, 1986).

Soil moisture plays an important role in developing and increasing the fungus inoculum in the soil (Xiao and Subbarao, 2000). Irrigation strongly influences the development of potato early dying in irrigated potato production systems. Cappaert et al. (1994) reported that disease severity in irrigated soils is greater than in soils that are water deficient or moderately moist. They also noticed that the reduction of the potato tuber yield is greater in wet than in moderate and deficient soil systems. Moist soils can be coupled with an aired atmosphere and resulting in rapid transpiration, which results in rapid movement of conidia with water in the stems and leaves (Cook, 1973). Gaudreault et al. (1995) suggested that wet soil can increase the disease severity by facilitating environments which help the fungus to penetrate the vascular tissue and it also can enhance the germination of microsclerotia.

2.3.7 Economic importance

Verticillium wilt, caused by *V. dahliae* and *V. albo-atrum* is a threat to a large number of plant species. In Canada, the disease has become a big issue in recent years. Verticillium wilt was reported for the first time in Canada by Sackston in 1964 when he found several infected alfalfa plants in Quebec and Vancouver (Sheppard, 1979). As a result of disease development throughout a field midway through the growing season and the severity of the disease during the period of maximum tuber bulking, reduction in the size and total marketable yield may occur (Rowe and Powelson, 2002). In Manitoba, Verticillium wilt and potato early dying are a major concern. The economic impact of Verticillium wilt in potato industry is significant due to the losses of potato yield and the brown ring in the vascular tissue in the tuber which reduces the quality of commercial potato cultivars for chip and French fry production.

Verticillium wilt in sunflower can reduce the yield in lighter soils by up to 50% (Manitoba Agriculture, Food and Rural Initiative, 2005). The disease affects sunflower in most production areas of the world such as Europe, Argentina, Mexico, and the former Soviet Union (USSR) (Gulya et al., 1997; Sackston and Martens, 1959). In the 1950s, Verticillium wilt threatened sunflower production in Canada. The first observation of the disease in Manitoba was in the early 1970s. A recent survey by the National Sunflower Association (NSA) showed that Verticillium wilt was present in sunflower fields with 19% and 13%, disease severity in the 2002 and 2003 respectively. A more recent survey by Rashid et al. (2006), however, showed that Verticillium wilt was present in 46 (87%) of 53 fields surveyed, with incidence ranging from trace to 20%. Disease incidence was higher in 2006 than in many previous years, but lower than in 2005. The major factor in reducing sunflower yield is reduction in head size. Oil concentration in the kernels and kernel density can be reduced in infected plants. In addition, seeds of a diseased head are smaller (Gulya et al., 1997). Verticillium wilt continues to cause significant damage in sunflower fields especially in confection hybrids, which lack genetic resistance (Hoes, 1972; Rashid and Platford, 1994).

2.4 Pathogenicity of *Verticillium* spp.

V. dahliae enters the plant roots via wounds which can be caused by other organisms such as nematodes. The fungus invades the cortex, passes through the endodermis, and invades the xylem vessels. The fungus moves through the older mature xylem vessels to the above ground part of the plant (Heinz et al., 1998). The symptoms start with chlorosis which later turns to necrosis. Vascular discoloration is obvious in the vascular tissue. In fact, the wilting that appears in infected plants may be caused by a complex interaction

between toxins and enzymes. However, the critical components in the pathogenicity of Verticillium species are poorly understood. There is no complete characterization of the functions of the toxins and cell-wall degrading enzymes.

2.4.1 Cell-wall-degrading enzymes (CWDE$_S$)

In general, plant pathogenic fungi produce a range of cell-wall-degrading extracellular enzymes which are important in infection by providing the fungus with the means to penetrate the host and release soluble components for nutrition. *Verticillium* species produce some enzymes that play a role in plant cell-wall-degradation (Dobinson et al., 2004; Novo et al., 2006). Most of the studies in this area have studied pectin degrading enzymes, because pectinases are among the first enzymes produced and are produced in large quantities. They are also the only cell wall degrading enzymes (CWDEs) that are able to macerate the plant cell wall and kill the plant tissues on their own (Annis and Goodwin, 1997). *Verticillium albo-atrum* releases several CWDEs. Endo-polygalacturonase (PG) and endo-pectin lyase (PL) have the ability to kill potato and tomato cells *in vitro* and produce symptoms in tomato cuttings including vascular discoloration. Endo-pectinases are important because of pectic gels occlude host xylem vessels. These gels are created from opening middle lamella at hole in the membranes. Consequently, this gel formation induces wilting and reduces vascular flow, and will respond to water stress (Durrands and Cooper, 1988).

Cellulases are one of the cell-wall degrading enzymes that are released by Verticillium species. Novo et al. (2006) showed that two *V. dahliae* isolates differing in aggressiveness and isolated from *Capsicum annuum L. var. annuum cv.* were able to produce cellulases which were capable of degrading soluble and crystalline cellulose.

Cellulases may not be the determining factor for aggressiveness and, as a result, the cause for the disease symptoms, but they can play an important role in the penetration process (Walton, 1994).

2.4.2 Toxins

Both *V. dahliae* and *V. albo-atrum* are reported to produce phytotoxins in plant tissues (Mace et al., 1981b). Phytotoxins can be of a high molecular weight such as protein-lipopolysaccharide (PLP) complexes, glycoproteins, and cell wall degrading enzymes (Buchner et al., 1982; Mansoori et al., 1995, Meyer et al., 1994; Palmer et al., 2005, Pu et al., 2007). These components are effective at very low concentrations (Fradin and Thomma, 2006).

The original explanation of why plants wilt when infected by a vascular pathogen is because of the physical blockage of the plant's xylem by the pathogen. Extracellular polysaccharides which are produced by a pathogen could play a role in the physical blockage of the plant xylem, where small amount of extracellular polysaccharides can cause plants to wilt. This wilting is due to the susceptibility of the plant to disruption of the water conducting system by embolism formation.

Also, pathogen increases the resistance to water conduction through leaves. This increased resistance to water flow reduced the leaf-water potential to the point where water stress happened daily. Stomata remained closed most of the day as a result of the low leaf-water potential, resulting in stunted, chlorotic plants. In alfalfa the pit membrane pores are not the same size throughout the plant parts. In the stem, the pores are larger than the leaf, thus, very small polysaccharides are able to plug the pores in the leaf as compared to in the stem (Durrands and Cooper, 1988).

Filtered cultures of *Verticillium* species contain toxin complexes, but exact nature of the components of the complexes is unclear (Fradin and Thomma, 2006). Protein-lipopolysaccharyde (PLP) isolated from a potato isolate of *V.dahliae* contain glycopeptides toxin which correlates with the symptoms of *Verticillium* wilt (Fradin and Thomma, 2006). Toxins are present in stems, cell walls, xylem and tubers (Nachmias et al., 1985). Protein-lipopolysaccharyde (PLP) isolated from culture filtrates of *V. dahliae* showed binding to the plasma membrane of various cotton tissues (root, hypocotyl and cotyledon tissue). Root tissue had the highest binding of PLP comparison with hypocotyl and cotyledon tissues (Meryer and Dubery, 1993). PLP complex filtrated from seven cultures of *V. dahliae* had polygalacturonase and cellulase enzyme activities. Cotton seedlings treated with PLP complex showed symptoms of wilting and necrosis (Meyer et al., 1994) suggesting that the symptoms of *Verticillium* wilt disease are the result of toxin activity.

Wilt pathogens also secrete glycopeptides as a toxin. These molecules induced wilting in plant tissues as a result of cell membrane damage, and when the membrane damage occurs, the osmotic properties of the cell wall are damaged too, resulting in the loss of electrolytes and water. Strobel and Hess (1968) tested root hairs by treating them with 2mg/ml of toxin for one hour. The hairs failed to plasmolyze when they were dipped in a hypertonic solution of mannitol (a sorbitolisomer which can act as an osmotic diuretic agent and a weak renal vasodilator). This result showed that the plasmolytic ability of the cell was damaged by the toxin and the electrolyte loss was higher in the treated plant than in the control.

Even though the exact nature of the components of *Verticillium* toxins is not clear, several studies indicated the impact of these components and their important role in inducing symptoms of wilt disease in a large number of hosts.

2.5 Other factors affecting Verticillium pathogenicity

2.5.1 Effects of the origin of isolates

Verticillium dahliae isolates are reported to lack host specificity (Douhan and Johnson, 2001). Several studies showed that *Verticillium* species vary in pathogenicity on different hosts. However, isolates from one particular host can infect and cause symptoms in other hosts (Bhat and Subbarao, 1999; Tsror et al., 2001). *Verticillium* isolates are pathogenic and very aggressive on the hosts from which they were originally isolated (Bhat and Subbarao, 1999; Bhat et al., 2003; Gordon et al., 2006; Goud and Termorshuizen, 2002, Resende et al., 1994). Moreover, the previous cropping history may play a role in the aggressiveness of *Verticillium* isolates. Tjamos (1981) showed that isolates from tomato that originated from areas where solanaceous plants were widely grown tend to be more virulent in tomato. However, most isolates from cotton, artichoke, and peach are nonpathogenic to mildly pathogenic in tomato (these isolates were obtained from fields in which tomatoes had not been grown, had been grown on a limited basis, or were introduced recently). Tjamos (1981) also reported that isolates of *V.dahliae* and *V. albo-atrum* from potato that originated from the highlands, where potatoes are cultivated for seed production, are pathogenic to tomato in spite of the diversity of the previous crops grown.

2.5.2 Vegetative compatibility groups and pathogenicity of *Verticillium* isolates

Verticillium species vary in pathogenicity, and *V. dahliae* and *V.albo-atrum* have a broad host range with no clear host specificity. Also, it is not known whether similar isolates are genetically associated to each other (Korolev et al., 1997). Because the sexual stage is absent in *Verticillium* species, parasexuality is the only known means of exchange of genetic material between individual mycelia. Vegetative compatibility occurs when the isolates can anastomose with one other and form a viable heterokaryon (Katan, 2000). Nitrate non-utilizing (nit) mutants are used to group *V. dahliae* strains in different vegetative compatibility groups. These nit isolates express mutant growth on minimal medium. Different isolates are allowed to grow on the media and isolates that are able to mate and produce wild-type growth are grouped in the same vegetative compatibility group. If the mutants do not anastomose or are not complementary, no wild-type growth can be achieved from their combination (Joaquim and Rowe, 1990). *Verticillium dahliae* was classified into four vegetative compatibility groups; VCG1, VCG2, VCG3, and VCG4. Katan (2000) grouped VCG3 with VCG4.

Several studies correlated vegetative compatibility groups (VCGs) of *V. dahliae* with pathogenicity on different hosts (Bao et al., 1998; Chen, 1994; Daayf et al., 1995; Elena and Paplomatas, 1998; Joaquim and Rowe, 1991; Puhalla, 1979). For instance, Joaquim and Rowe, 1991, deliniated two subgroups of VCG4 based on the pathogenicity of the isolates on potato, in which they found that VCG 4A isolates are more aggressive on potato than VCG 4B isolates. Moreover, the pathogencity linking with VCGs was reported in some American VCGs. Daayf et al. (1995) reported that VCG1 contains all cotton-defoliating strains which are nonpathogenic on tomato, and VCG 2 and VCG 4

contain all cotton non-defoliating strains that belonged to different races on cotton and on tomato. Most isolates from potato are VCG 4. Corsini et al. (1985) examined (2 of VCG4 and 1 of VCG3) isolates obtained from potato and a defoliating strain from cotton assigned to VCG 1 for pathogenicity on potato cv. Russet Burbank. These isolates showed that the two strains of *V. dahliae* assigned to VCG 4 were more virulent to potato than the two strains in VCG 1 and VCG 3. Strains of a more virulent pathotype on potato are most likely to be found in VCG 4A, while strains from VCG 2 and VCG 4B are more likely to be of a less aggressive or virulent pathotypes on potato (Joaquim and Rowe, 1991; Tsror et al., 2001).

Vegetative compatibility is a useful marker for determining the genetic structure of some fungal populations (Leslie, 1993). Including the identification of genetic diversity of *Verticillium* strains, VCGs allow for the identification of variability of pathogenicity of the population (Chen, 1994).

2.5.3 Effects of serial passages on hosts

Verticillium species have not been grouped into special forms because an isolate from one host often attacks several other unrelated plant species. However, some isolates of *Verticillium* are rather specialized. *Verticillium dahliae* from peppermint, pepper, strawberry and *V. albo-atrum* from Lucerne and hops have limited host ranges (Puhalla and Bell, 1981). Moreover, host specificity appears not to be a rigid character (Jeger et al., 1996). In some cases, *Verticillium* strains, which are initially avirulent to a particular host, become virulent after serial passages through that host. Certain plant parasites show increased virulence (Fordyca and Green, 1963). Mills, (1940) showed that *Phytophthora infestans* isolated from potato was initially only mildly pathogenic to tomato, but

increased in virulence after 2-3 serial passages through tomato. Fargette et al, (2002) tested two highly aggressive isolates of the virus *Sobemovirus* that causes the disease Rice Yellow Mottle Virus (RYMV). The virus induces intermediate symptoms in the susceptible cultivar, inconspicuous symptoms on the partially resistant and no symptoms on the resistant rice cultivar. The pathogenicity of the two isolates changed rapidly after serial passages into the host, where they became conspicuous on the partially and resistant cultivars.

Fordyce and Green (1963) studied the effect of serial passages of *Verticillium albo-atrum* isolated from mint (*Menthae piperita* L.) on the pathogenicity of the nonhost tomato. The study showed that all isolates initially were nonpathogenic to tomato; however, two isolates became pathogenic after one passage and one isolate after five serial passages. Concurrently, with the increase in pathogenicity to tomato, all three isolates became nonpathogenic to *Menthae piperita* L. Certain phenotypic changes were also observed.

2.6 Disease control and management strategies

Microsclerotia are one of the inoculums sources and form in infected potato vines (Schnathorst, 1981). Infected seed tubers are also a major source of contamination of soils and potato storage areas (Adams et al., 1980; Bang, 1986; Hide et al., 1977). Irrigation water, wind or contaminated equipment can cause of infesting clean fields. However, introducing the fungus to the surface of the tubers or within tissues of seed tubers seems to be the primary source of infection (Rowe and Powelson, 2002). The ability of Verticillium wilt fungi to survive in the soil for long periods with or without a host plant and the colonization of the vascular tissues limit any method of eradicating the

pathogen and make the disease difficult to control. Management practices for control of this disease are aimed at reducing the inoculums in the soil by using resistant or tolerant varieties. Soil fumigation and cultural controls, which include crop rotation, soil solarization, proper water and fertility management, and the application of biological control, are also used as control methods for Verticillium wilt (Powelson and Rowe, 1993; Tjamos et al., 2005).

2.6.1 Chemical soil fumigation control

Soil fumigation is an effective method to control Verticillium wilt (Powelson and Rowe, 1993). Soil fumigants such as metham sodium and chloropicrin are effective and easy to apply. Fumigation can be accomplished either by injecting the fumigant into the soil or by application in water directly through a sprinkler irrigation system. The use of soil fumigation has declined in the past few years because of the cost of the application and the negative environmental side-effects.

2.6.2 Cultural control

2.6.2.1 Crop rotation

Crop rotation is one of the important practices for controlling Verticillim wilt. However, crop rotation may not successfully control the disease because of the ability of microsclerotia to survive in the soil for many years and the ability of *V. dahliae* to persist on the roots of non-host cultivars. Verticillium wilt is a serious problem in Manitoba (Gulya et al., 1997), because of the lack of resistant in sunflower cultivars and the short crop rotations, long rotations that include non-host crops of Verticillium can reduce the population of the fungus in the soil. Several crops are recommended for rotation. Cereals (barley, wheat, corn, and oats), legumes (clover, bean, and pea), carrot, and onion which

are not hosts to Verticillium can be used in rotation with potato and sunflower (Davis et al., 2000).

2.6.2.2 Soil solarization

Soil solarization was reported in 1976 as a method to elevate soil temperature for soilborne pathogen control (Katan et al., 1976). Soil solarization utilizes the sun's energy to heat moist soil. Transparent polyethylene film allows the solar radiation to be transmitted directly to the soil and also reduces moisture loss from the soil through evaporation. Higher soil temperatures may be obtained with dark-colored soils since they absorb more solar radiation than light-colored soils (Stapleton and DeVay, 1986). The primary advantage of soil solarization is that it is a nonchemical method of soil disinfestation so worker and environmental exposure to chemicals are reduced. In addition, it may reduce the cost of soilborne pest management by eliminating or reducing the amount of pesticide used (Gruenzweig et al., 1993). This method is not feasible in Manitoba due to the cold weather.

2.6.3 Irrigation practice

Verticillium wilt has been pronounced to be a disease of threat in North America. The severity of disease and associated yield loss are affected by irrigation (Davis and Everson, 1986). Disease severity of Verticillium may increase in wet soil by providing the fungus with favorable conditions to penetrate the root tissue (El-Zik, 1985). Wet soil may indirectly cause tuber-disease problems by increasing the tuber water potential and as a result, increasing lenticel swelling and the susceptibility of the tuber to other soilborne pathogens such as soft-rot Erwinias (Cappaert et al., 1992). Moreover, under irrigation conditions, the microsclerotia can germinate and sporulate many times thereby

increasing the population density under the high soil moisture, resulting in an increase in the disease severity (Nicot and Rouse, 1987). The amount and time of irrigation are important to manage the disease (Rowe and Powelson, 2002). In irrigated areas, the amount of applied water can be controlled by regularity and time of irrigation to reduce Verticillium wilt. The modifications of irrigation practices should take place before the tuber initiation stage to reduce the chance of malformed tubers (Cappaert et al., 1992a, 1992b).

2.6.4 Host resistance

Developing resistant cultivars seems to be the most effective, efficient, economical and environmentally sound strategy to control Verticillium wilt disease. Studies in the US show that planting highly resistant cultivars for five seasons reduced the population of pathogen by 60-70% (Rangahau, 2003). There are varying levels of Verticillium wilt resistance among commercial potato varieties. Some of the cultivars with high levels of Verticillium wilt resistance are not acceptable for chipping and frozen product processing because of low solids and dark frying color (Mosley et al., 2000). Data from North America listing the cultivars that grow in a particular region indicated that all cultivars, except Ranger Russet, are moderately to highly susceptible to Verticillium wilt (Rowe and Powelson, 2002). The use of dominant gene resistance is widely employed to produce wilt-resistant hybrids in sunflower and as a result, the oilseed hybrids are resistant to the North American race of *V. dahliae* (Dedio and Rashid, 1991). There are nine inbred lines with resistance to Verticillium wilt which were released by Dedio and Rashid in 1991 in Manitoba. Hoes (1973) noted that the most resistant sunflower species to Verticillium wilt are wild *H. annuus.* Collections from Manitoba, Saskatchewan, and

North Dakota are less resistant than those from Colorado, and Kansas (Gulya et al., 1997).

2.6.5 Biological control

For many field and vegetable crops, cultural practices and use of resistant cultivars are alternatives to soil eradication (Davis et al., 1996; Ioannou et al., 1977; Pullman and DeVay, 1981). The nature of pathogenesis by *V.dahliae* makes the potential use of biological control difficult because continuous defense of feeder root apices is necessary to prevent vascular invasion of host plants over most of their vegetative growth period (Huisman, 1982). However, several organisms have shown abilities to suppress the growth of soilborne pathogens (Martin and Bull, 2002). The Bacterium *Pseudomonas* spp. can aid in controlling soilborne pathogens. *Pseudomonas* produce antibiotic or pyoverdine siderophores (Loper and Buyer, 1991) and also can compete for nutrients (Andrews, 1992) thereby suppressing the fungus. *Bacillus* is another organism that can suppress *V. dahliae.* Tjamos et al. (1998a and b) showed that strains of Bacillus sp. obtained from the root tip were capable of living in the rhizosphere and growing in susceptible hosts and reducing the disease. Uppal et al. (2008) found that the bacteria *Rahnella aquatilis*, an unknown bacteria, a strain of *Pseudomonas fluorescens*, *Bacillus. pumilus*, and *Bacillus. amyloliquefaciens* had a high level of inhibition on the *in vitro* growth of *V. dahliae*. *Pseudomonas fluorescenss and B. pumilus* were examined for their abilities to suppress the fungus under the growth room and field condition on two potato cultivars; Kennebec (susceptible) and Russet Burbank (moderately susceptible). The bacteria were significantly effective in reducing the disease and increasing the yield for cultivar Kennebec, while only *P. fluorescens* was effective with Russet Burbank.

The hypotheses for these studies are that

1- *Verticillium dahliae* isolates from a particular host are able to infect other hosts.

2-*Verticillium dahliae* isolates are more aggressive on the original host than on alternative hosts.

3-The disease incidence and severity are more pronounced on susceptible cultivars than on moderately resistant ones.

4-Serial passages of *V. dahliae* can affect the fitness of the pathogen, in terms of gain or loss of pathogenicity on specific host species.

VERTICILLIUM DAHLIAE CROSS PATHOGENICITY BETWEEN POTATO AND SUNFLOWER

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Key words: Alternative host, cross-pathogenicity, disease assessment, host of origin, potato, rotation, sunflower, Verticillium wilt

3.0 *Verticillium dahliae* Cross Pathogenicity Between Potato and Sunflower

3.1 Abstract

This study examined cross-pathogenicity of the soilborne pathogen *Verticillium dahliae* between potato and sunflower. Four-wk-old potato and sunflower seedlings were inoculated with 10 isolates from each of the two host species. Potato cultivars (Kennebec, susceptible, and Ranger Russet, moderately resistant) and sunflower hybrids (IS8048, susceptible, and 6946, moderately resistant) were assessed for disease severity and percent infection at 2, 3, 4, 5, and 6 weeks after inoculation (wai), and for vascular discoloration at 6 w.a.i., using visual scales developed for each host species. The experiments were conducted in 2006 and repeated in 2007. Based on percent infection and disease severity, most *V. dahliae* isolates were highly aggressive on both host species. The tested isolates caused higher disease levels in the susceptible than in the moderately resistant phenotypes. They also caused more vascular discoloration in their original than in the alternative host. However, the isolates originating from sunflower caused less infection and lower disease severity on both hosts, as compared to their potato counterparts. Cluster analysis based on all of the criteria used to assess pathogenicity led to three groups of isolates: (i) most *V. dahliae* potato isolates, which ranged with the highly aggressive control isolates, (ii) one *V. dahliae* sunflower isolate, which showed a similar pathogenicity level as the weakly-aggressive *V. albo-atrum* sub-group II control isolate, with no more symptoms than in the non-inoculated plants, and (iii) most *V. dahliae* sunflower isolates with mildly- to weakly-aggressive levels of pathogenicity. Based on these results, *V. dahliae* cross-pathogenicity is very effective between potato and sunflower. Therefore, rotations involving these species should be avoided, especially when sunflower follows potato.

3.2 Introduction

Potato and sunflower are important crops worldwide. Both are hosts to *Verticillium* spp. (i.e., *V. dahliae* and *V. albo-atrum*), which reduce yield and tuber quality in potato (Rowe and Powelson, 2002) and both head size and oil content in sunflower (Hoes, 1972). These fungal pathogens are soil-borne and cause wilts in several other crops worldwide (Pegg and Brady, 2002). Rotation is one of the major components of Verticillium wilt management. However, many field crops and vegetables are susceptible to the pathogens causing this disease. In addition, *V. dahliae*'s resting structures (microsclerotia) can survive in the soil for more than 10 years (Heale and Karapapa, 1999). Its disease cycle starts with the germination of microsclerotia after stimulation by root exudates (Mol, 1995). The germinated hyphae penetrate the root tip, colonize the cortex tissues and move upward through the vascular system to the above-ground part of the plant (Bowers et al., 1996). The ability of microsclerotia to germinate and produce secondary microsclerotia is a significant factor that can maintain a high inoculum load in the soil for several years (Coley-Smith and Cooke, 1971).

V. dahliae varies in its level of pathogenicity on different hosts even though it has a wide host range (Bhat and Subbarao, 1999). Isolates from one host are able to cause disease on other plant species but symptoms are often more severe on the host of origin. For instance, isolates from cocoa or strawberry were shown to be more aggressive on these host plants than on other tested crops (Resende et al., 1994; Gordon et al., 2006). Cross-pathogenicity was also reported in other pathogens, i.e., *Fusarium oxysporum* f. sp. *cucumerinum* on cucumber and melon (Cafri et al., 2005), *Gaeumannomyces graminis* var. *graminis* on bermudagrass, St. Augustine grass, and rice (Datnoff et al., 1997), and

Fusarium moniliforme on corn and asparagus (Damicone et al., 1988). *Verticillium dahliae* cross-pathogenicity was also reported on artichoke, bell pepper, broccoli, cabbage, cauliflower, chili pepper, cotton, eggplant, mint, lettuce, potato, strawberry, tomato, and watermelon (Qin et al., 2006). However, it is not known whether sunflower isolates cause disease on potato, to what extent, and *vice-versa*. This information is important in locations where both crops are widely grown and could be considered for rotation such as in Manitoba, Canada.

The objectives of this study were to: (i) isolate *Verticillium* spp. from infected potato and sunflower tissues grown in commercial or experimental fields in Manitoba; (ii) determine the prevalent species of *Verticillium* spp. across the sampled potato and sunflower fields; (iii) develop an accurate rating scale for both potato and sunflower in order to assess both external and internal symptoms; and (iv) evaluate the cross-pathogenicity of *V. dahliae* isolates recovered from potato on sunflower and *vice-versa*.

3.3 Material and Methods

Plant material and growth conditions

Two potato cultivars and two sunflower hybrids were selected for this study, based on their level of susceptibility to *V. dahliae*. Potato cultivar Kennebec and sunflower hybrid IS8048 are susceptible while potato cultivar Ranger Russet and sunflower hybrid 6946 are moderately resistant. Potato seed pieces and sunflower seeds were sown in 10 cm diameter plastic pots filled with a pasteurized mixture of sand and soil (1:1, v/v) containing NPK fertilizer granules (16:20:16). Pots were incubated for four weeks in a growth room set at 20/16 ± 2°C day/night under 16h-photoperiod with a light intensity of

350 $\mu mol.m^{-2}.s^{-1}$. After inoculation, seedlings were transplanted into 15-cm-diameter pots filled with pasteurized soil mixture composed of soil, sand, peat and perlite (4:4:4:1, v/v/v/v) and received NPK treatment (20:20:20). Plants were watered regularly and kept clean from aphids, spider mites and other pests that may interfere with Verticillium wilt assessment during all experimentation.

***Verticillium* spp. characterization**

All *Verticillium* spp. used in this study were single-spore isolates recovered in 2004, 2005 and 2006, from potato and sunflower tissues collected from commercial and experimental fields across the province of Manitoba, Canada. Altogether, 23 *V. dahliae* isolates were used, 10 from each plant species (potato: 04-07, 04-09, 04-17, 04-21, 04-28, 04-25, 04-35, 04-38, 04-41, 04-47; sunflower: 06-01, 06-02, 06-03, 06-06, 06-07, 06-09, 06-11, 06-13, 06-14, 06-20), two highly aggressive *V. dahliae* control isolates from potato (Vd1396-9 and Vd1398-21, Uppal et al., 2007), and one weakly aggressive *V. albo-atrum* sub-group II control isolate from potato (V104b, P.E.I.-Canada).

Mycelia originating from three-wk-old cultures on PDA were examined under the microscope for their typical features such as the presence of microsclerotia and their shape, and the verticillate branching of phialides on the conidiophores for morphological identification of the isolates.

Once single-spore cultures were produced, DNA was extracted from each isolate following the protocol described by El Hadrami et al. (2007). Briefly, harvested mycelia (~50-100 mg of fresh weight) placed in a sterile 1.5mL Eppendorf tube were mixed with 125µL of 0.5 M NaOH and ground using a plastic micro-pestle. After 5 min of incubation at room temperature, 500 µL of 0.1 M Tris-HCl buffer containing 0.5 M EDTA (pH 8.0)

were added to the extracts and the mixture was centrifuged for 6 min at 14000 r.p.m. Three hundred µL of the supernatant, representing the DNA solutions, were transferred to new tubes and stored at -20 °C until further analysis by PCR. PCR identification of *Verticillium* spp. consisted of using a universal primers' set (UVd-F: 5'-CTCATAACCCTTTGTGAACC-3': UVd-R: 5'- CCGAGGTCAACCGTTGCCG-3') that is *Verticillium* genus-specific and amplifies a 452bp product, as well as a *V. dahliae*-specific primers' set (Vd-F: 5'-CCGGTCCATCAGTCTCTCTG-3': Vd-R: 5'-ACTCCGCATCAGTCTCTCCTG-3') that yields a 334bp (Nazar et al., 1991; Robb et al., 1994). One µL DNA was used as a template in 25 µL PCR reaction mixture containing 1X PCR buffer, 1 mM dNTPs mix, 1 µM of each primer, 5 mM $MgCl_2$, 0.1 U Taq DNA polymerase and H_2O. PCR amplification was carried out in a Flexigene thermocycler (Techne, Princeton, N.J.) with an initial denaturation at 94°C for 5 min followed by 35 reaction cycles consisting of 30 s denaturation at 94°C, 45 s annealing at 60°C and 1 min elongation at 72°C and a final extension (for 10 min) at 72°C. PCR products were then analyzed by electrophoresis using a 1.5% agarose gel containing 1% ethidium bromide at 105 V for 30 min. The gels were visualized using an AlphaImager HP version 6 (Alpha Ease FC software, Alpha Innotech, San Leandro, U.S.A).

Inoculation of *V. dahliae* isolates and evaluation of their pathogenicity

Verticillium dahliae cultures incubated at 25 °C for three weeks on PDA were used to produce inoculum. Conidial suspensions were made in sterilized sterile distilled water (SDW) and calibrated at 10^6 conidia.ml^{-1}. Four-wk-old seedlings grown in a pasteurized sandy soil mixture were gently removed and washed under running tap water. Following the rinsing, a few mm of the root tips were trimmed then dipped for approximately 75 sec

into the inoculum solutions prepared from each tested isolate as described by Daayf et al. (1998).

Development of disease rating scales

Both sunflower and potato seedlings were inoculated as described above In a preliminary study, using a reference *V. dahliae* isolate Vd1396-9 in order to accurately develop rating scales for percent infection and disease severity using the image analysis software Assess 2.0 (Lamari, 2002). For that purpose, 10 symptomatic leaves with various degrees of chlorosis and necrosis were chosen from infected potato and sunflower seedlings at different stages after inoculation. The percentage of infected area (chlorosis and necrosis) was deduced from the ratio of infected area over the total leaf area. Based on these percentages, a 0-5 qualitative visual scale was developed for both potato and sunflower (Fig. 1). In these scales, 0 represents no necrosis or chlorosis, 1: visible chlorosis with less than 1% necrosis, 2: up to 40% chlorosis and 1-20% necrosis, 3: up to 65% chlorosis and 20-35% necrosis, 4: 100% chlorosis, 35-70% necrosis, 5: 100% chlorosis, 70-100% necrosis.

Similarly, a scale was also developed to assess the degree of discoloration of the vascular system. Cross-sections taken from the lower, middle and upper parts of the stems of infected seedlings were chosen to represent various degrees of vascular discoloration at 6 w.a.i. Using the software Assess 2.0 (Lamari, 2002), the vascular discoloration percentages were calculated and a visual scale 0-5 was developed according to the following: 0= no vascular discoloration, 1= trace to less than 9% of the stems cross-section showing discoloration, 2= 10-24%, 3= 25-49%, 4= 50-74%, and 5= 75-100% discoloration (Fig. 1). Due to the hollow nature of the upper part of sunflower

seedlings, the vascular discoloration was assessed using another scale that was developed for this particular section based on the same principle (Fig. 1).

Experimental design and data analysis

All trials were conducted following a randomized complete block design. Each experiment consisted of four individual replicates per treatment isolate x cultivar/hybrid and each trial was conducted in 2006 and repeated in 2007 in the geenhouse. Both non-inoculated control plants and plants inoculated with highly-aggressive *V. dahliae* isolates Vd1396-9 and Vd1398-21 or weakly-aggressive *V. albo-atrum* isolate V104b were included in all trials. In each experiment, plants were evaluated for disease development and progress at 2, 3, 4, 5, and 6 weeks after inoculation (w.a.i.). Percent infection and disease severity were calculated as follows: $\text{Percent Infection} = \left(I_L / T_L\right) \times 100$; where I_L is the number of leaves exhibiting external Verticillium wilt symptoms (i.e. chlorosis, necrosis, wilting), and T_L is the total number of leaves of the rated plant; $\text{Disease Severity} = \left[\sum_{i=0}^{n} (n \times b)\right] \times 100 \Big/ T \times (N - 1)$ (Gauhl et al., 1993), where b is the chlorosis/necrosis grade (0-5 referring to the pre-developed scale) and n is the number of leaves with necrosis grade b, N is the total number of chlorosis/necrosis grades used on the scale and T is the total number of leaves. Based on the over time percent of infection and disease severity, the areas under disease progress curve (AUDPC) were calculated using the following formula (Campbell and Madden, 1990): $\text{AUDPC} = \sum_{i=1}^{n-1} [((y_i + y_{i+1})/2)(t_{i+1} - t_i)]$, where n is the total number of assessments in weeks, y_i is the percent infection or disease severity at the i^{th} assessment week, and the term $t_{i+1} - t_i$ is the time duration between two assessments.

Internal Verticillium wilt symptoms were also determined by assessing the degree of vascular discoloration in the plant stems. Since this is a destructive method, cross-sections from the lower, middle, and upper stem parts were assessed for vascular discoloration only at the end of each experiments (6 w.a.i.).

All collected data was statistically analyzed using the General Linear Model (GLM) in S.A.S. Package (Statistical Analysis Systems Institute Inc., Cary, N.C, and U.S.A.). Differences among *V. dahliae* isolates in terms of total AUDPC for percent infection and disease severity were determined by analysis of variance followed by means comparison using Newman-Keul's test ($P < 0.05$). Comparing the data gathered in 2006 and 2007 showed significant differences ($P < 0.05$) between the two years with higher disease ratings in 2007. Although, the overall trend remained similar between the two years data is presented in separate graphics.

Cluster analysis of Statistica v. 8 (statSoft, 1999) was also used to classify the tested isolates and determine similarities among them. In these analyses, all criteria used to assess the disease (percent infection, disease severity and vascular discoloration) in both 2006 and 2007 were used to generate a horizontal hierarchical tree plot representing similarity among the tested isolates. Linkage distances were calculated based on the Euclidean distance ($\text{Distance}(x,y) = \left\{\sum_i (x_i + y_i)^2\right\}^{1/2}$), which is the geometric distance in the multidimensional space. Isolate clusters were generated using unweighted pair-group averages, in which the distance between two clusters was calculated as the average distance between all pairs of isolates in different clusters. This method was chosen because it performs equally well with elongated "chain" type clusters such as variation of pathogenicity among isolates.

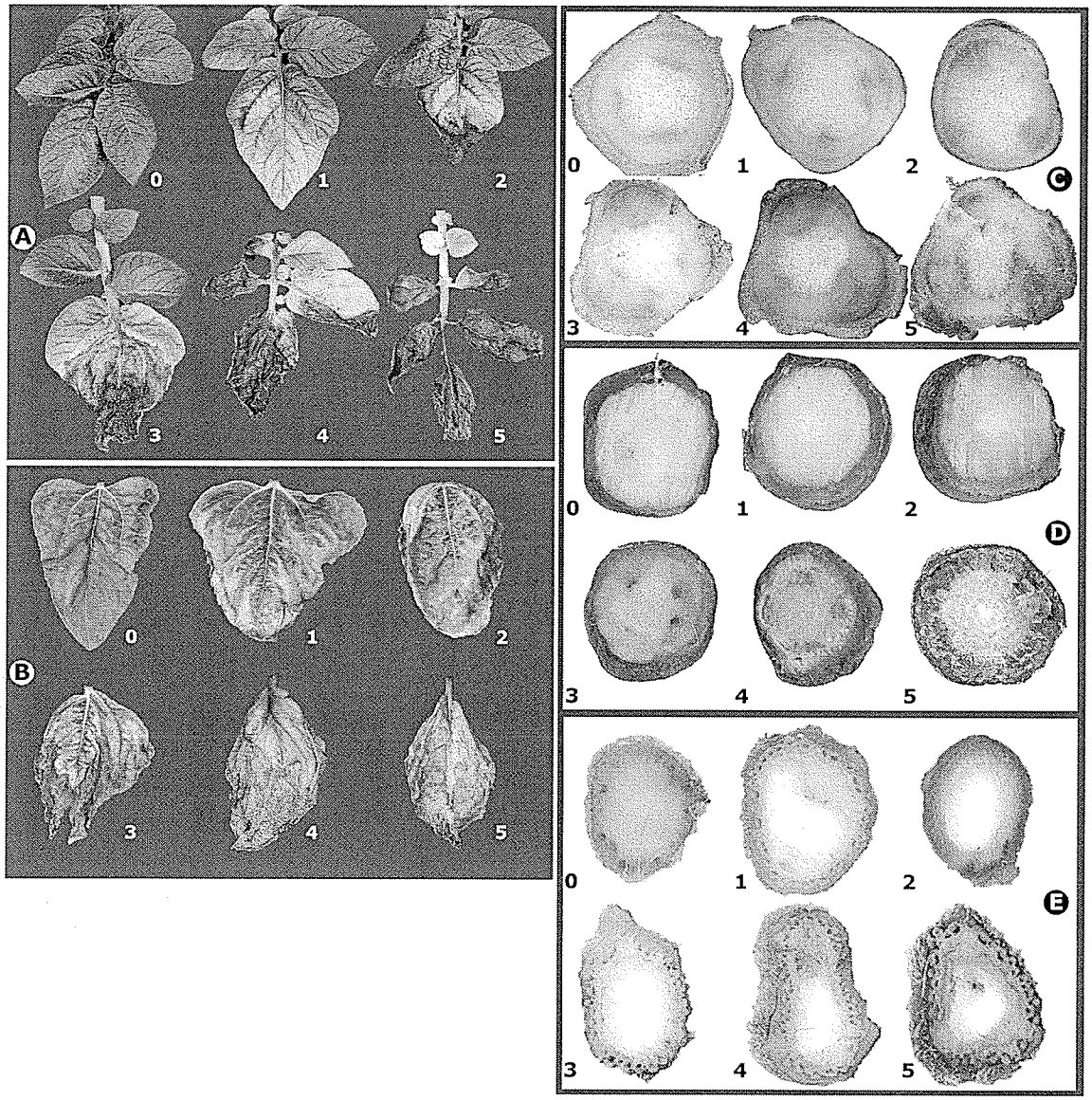

Figure 3.1. Visual scales (0-5) used to assess the external (chlorosis and necrosis in A and B) and internal symptoms (vascular discoloration in C, D and E) of Verticillium wilt in potato (A, C) and sunflower (B, D, E). D and E represent the scales used to rate vascular discoloration of sunflower cross-section from the lower and upper stem parts, respectively. In A and B, 0 represents no necrosis or chlorosis, 1: visible chlorosis with less than 1% necrosis, 2: up to 40% chlorosis and 1-20% necrosis, 3: up to 65% chlorosis and 20-35% necrosis, 4: 100% chlorosis, 35-70% necrosis, 5: 100% chlorosis, 70-100% necrosis. In C, D and E, 0 represents no vascular discoloration (VD); 1: traces to less than 9% VD; 2: 10-24%; 3: 25-49%; 4: 50-74%; and 5: 75-100%. These percentages were calculated using the image analysis software Assess (Lamari, 2002).

3.4 Results

Morphological and PCR identification of *V. dahliae*

All tested isolates were morphologically identified *in vitro* as *V. dahliae*. Their septated mycelia harbored 3-6 phialides in verticillate arrangement on the conidiophores (Fig. $2A_1$). Microsclerotia were also observed on all tested cultures as globose to oblongate dark melanized structures throughout the colonies with no presence of dark mycelia around (Fig. $2A_{2,3}$). PCR analysis confirmed the identification of all tested isolates to the genus *Verticillium*, since a 452 bp specific fragment was positively amplified from their DNA using *Verticillium* universal primers. When this first PCR product was used as a template for a nested PCR with *V. dahliae*-specific primers, a 334 bp product was amplified, confirming the identification of the tested isolates to the *V. dahliae* species (Fig. 2B).

Plant responses to the infection with *V. dahliae* isolates

Potato cultivars and sunflower hybrids all exhibited typical Verticillium wilt symptoms in response to inoculation with the tested *V. dahliae* isolates (Fig. 3A,B). The disease was much more pronounced, and progressed faster, on the susceptible cultivars/hybrids than on the moderately resistant ones. In potato, disease symptoms were visible 2 w.a.i. and developed over time from chlorosis to necrosis and wilting. At late stages ($\geq$ 6 w.a.i.) disease severity was high and stunting was apparent on the rated plants, especially the susceptible cultivar Kennebec. When lower, middle and upper stem sections of this cultivar were dissected, they all showed vascular discoloration and most of them had microsclerotia (Fig. 3D). Cultivar Ranger Russet, on the other hand, exhibited chlorosis only on the lower leaves 3 w.a.i. Six w.a.i., plants of this cultivar

dropped their mostly affected leaves and produced new branches. At 8 w.a.i., only a few plants displayed vascular discoloration in response to highly aggressive isolates.

Verticillium wilt symptoms observed on sunflower hybrids were different in shape and appearance from those seen on potato cultivars (Fig. 3A), and were generally visible before the second week after inoculation. Generally, wilting and stunting accompanied chlorosis and necrosis and all analyzed cross-sections displayed vascular discoloration (Fig. 3C). Significant differences ($P < 0.05$) were recorded among data sets collected in 2006 and 2007, although a similar trend was observed between the two years (Fig. 4).

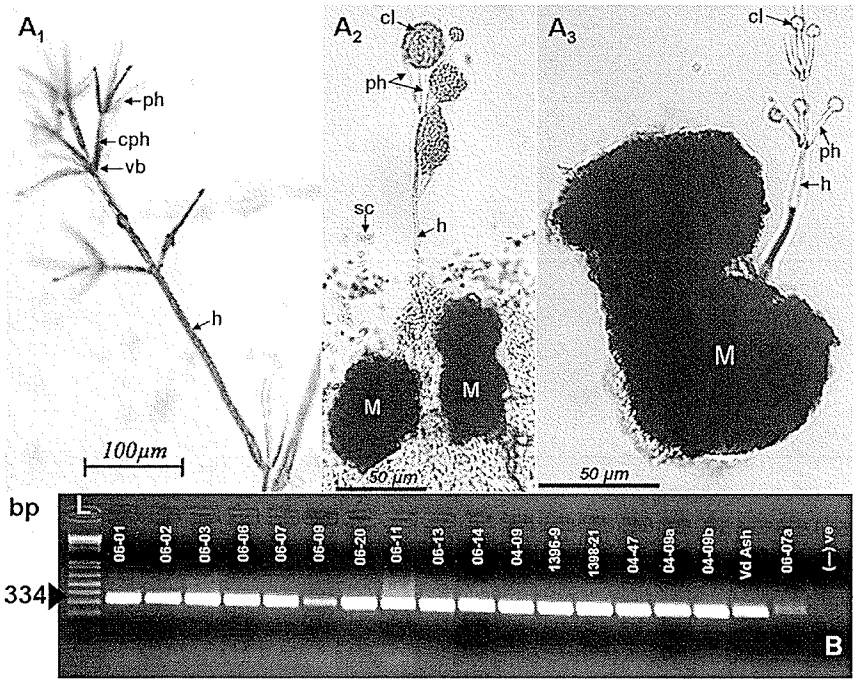

Figure 3.2. Morphological characteristics of a potato *V. dahliae* isolate showing the number of phialides (ph) per conidiophores (cph) and their arrangement in verticillate (whorled) branching (vb) on the conidiophores (A_1) (magnification x125) as well as the presence of microsclerotia (M) when grown on PDA ($A_{2,3}$) (magnification x400). $A_{2,3}$ show also branches of phialides carrying apical clusters of conidia (cl). The size of these phialides depends on the number of carried conidia. Single conidia (sc) are also observed. h: septated hyphae. B represents an agarose gel showing the amplification of a 334bp fragment using *V. dahliae*-specific primers set Vd-F and Vd-R. 06-0x isolates were recovered from sunflower while 04-0x were recovered from potato. Vd Ash: *V. dahliae* control isolate from an ash tree. L: 1Kb+ ladder (Invitrogen Inc.). (-)ve: negative control.

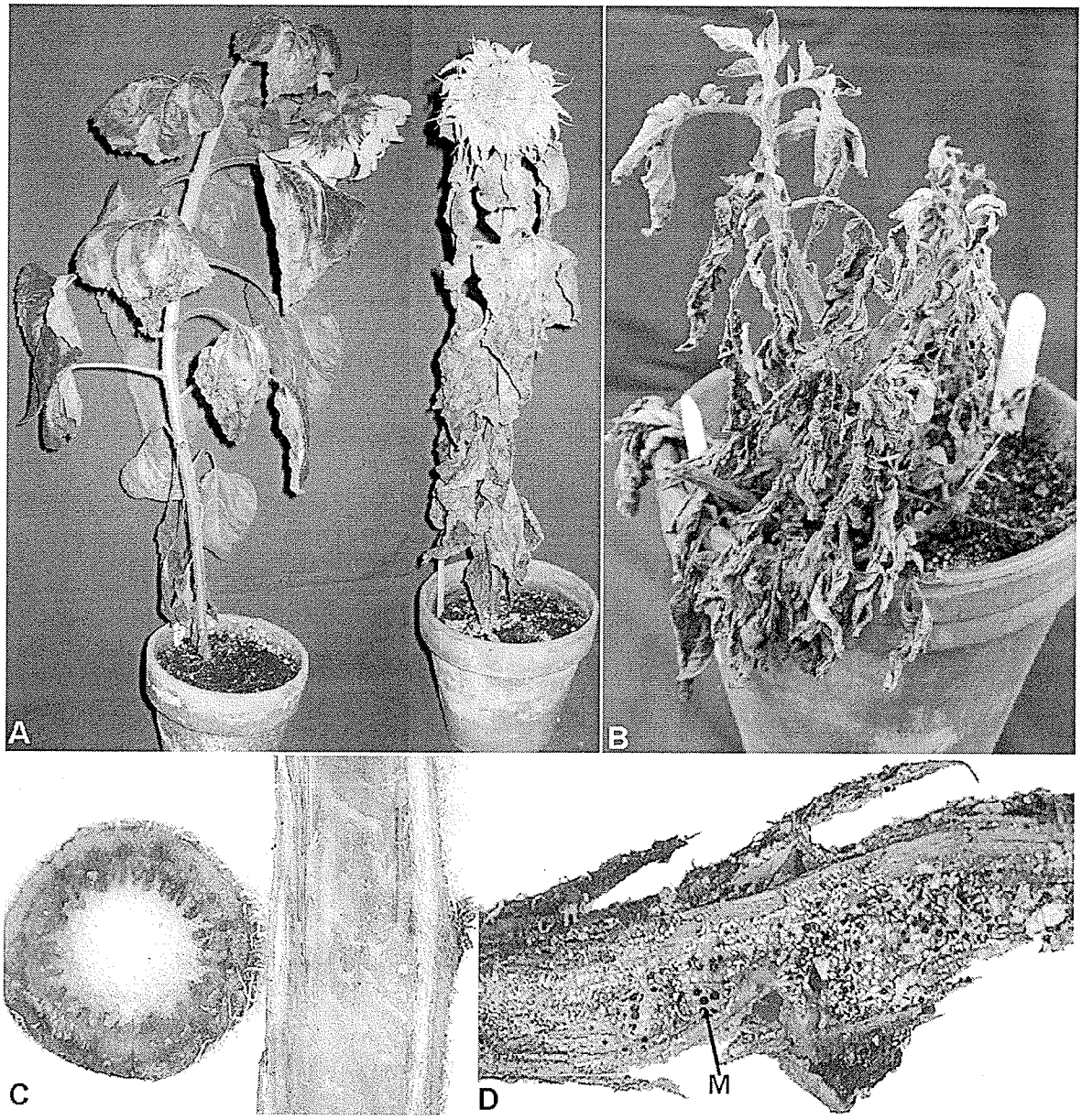

Figure 3.3. Verticillium wilt symptoms on sunflowers (A, C) and potatoes (B, D). A and B represent the chlorosis and necrosis as well as wilting observed on the foliage; C represents the vascular discoloration observed in cross- and longitudinal-sections of sunflower hybrid IS8048 stems; D shows the presence of microsclerotia in potato stems sections of cultivar Kennebec.

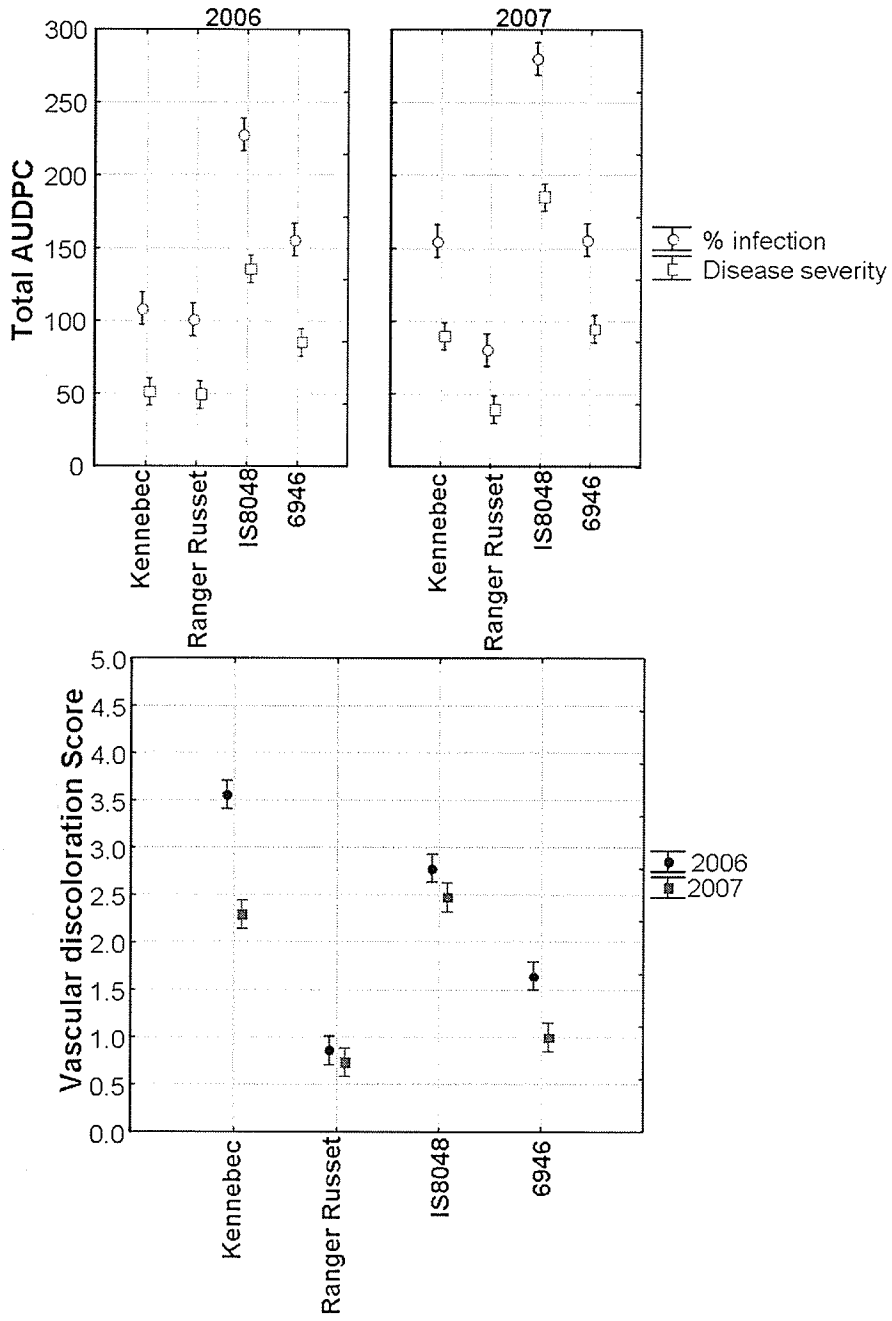

Figure 3.4. Percent infection, disease severity, and vascular discoloration recorded in 2006 and 2007 on two potato cultivars Kennebec (susceptible) and Ranger Russet (moderately resistant) and two sunflower hybrids IS8048 (susceptible) and 6946 (moderately resistant) infected with 22 *V. dahliae* isolates (12 originating from potato and 10 from sunflower). Each data point represents the mean across isolates x replicates. A significant year effect was observed at $P < 0.05$ even if the trend remains similar.

Pathogenicity of potato isolates on potato cultivars (P/P)

In 2006, no significant differences ($P < 0.05$) were observed among the tested potato isolates, based on the percent infection and disease severity they caused on either potato cultivar (Figs 5 and 6). In 2007, however, differences ($P < 0.05$) were detected among the same isolates, especially on the susceptible cultivar Kennebec (Figs 5 and 6). With the exception of isolates 04-09, 04-47 and 04-25, all other tested potato isolates displayed high disease levels on cultivar Kennebec, similar to the highly-aggressive control isolates Vd1396-9 and Vd1398-21 (Figs 5 and 6). Based on vascular discoloration ratings, most potato isolates ranked 4.5-5 (55-100% discoloration) and was comparable to the highly-aggressive control isolates Vd1396-9 and Vd1398-21 on cultivar Kennebec in both trials (Fig. 7). Isolates Vs04-09, Vs04-25 and Vs04-47, on the other hand, induced ($P < 0.05$) significantly lower vascular discoloration ratings (level 2-3: 10-49% discoloration) as compared to the rest of the isolates in both years. Significant differences were also recorded among potato isolates on cultivar Ranger Russet, which displayed lower levels of vascular discoloration in comparison to Kennebec (Fig. 7).

Pathogenicity of potato isolates on sunflower hybrids (P/S)

Overall, potato isolates induced higher percent infection and disease severity on sunflower hybrids than on potato cultivars, especially on the susceptible hybrid IS8048 (Figs 5 and 6). No significant differences ($P < 0.05$) were detectable among isolates for percent infection, disease severity, or vascular discoloration in either sunflower hybrid (Fig. 7).

Pathogenicity of sunflower isolates on sunflower hybrids (S/S)

Significant differences ($P < 0.05$) were observed among the tested sunflower isolates depending on the hybrid used for inoculations (Figs 5 and 6). Lower percent infection and disease severity scores were recorded on the moderately tolerant hybrid 6946, as compared to the susceptible IS8048, especially in 2007. Differences among isolates were more prominent in terms of the vascular discoloration levels they caused on the two sunflower hybrids (Fig. 7). The majority of the tested sunflower isolates led to vascular discoloration levels ranging from 40 to 100% in the susceptible hybrid, whereas these levels ranged from traces to 24% discoloration in the moderately resistant hybrid (Fig. 7).

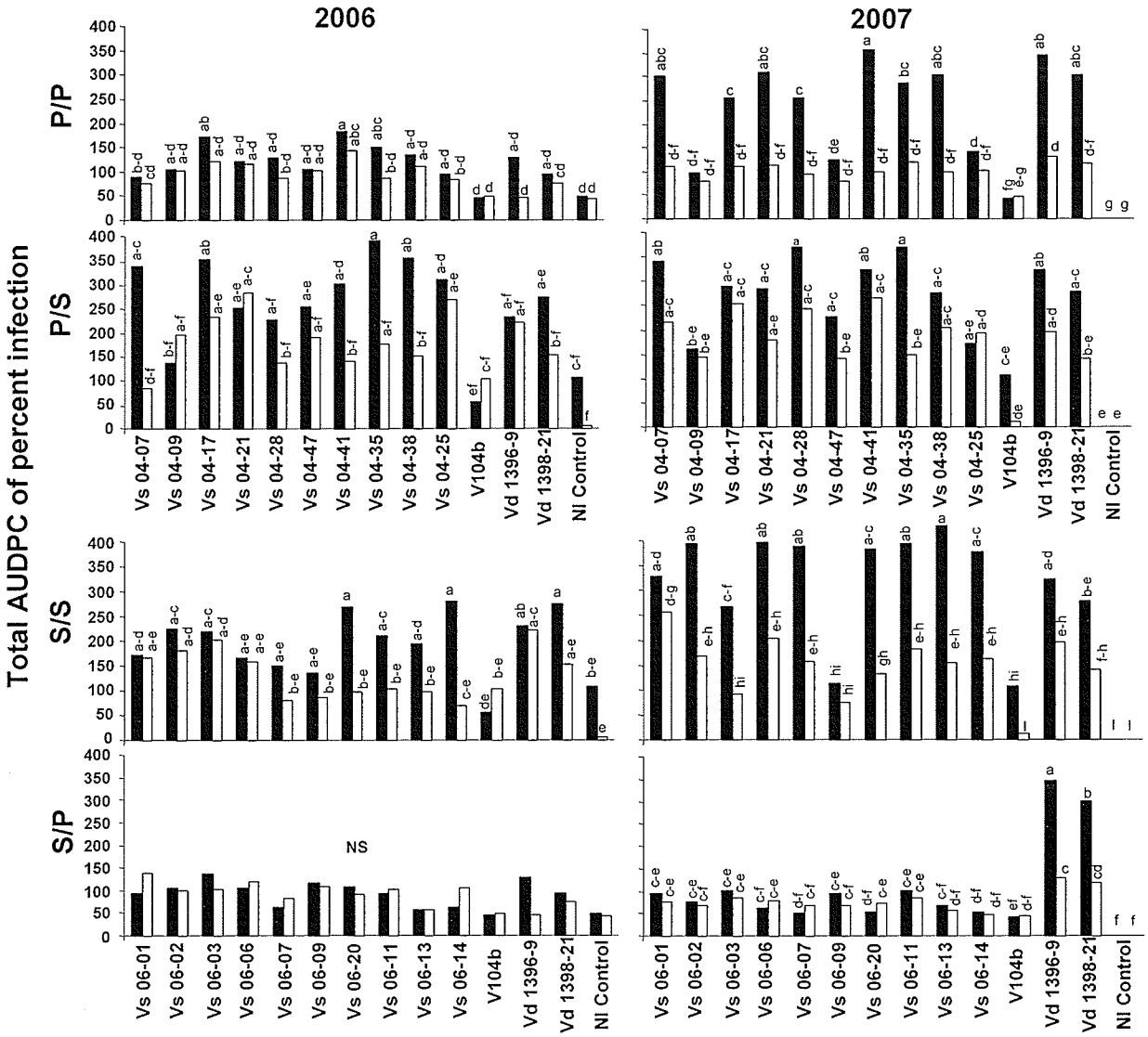

Figure 3.5. Total AUDPC calculated based on the percent infection of potato cultivars and sunflower hybrids inoculated with *V. dahliae* isolates recovered from potato (Vs04s) or from sunflower (Vs06s) in 2006 and 2007. NI Control represents the non-inoculated control. P/P represents potato isolates on susceptible Kennebec (■) and moderately resistant Ranger Russet (□) potato cultivars; P/S represents potato isolates on susceptible IS8048 (■) and moderately resistant 6946 (□) sunflower hybrids; S/S shows sunflower isolates on susceptible IS8048 (■) and moderately resistant 6946 (□) sunflower hybrids; S/P is sunflower isolates on susceptible Kennebec (■) and moderately resistant Ranger Russet (□) potato cultivars. Columns having the same letter are not significantly different according to Newman Keul's test at $P< 0.05$.

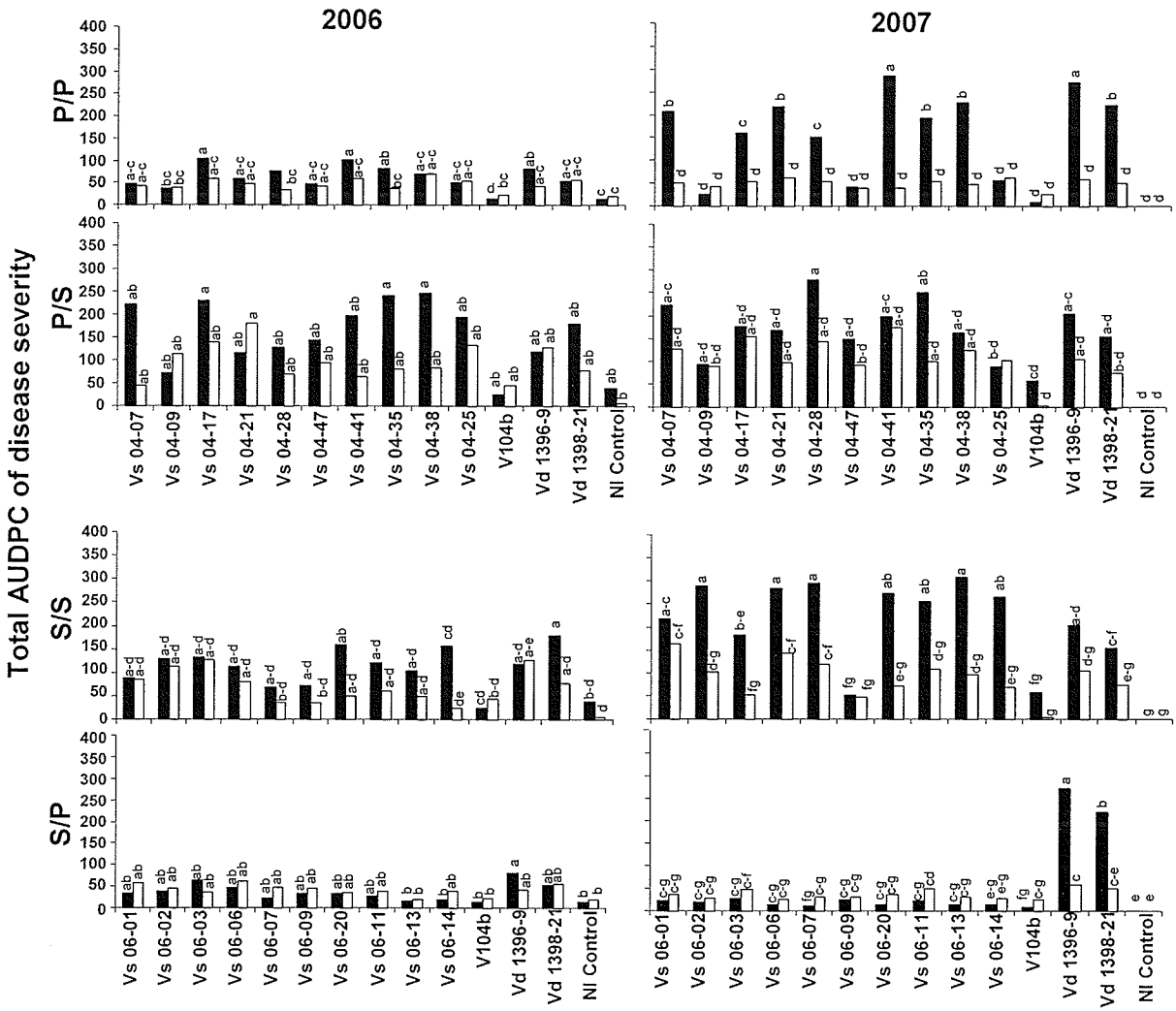

Figure 3.6. Total AUDPC calculated based on disease severity of potato cultivars and sunflower hybrids inoculated with *V. dahliae* isolates recovered from potato (Vs04s) or from sunflower (Vs06s) in 2006 and 2007. NI Control represents the non-inoculated control. P/P represents potato isolates on susceptible Kennebec (■) and moderately resistant Ranger Russet (□) potato cultivars; P/S represents potato isolates on susceptible IS8048 (■) and moderately resistant 6946 (□) sunflower hybrids; S/S shows sunflower isolates on susceptible IS8048 (■) and moderately resistant 6946 (□) sunflower hybrids; S/P is sunflower isolates on susceptible Kennebec (■) and moderately resistant Ranger Russet (□) potato cultivars. Columns having the same letter are not significantly different according to Newman Keul's test at $P < 0.05$.

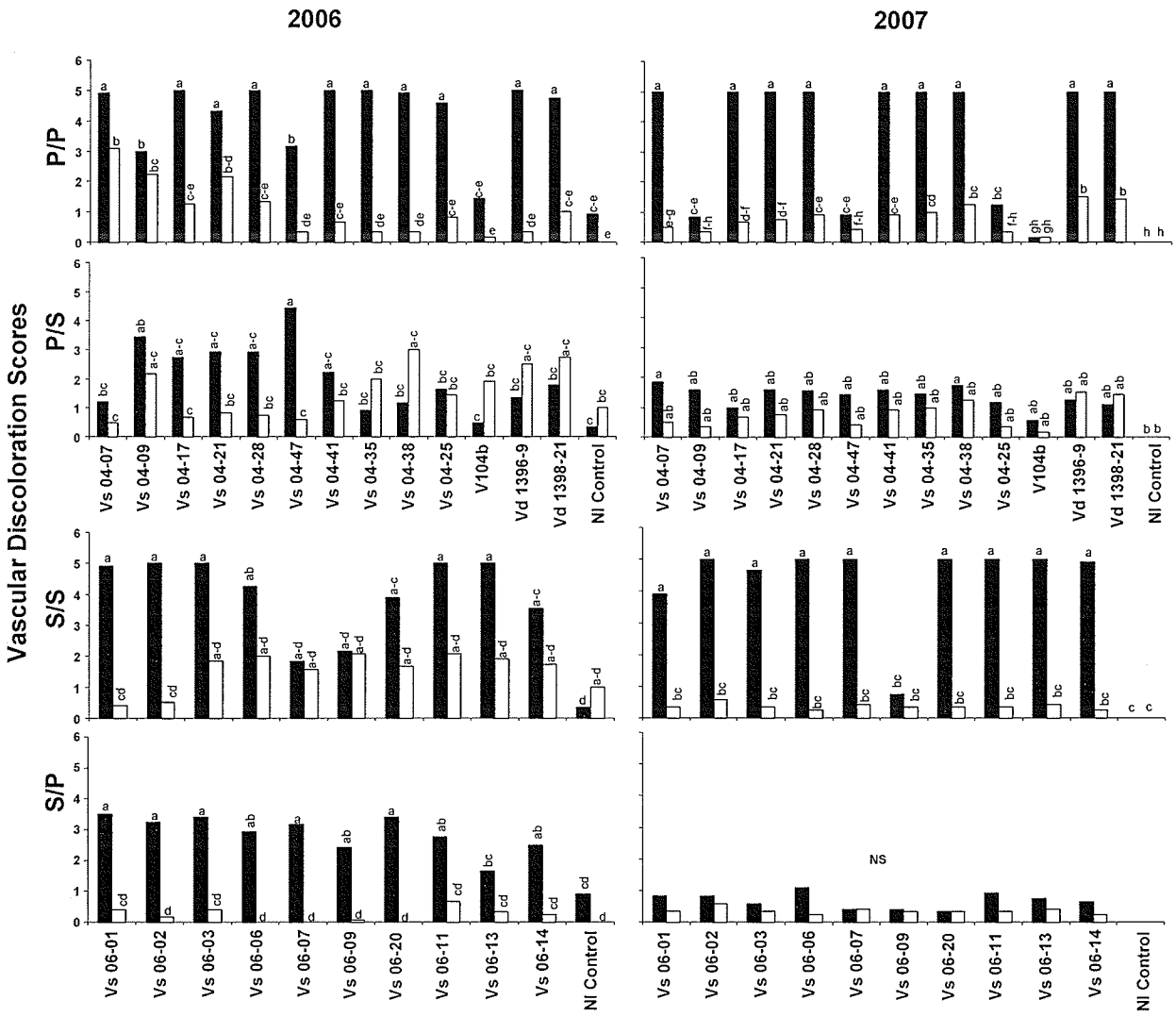

Figure 3.7. Vascular discoloration scores recorded as an average in the lower, middle and upper cross-sections of the stems of two potato cultivars and two sunflower hybrids 6 w.a.i. with *V. dahliae* isolates recovered from potato (Vs04s) or from sunflower (Vs06s) in 2006 and 2007. NI Control represents the non-inoculated control. P/P represents potato isolates on susceptible Kennebec (■) and moderately resistant Ranger Russet (□) potato cultivars; P/S represents potato isolates on susceptible IS8048 (■) and moderately resistant 6946 (□) sunflower hybrids; S/S shows sunflower isolates on susceptible IS8048 (■) and moderately resistant 6946 (□) sunflower hybrids; S/P is sunflower isolates on susceptible Kennebec (■) and moderately resistant Ranger Russet (□) potato cultivars. Columns having the same letter are not significantly different according to Newman Keul's test at $P< 0.05$.

Pathogenicity of sunflower isolates on potato cultivars (S/P)

Percent infection and disease severity scores recorded with sunflower isolates inoculated on either susceptible or moderately resistant potato cultivars were comparable (Figs 5 and 6) in both 2006 and 2007. Low levels of vascular discoloration were observed in cultivar Ranger Russet in response to all tested sunflower isolates in both years. Kennebec's response to the isolates was significantly different between 2006 and 2007 (Fig. 7). However, no significant differences were recorded among isolates in terms of the amount of vascular discoloration they induced.

Relative aggressiveness similarity among *V. dahliae* isolates

Hierarchical analysis was used to compare all of the tested isolates based on the percent infection, disease severity, and vascular discoloration they caused in both susceptible and moderately resistant cultivars/hybrids from both species. Using the Euclidean distance analysis, the tested isolates were ranged into three pathogenicity groups (Fig. 8). Group 1 included *V. albo-atrum* V104b, which was used as a weakly-aggressive isolate control on potato, and isolate vs06-09, which is a sunflower isolate that induced limited symptoms on both potato and sunflower, similar to non-inoculated wounded and non-wounded controls. Group 2 had most of the tested potato isolates, including the two highly-aggressive control isolates on potato Vd1398-21 and Vd1396-9. Group 3 encompassed all sunflower isolates except the one found with group 1 (06-09). It also included three potato isolates (Vs04-09, Vs04-25 and Vs04-47) that were similar to the sunflower isolates in terms of their aggressiveness on both potato and sunflower.

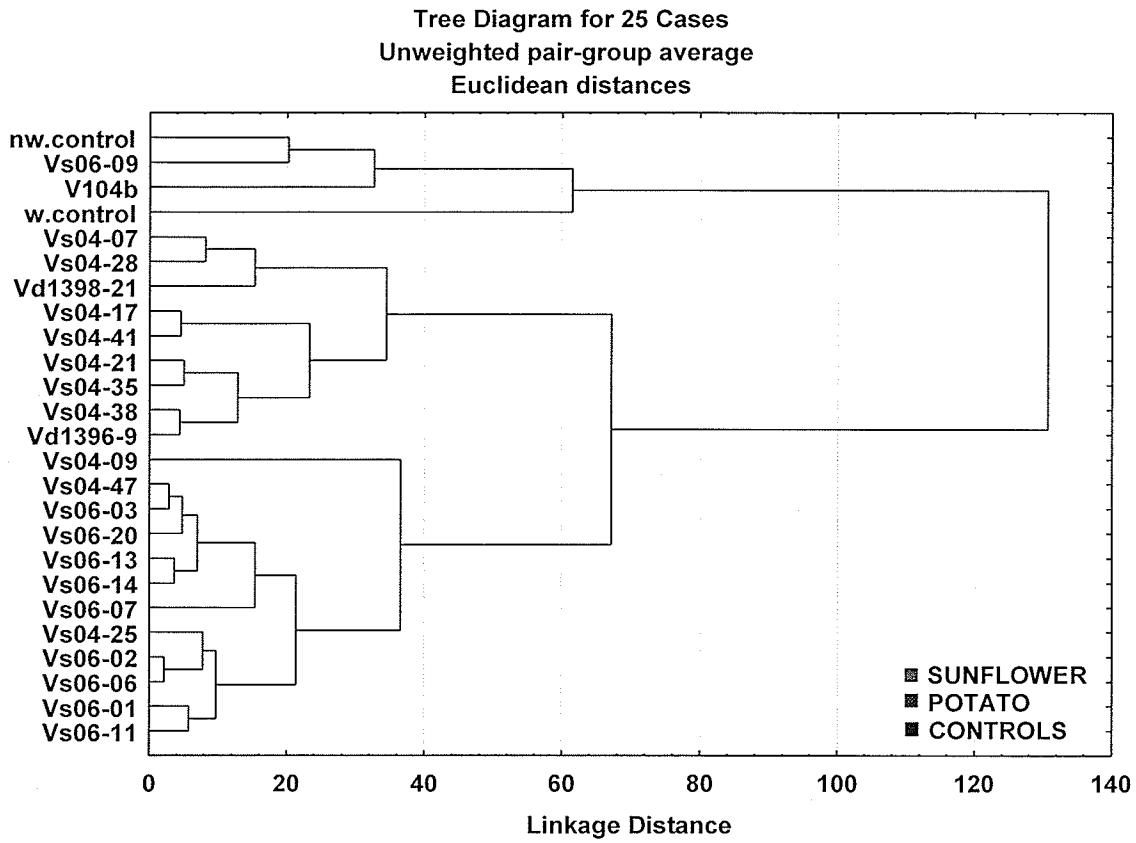

Figure 3.8. Dendrogram representing disease assessment on control plants and those inoculated with *V. dahliae* isolates. The dendrogram was generated from cluster analysis based on unweighted pair-group average method and the Euclidean distances calculated out of percent infection, disease severity and vascular discoloration scores observed on two potato cultivars and two sunflower hybrids either susceptible or moderately resistant to *V. dahliae*. NW control represents the unwounded healthy control plants; W control corresponds to the healthy control that was wounded by trimming the root tips but non-inoculated; Vd1396-9 and Vd1398-21 are two highly aggressive *V. dahliae* that were used in this study as control isolates; V104b is a *V. albo-atrum* Sub-group II represents the weakly aggressive control isolate. Vs04's isolates shown in brown color on the diagram were originally recovered from potato. Vs06's isolates shown in green on the diagram represent the isolates being isolated from infected sunflowers.

3.5. Discussion

We report on *V. dahliae* cross-pathogenicity between potato and sunflower in this study. Higher disease severity was recorded when the isolates were inoculated on their original host, as opposed to the alternative one, which concurs with previous reports in other host species (Bhat and Subbarao, 1999; Resende et al., 1994). Regardless of the isolates' host of origin, variability was observed in their pathogenicity on each host plant, as well as between the two hosts, as shown in other pathosystems involving this pathogen (Daayf et al, 1995; Katan, 2000; Korolev et al., 2001; Sebastjan et al., 2006). In *V. dahliae*, pathogenic variability is related to several factors, including the ability to produce pathogenicity factors such as toxins, i.e., glycoproteins and protein-lipopolysaccharide complexes, which induce necrosis and wilting in host plants (Mansoori et al., 1995, Meyer et al., 1994; Palmer et al., 2005). Pathogenicity was also reported to be dependent on the host of origin and the vegetative compatibility groups (VCGs). For instance, most *V. dahliae* isolates from potato were grouped in VCG_4 (Rowe, 1995) while cotton-defoliating strains were ranged into VCG_1, and cotton non-defoliating strains in either VCG_2 or VCG_4 (Daayf et al., 1995).

Trials in both 2006 and 2007 have shown that most *V. dahliae* isolates obtained from potato were highly aggressive on potato cultivar Kennebec (susceptible) and less aggressive on Ranger Russet (moderately resistant). These isolates were also very aggressive on sunflower hybrids, particularly on the susceptible phenotype. Verticillium wilt symptoms appeared on sunflower hybrids after about 10 days following inoculation, whereas a full 2 w.a.i were necessary to see the earliest symptoms on potato. In some cases, symptoms on the leaves do not reflect the true potential of an isolate to cause

disease, because the symptoms could be induced by other stress factors that often occur in growth rooms. On the other hand, vascular discoloration in stem cross-sections seemed to be a good criterion to discriminate highly- from weakly-aggressive isolates, probably because vascular discoloration is permanent and can extend beyond infected tissues (Mace, 1989). Even when plants recover quickly enough from the disease, as noticed on cultivar Ranger Russet, by forming new vascular tissues, vascular discoloration remains visible in their old xylem tissues. For instance, both sunflower hybrids developed strong vascular discoloration at the upper part of their stems in response to most *V. dahliae* isolates whereas in potato, such a response was apparent only in the susceptible cultivar infected with highly-aggressive potato isolates. This may be partly due to the anatomical differences between the two hosts. Sunflower has broader leaves and larger stems with wider vessels, denoting a stronger sap flow and suggesting a greater up-ward movement of the conidia to the top part of the plant (Beckman et al., 1976). Potato, on the other hand, displays a greater ability to ramification, which can possibly hold these spores from quickly reaching the top part of the plant.

The higher level of aggressiveness on potato cultivars, of *V. dahliae* isolates originating from potato, as compared to those from sunflower based on percent infection and disease severity, suggests that selection or adaptation of the pathogen to the host may have occurred over time (Okoli et al., 1994; Resende et al., 1994). This seems also to be true for sunflower isolates inoculated to sunflower. However, cross-infection of sunflower with potato isolates has shown that these ones maintained a higher level of aggressiveness, even on an alternative host, and suggests that their acquired pathogenicity factors are effective at the multi-host level. Oppositely, cross-infection of potato cultivars

with sunflower isolates has shown that these ones were weakly-aggressive. This may be due to a loss of certain essential pathogenicity factors by these isolates while adapting to sunflower, or to their initial transmission to sunflower from another alternative host, which had attenuated their aggressiveness. Another possible explanation to these findings is that potato defenses are much more sophisticated for non-adapted isolates coming from sunflower to potato, while potato isolates probably have already acquired counter-defenses against these mechanisms. It is important to keep in mind that if isolates were collected from diseased plants, they were possibly the most adapted among the ones that started the infection.

Verticillium dahliae isolates from sunflower were in general mildly- to weakly-aggressive on potato cultivars, where they induced symptoms about 2-3 w.a.i. These symptoms were mainly in the form of chlorosis, rather than necrosis, in comparison with the ones caused by potato isolates. This suggests that infections by these isolates are restricted rather early by the various mechanisms that potato puts in place to reduce the impact of diseases in general (Wang et al., 2004; 2005; 2008b). The presence of chlorosis implies that, if toxins are involved (Pu et al., 2007), they possibly reach the top of the plant through the vascular system even though the progress of the pathogen is blocked early by the host. The ability to induce more or less of these symptoms may depend on the differential expression of genes controlling pathogenicity factors in *V. dahliae* isolates (El-Bebany et al., 2008).

Trials in both 2006 and 2007 showed high levels of vascular discoloration induced by each group of isolates in their original host. Significant differences occurred between the susceptible and moderately resistant potato cultivars and sunflower hybrids, respectively.

Most *V. dahliae* potato isolates had the ability to cause high levels of vascular discoloration (level 5: 75-100% discoloration) on the susceptible potato cultivar Kennebec. However, these levels were significantly lower in the moderately resistant cultivar Ranger Russet. Similar results were observed in sunflower hybrids when infected with sunflower isolates, which supports previous studies on host adaptability in *V. dahliae* and *V. albo-atrum* (Horiuchi et al., 1990; Okoli et al., 1994; Resende et al., 1994; Bhat and Subbarao, 1999). This suggests that vascular discoloration is perhaps the best marker to assess Verticillium wilt (Pegg and Brady, 2002). External symptoms are sometimes induced by factors other than the soilborne pathogen and may be overestimated. *Verticillium dahliae* has a wide host range and is able to cause both external and internal symptoms, sometimes similar to those induced by other biotic and abiotic stresses. In an attempt to accurately assess Verticillium wilt on both tested plant species, we have developed assessment scales for each of the two hosts. These scales were based on both external (chlorosis and necrosis) and internal symptoms (vascular discoloration). Even though both symptoms have been previously described (Church and McCartney, 1995; Rowe and Powelson, 2002; Fradin and Thomma, 2006), we have observed differences, between the two plant species, that were not reported in previous studies. For instance, necrosis in potato leaves appears gradually from the edge after chlorosis, then areas increase to produce a typical V-shaped lesion (Fig. 1). On the contrary, in sunflower leaves, chlorosis appears as flecks that increase in size with time. The necrosis appears as tiny dark brown spots in the chlorotic areas which are located in inter-veinal tissues. Later, these spots become larger and united to form larger necrotic areas. Scales developed for external symptoms were based on the symptomatic area

(chlorosis and necrosis) on the leaves in each plant species. Although we have developed this assessment system under growth room conditions, we cannot rule out that such symptoms could be slightly different under field conditions. Given the many factors prevailing in the field, our assessment system may be used as a tool to both quantitatively and qualitatively evaluate symptoms that are caused by the pathogen and with a minimal influence from other external factors, especially when using vascular discoloration. Vascular discoloration scales were developed for each plant species, and measurements were made on cross-sections of the lower, middle and upper parts of the stems. The appearance of vascular discoloration in the upper part of the sunflower hybrids infected with *V. dahliae* that was not noticed in potato cultivars, led to the development of another vascular discoloration scale for this particular section of sunflower plants having a hollow stem.

Cluster analysis used in the present study allowed for the grouping of the tested isolates into pathogenicity groups with comparable aggressiveness levels. This analysis showed that isolates originating from sunflower were distinctly separated from the ones initially isolated from potato and closely related to weakly-aggressive control isolates from *V. albo-atrum*. These isolates, like *V. albo-atrum,* were weakly aggressive on both potato and sunflower and caused a low level of vascular discoloration. This result concurs with the findings of Bhat and Subbarao (1999), who showed that *V. albo-atrum* from alfalfa, was able to cause vascular discoloration in potato but the plants never showed wilting. Isolates originating from potato were, on the other hand, clustered with highly-aggressive control isolates Vd1396-9 and Vd1398-21 from potato. The high level of aggressiveness of these isolates on their original host may be assigned to short rotations

with alternative hosts. This may have had increased selection pressure in favor of the isolates that colonize better, and effectively reproduce on the original host. Vigouroux (1971) suggested that the introduction of a preferential host in a rotation sequence could lead to an increase in the amount of the inoculum with similar pathogenicity. He also reported that isolates from regions with permanent monoculture are similar, and are all highly aggressive on the particular monoculture crop but weakly aggressive on other host crops. The question is: when should an isolate be considered to originate from a given host and how to define "the host of origin"? This refers to whether it spent several or only the last generation on the same host.

Even if each group of isolates was more aggressive on its original host, it caused various levels of infection on the alternative host. This suggests that the tested hosts are potential reservoirs to both of these isolates and in any case should not be considered in a rotation sequence, especially where sunflower follows potato, and knowing that most confection sunflower hybrids lack genetic resistance to Verticillium wilt (Rashid and Platford, 1994). In this case, other biological control methods including the use of PGPRs (Ongena et al., 1999, El Hassni et al., 2007), Rhizobium (Arfaoui et al., 2007), and plant extracts (Uppal et al, 2008), or cross-protection using hypo-aggressive isolates (Daayf et al., 2003; El Hassni et al., 2004), may be more efficient in controlling this disease.

PATHOGENIC VARIABILITY OF FOUR *V. DAHLIAE* ISOLATES AFTER SERIAL PASSAGES IN POTATO AND SUNFLOWER PLANTS

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Key words: *Verticillium dahliae*, Potato, Sunflower, Host of origin, Alternative host, Successive passages, Pathogenicity gain/loss.

4.0 PATHOGENIC VARIATION OF *V. DAHLIAE* AFTER SERIAL INOCULATIONS ON POTATO AND SUNFLOWER

4.1. Abstract

Verticillium wilt is a limiting factor in many economic crops, including potatoes and sunflowers. The causal agent, *Verticillium dahliae* Kleb., maintains a high level of diversity and of pathogenic variability. In an earlier study, we showed such a high variability among isolates recovered from either potato or sunflower when inoculated on their original or alternative hosts. *V. dahliae* isolates from potato were more aggressive on both potato and sunflower while the ones from sunflower were to certain extent more adapted to sunflower than they were to potato. In the present study, we selected those with the lowest aggressiveness levels and showed that those from potato are able to gain pathogenicity on either potato or sunflower when inoculated to a susceptible potato cultivar (Kennebec) for four successive generations. In contrast, some isolates showed a loss in pathogenicity, after four successive passages on the susceptible potato cultivar, especially towards the moderately resistant potato cultivar (Ranger Russet). Results in terms of gain or loss of pathogenicity by potato and sunflower isolates are discussed in relation to the effect of successive passages through their original or alternative hosts.

4.2. Introduction

Verticillium wilt is mainly caused by the soilborne pathogens *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke & Berth. It is a limiting factor in the production of many economically important crops, including potato and sunflower. Verticillium was found in most sunflower fields surveyed in Manitoba in recent years (Rashid et al., 2006). Unfortunately, there is no or very little resistance to Verticillium in potato cultivars or in most oilseeds sunflower hybrids and no resistance in most confection sunflower hybrids. In addition, the resting structures of *V. dahliae* can survive in the soil for more than 10 years (Heale and Karapapa, 1999).

Verticillium dahliae is known for its high pathogenic diversity and variability of other traits (Daayf et al. 1995). Such variability has been shown among isolates collected from potato and sunflower (Alkher et al. 2009). *Verticillium dahliae* isolates from potato were more aggressive on both potato and sunflower than the ones isolated from sunflower (Alkher et al. 2009). However, when an isolate is labeled to originate from a given host, the number of generations it has infected such a host remains unknown. Therefore a potato isolate is called so only because it had spent at least the last generation in that host. Fordyce and Green (1963) studied changes in the pathogenicity of *V. albo-atrum,* originating from peppermint and spearmint, on tomato, since it was previously reported that isolates of *V. albo-atrum* from *Mentha* spp. were nonpathogenic on tomato. The study showed that two of the tested isolates became pathogenic after 1 passage, and 1 isolate after 5 serial passages, on tomato. Simultaneously with the increase in pathogenicity to tomato, all 3 isolates became nonpathogenic to *Mentha piperita* L. (Fordyce and Green, 1963).

Serial passage experiments, in which pathogens are successively transferred from one plant to another, within the same or different species, is an approach developed to investigate the causes and consequences of microbial evolution in terms of pathogenicity. It has been successfully used towards understanding the evolution of virulence and fitness of pathogenic fungi, such as *Fusarium graminearum* Schwabe, Group II [anamorph] and *F. pseudograminearum* O'Donnell et. T. Aoki sp. nov. (Akinsanmi et al. 2006), *Rhynchosporium secalis* (Oudem.) J. J. Davis (Abang et al. 2006), *Septoria nodorum* (Berk.) Berk. in Berk. & Broome (Cunfer, 1994), and *Verticillium albo-atrum* (Fordyce and Green, 1963). More recently, Wang et al. (2008a) found an increase in the virulence of *F. oxysporum* Schlechtend.:Fr. f.sp. *vasinfectum* (Atk.) W.C. Snyder & H.N. Hans. isolates generated after 10 successive passages on cotton. On the other hand, Akinsanmi et al. (2006) showed a reduction in the fitness of *F. graminearum and F. pseudograminearum* after their passage through 10 alternative plant hosts.

In order to better understand the behavior of *V. dahliae* populations from potato and sunflower in these two important crops, the objective of this study was to determine the aggressiveness of selected isolates of *V. dahliae* isolated from potato or sunflower after serial passages on susceptible and moderately resistant potato and sunflower plants.

4.3. Materials and methods

Plant material and growth conditions

Two potato cultivars, Kennebec (susceptible) and Ranger Russet (moderately resistant), and two sunflower hybrids, IS8048 (susceptible) and 6946 (moderately resistant) were used in this study. Cultivar Kennebec and hybrid IS8048, both susceptible to *V. dahliae*, were used during the successive passages of selected *V. dahliae* isolates, as

well as in the final step of comparing pathogenicity between original isolates and their forth generation counterparts. Moderately resistant potato cultivar Ranger Russet and sunflower hybrid 6946 were included only in the final comparison of isolates. These cultivars were grown out of seed pieces (potato) or true seed (sunflower) in 10 cm diameter clay pots filled with a pasteurized sandy soil mixture (1:1, v/v) amended with NPK fertilizer granules (16:20:16). After sowing, pots were transferred into a growth room with 20/16±2°C day/night, 16h-photoperiod and a light intensity of 350 $\mu mol.m^{-2}.s^{-1}$. Four weeks later, plants were inoculated and transplanted into 15-cm-diameter pots filled with a pasteurized mixture of soil-sand-peat-perlite (4:4:4:1, v/v/v/v) amended with NPK (20:20:20). Plants regularly received water and necessary care to prevent damage by aphids, spider mites and other pests that may interfere with Verticillium wilt assessment during all experimentation.

Fungal isolates

Four single-spore *V. dahliae* isolates were selected from our collection based on their low levels of aggressiveness on potato and sunflower, as determined in our earlier studies (Alkher et al. 2009). Two were originally isolated from potato (04-47, 04-09) and two from sunflower (06-07, 06-09). Serial passage assays on potato and sunflower were conducted on the highly susceptible potato cultivar (Kennebec) and sunflower hybrid (IS8048) (Table 1). This choice was based on the greater likely hood of re-isolating the pathogen after several passages through a susceptible host than a resistant one. The isolates recovered from potato were assigned a letter 'p' (p47, p09). The recovered isolates, after four passages through potato were labeled p47-p4 and p09-p4, and through sunflower p47-s4 and p09-s4. A similar labeling was used for isolates originating from

sunflower s07 (s07-p4, s07-s4), s09 (s09-p4, s09-s4). All tested *V. dahliae* isolates successfully infected both the original and alternative hosts and produced substantial amounts of spores in all generations.

Table 4.1. Characteristics of the original potato and sunflower isolates as well as their counterparts isolated from four consecutive passages on either potato cultivar Kennebec (P) or sunflower hybrid IS8048 (S). Isolates from the forth passage were compared for their pathogenicity to the original ones on two potato cultivars (Kennebec; susceptible and Ranger Russet; moderately resistant) and two sunflower hybrids (IS8048; susceptible and 6946; moderately resistant).

Host of Origin		Potato				Sunflower			
Original Isolates		P47		P09		S07		S09	
Host of Passage		P	S	P	S	P	S	P	S
Passage	1	P47-p1	P47-s1	P09-p1	P09-s1	S07-p1	S07-s1	S09-p1	S09-s1
	2	P47-p2	P47-s2	P09-p2	P09-s2	S07-p2	S07-s2	S09-p2	S09-s2
	3	P47-p3	P47-s3	P09-p3	P09-s3	S07-p3	S07-s3	S09-p3	S09-s3
	4	P47-p4	P47-s4	P09-p4	P09-s4	S07-p4	S07-s4	S09-p4	S09-s4

Serial passage assays on potato and sunflower

The incubation, inoculum preparation, and inoculation methods used in this study were previously described (Alkher et al. 2009). Briefly, *V. dahliae* isolates were grown on potato dextrose agar in an incubator at 25°C for three weeks. Inocula were prepared by adding 15ml of sterile distilled water (SDW) onto the cultures. Based on the hemacytometer counts, final spore concentrations were adjusted to 10^6 conidiospores per ml. Four-week-old potato and sunflower seedlings were inoculated by dipping their roots into inoculum suspensions for 2-3 min. In each passage, two additional treatments, where plants were treated with sdH_2O or inoculated with a conidial suspension prepared from our highly aggressive reference *V. dahliae* isolate Vd1396-9, were used as non-inoculated and inoculated controls, respectively. Treated plants were then transplanted and re-incubated as described above. Diseased plants were identified by the appearance of

yellowing, wilting and plant death. The vascular discoloration in stems was observed six weeks after inoculation (w.a.i.) according to Alkher et al. (2009). Re-isolation was carried out from the most diseased plants. Subsequently, all of the recovered isolates were maintained separately and used to start the next serial passage, following the steps described above.

Comparison of pathogenicity between original isolates and their fourth passage counterparts

After four successive inoculation cycles, representative isolates of the final passage (fourth passage) were compared to their corresponding initial isolates in terms of their pathogenicity levels. The comparison was conducted on potato cultivars Kennebec and Ranger Russet and sunflower hybrids IS8048 and 6946, following the same aforementioned procedure.

Verticillium wilt assessment

Plants were evaluated for disease progress at 2, 4, and 6 w.a.i in each experiment. Percent infection, disease severity, and vascular discoloration were calculated as described by Alkher et al. (2009). Briefly, percent infection (PI) and disease severity (DS) were calculated as follows: $PI = \left(I_L / T_L\right) \times 100$; where I_L is the number of leaves exhibiting external Verticillium wilt symptoms (i.e. chlorosis, necrosis, wilting), and T_L is the total number of leaves of the rated plant; $DS = \left[\sum_{i=0}^{n} (n \times b)\right] \times 100 \Big/ T \times (N-1)$ (Gauhl et al. 1993), where b is the chlorosis/necrosis grade (0-5 referring to the pre-developed and calibrated (Alkher et al. 2009) visual scale using the image analysis software Assess 2.0 (Lamari, 2002), where 0 represents no necrosis or chlorosis, 1: visible chlorosis with

less than 1% necrosis, 2: up to 40% chlorosis and 1-20% necrosis, 3: up to 65% chlorosis and 20-35% necrosis, 4: 100% chlorosis, 35-70% necrosis, 5: 100% chlorosis, 70-100% necrosis.) and n is the number of leaves with necrosis grade b. N is the total number of chlorosis/necrosis grades used on the scale and T is the total number of leaves. Based on the over time percent of infection and disease severity, the areas under disease progress curve (AUDPC) were calculated using the following formula (Campbell and Madden, 1990): $\text{AUDPC} = \sum_{i=1}^{n-1}[((y_i + y_{i+1})/2)(t_{i+1} - t_i)]$, where n is the total number of assessments in weeks, y_i is the PI or DS at the i^{th} assessment week, and the term $t_{i+1} - t_i$ is the time duration between two assessments. An additional assessment of DS was conducted through the evaluation of vascular discoloration at the end of the experiments (6 w.a.i.). This parameter was assessed in cross-sections taken from the lower, middle and upper parts of the stems of infected plants according to a pre-established visual scale 0-5 calibrated using the image analysis software Assess 2.0 (Lamari, 2002), where 0 represents no vascular discoloration, 1: trace to less than 9% of the cross-section showing discoloration, 2: 10-24%, 3: 25-49%, 4: 50-74%, and 5: 75-100% discoloration (Alkher et al. 2009).

Evaluation of variation in *Verticillium dahliae* pathogenicity

To assess pathogenicity among the original isolate and their counterparts recovered after four passages on either potato cultivar Kennebec or sunflower hybrid IS8048, a pathogenicity index was established (El Hadrami, personal communication), based on the following formula: $\text{Ipath} = \left(\frac{PI_4}{PI_i}\right) + \left(\frac{DS_4}{DS_i}\right) + \left(\frac{VD_4}{VD_i}\right)$, where PI, DS and VD represent the percent infection, disease severity and vascular discoloration recorded either initially (i)

or at the fourth passage through either potato or sunflower (*4*). This index was used either in its absolute or relative forms. The Ipath absolute represents the sum of the three ratios included in the index, which equaled 3 in the original isolates and lower or higher than 3 if there was a loss or a gain in pathogenicity, respectively. In the relative form of the Ipath, the sum of the ratios was divided by the number of ratios included in the formula, which led to values equal to 1 for the original isolates or either higher or lower than 1, based on a gain or a loss in pathogenicity, respectively. The relative form can be used for universal comparison and can include as many ratios as possible to describe the pathogenicity of *V. dahliae* isolates.

Data analysis

All trials were conducted following a randomized complete block design. The experiment consisted of four individual replicates per treatment isolate x cultivar/hybrid and each trial was conducted one time for the serial passage and twice for the final pathogenicity assessment. All data including the Ipath indices were analyzed statistically using General Linear models (GLM) in SAS Package (Statistical Analysis Systems Institute Inc., Cary, N.C, U.S.A.). The means of each of the initial isolates were compared to their counterparts recorded after the fourth passage on potato or sunflower using the least significant differences (LSD) test ($P< 0.05$). Means calculated from four replicates of the vascular discoloration observed in the lower, middle, and upper stem sections were compared among treatments using the same test. Cultivar effect was high, and in order to reduce its effect on pathogenicity we analyzed the isolates per cultivar.

4.4. Results

Pathogenicity variation among potato isolates after serial passages on their original and alternative hosts

In both the original and alternative hosts, potato isolate p47 exhibited higher levels of pathogenicity on susceptible cultivars/hybrids in comparison to the moderately tolerant ones (Fig. 1). Disease levels recorded with this isolate were higher on sunflower compared to potato (Fig. 1). After four passages on potato cv. Kennebec, this isolate (p47-p4) showed a significant increase in pathogenicity on cv. Kennebec, where it caused more infection and disease severity as compared to the initial isolate p47 (Fig. $1A_1$ and $1A_2$). No such increase was observed in vascular discoloration (Fig. $1A_3$). In sunflowers, a significant increase in pathogenicity levels was recorded only in vascular discoloration on both tested hybrids (Fig. $1B_3$). After four passages on susceptible sunflower hybrid IS8048, potato isolate p47 (p47-s4) exhibited increased levels of infection and disease severity in potato cv. Kennebec (Fig. $1A_1$ and $1A_2$) and in both sunflower hybrids in terms of vascular discoloration (Fig. $1B_3$).

The second tested potato isolate (p09) showed similar patterns of pathogenicity to those recorded with isolate p47 on both potato cultivars and sunflower hybrids (Fig. 1). After the forth passage on the susceptible cultivars/hybrids, this isolate (p09-p4 and p09-s4) exhibited a significant increase in pathogenicity in both hosts especially on the susceptible cultivars/hybrids. However, there were no significant changes between p09 and p09-s4 on sunflower susceptible hybrid (Fig. 1).

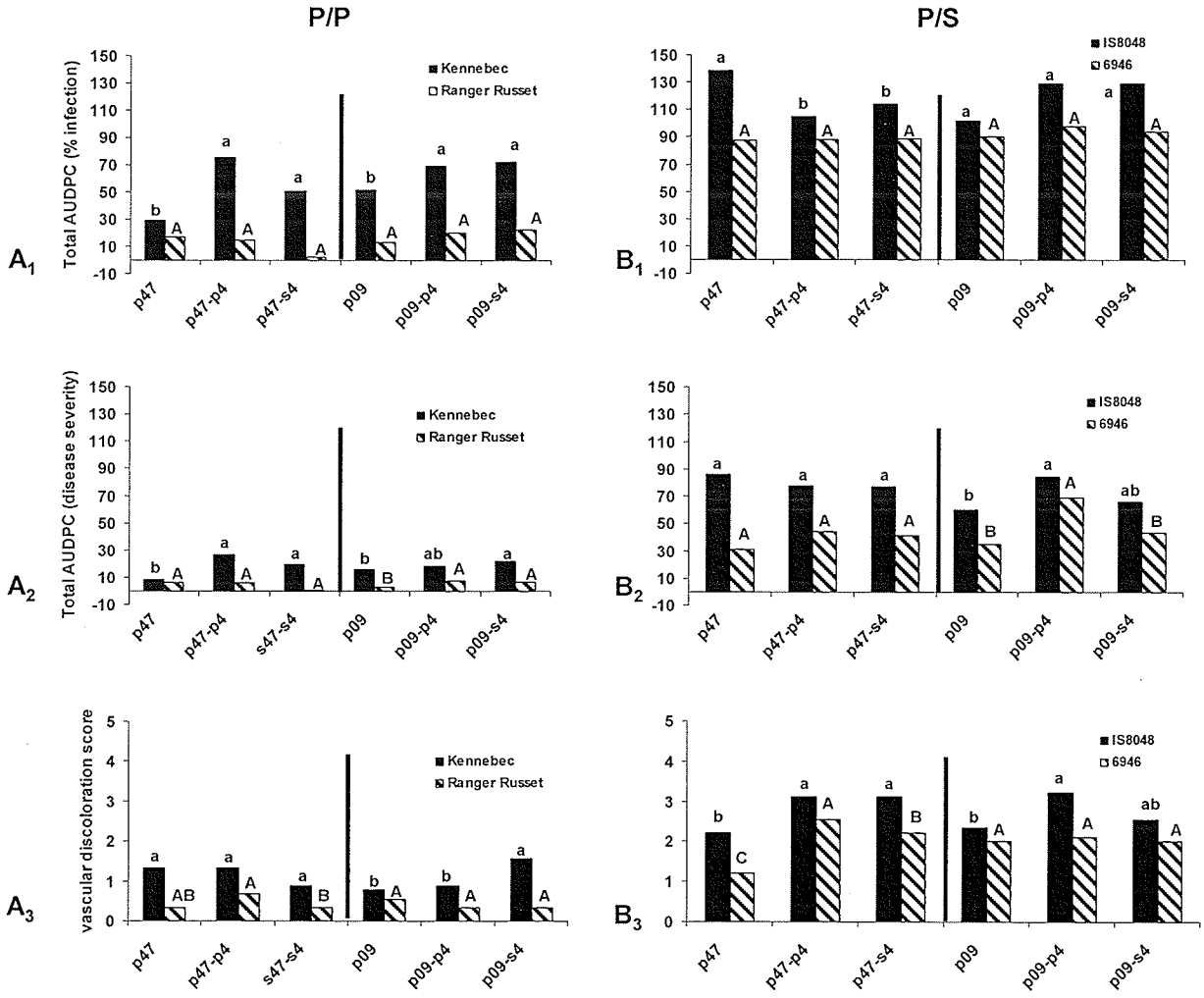

Figure 4.1. Percent infection total AUDPC (A_1, B_1), disease severity total AUDPC (A_2, B_2), and vascular discoloration in lower, middle and upper stem sections (A_3, B_3) of Kennebec, Ranger Russet potato cultivars (P/P) and IS8048, 6946 sunflower hybrids (P/S) inoculated with *V. dahliae* potato isolates, respectively. The isolates are represented by the initial potato isolates (p47, p09) and their fourth passage counterparts on either potato (p47-p4, p09-p4) or sunflower (p47-s4, p09-s4), respectively. Significant differences were recorded among cultivar reactions (lower *versus* upper case letters). To exclude any masking effect of the resistance of the tested cultivars/hybrids, isolates were compared separately for their pathogenicity either on susceptible cultivar/hybrid (lower case letters a, b...) or on moderately resistant cultivar/hybrid (upper case letters A, B...). Values with same letters, either in lower or upper case, are not significantly different according to LSD test at $P< 0.05$.

Pathogenicity variation among sunflower isolates after serial passages on their original and alternative hosts

Sunflower isolate s07 produced higher levels of disease on tested sunflower hybrids particularly IS8048 than on potato cultivars (Fig. 2). After the fourth passage on potato (s07-p4), this isolate exhibited reduced levels of pathogenicity in terms of disease severity and vascular discoloration on sunflower hybrid IS8048 (Fig. $2D_{2,3}$). Isolate s07-p4 was 2 of 3 measurements were significantly increased from its original counterpart s07 in terms of its pathogenicity levels on potato cultivars, except for a slight increase in disease severity and percent infection on cv. Kennebec (Fig. $2C_{1,2}$). The same seemed to be true for isolate s09, where an increase in pathogenicity levels were recorded on sunflower hybrids, especially 6946 and on cv. Kennebec (Fig. 2, $C_{1,2,}$ $D_{1,2}$).

Isolate s07, after the forth passage on sunflower (s07-s4), showed no significant changes in pathogenicity on potato but showed an increase in percent infection and vascular discoloration on hybrid 6946 (Fig. $2D_{2,3}$). In hybrid IS8048 a decrease was observed in AUDPC and vascular discoloration as compared to the original isolate s07 (Fig. 2D). Similar results were observed for isolate s09 and its fourth generation counterpart s09-s4 on both potato and sunflower (Fig. 2).

Variation of pathogenicity indices among isolates

The pathogenicity index (Ipath) established in this study allows comparison of isolates recovered after several passages on either the original or the alternative hosts, in terms of pathogenicity gain or loss. Table 2 summarizes the relative values of Ipath indices for the selected isolates after inoculation on susceptible and moderately resistant potato cultivars and sunflower hybrids.

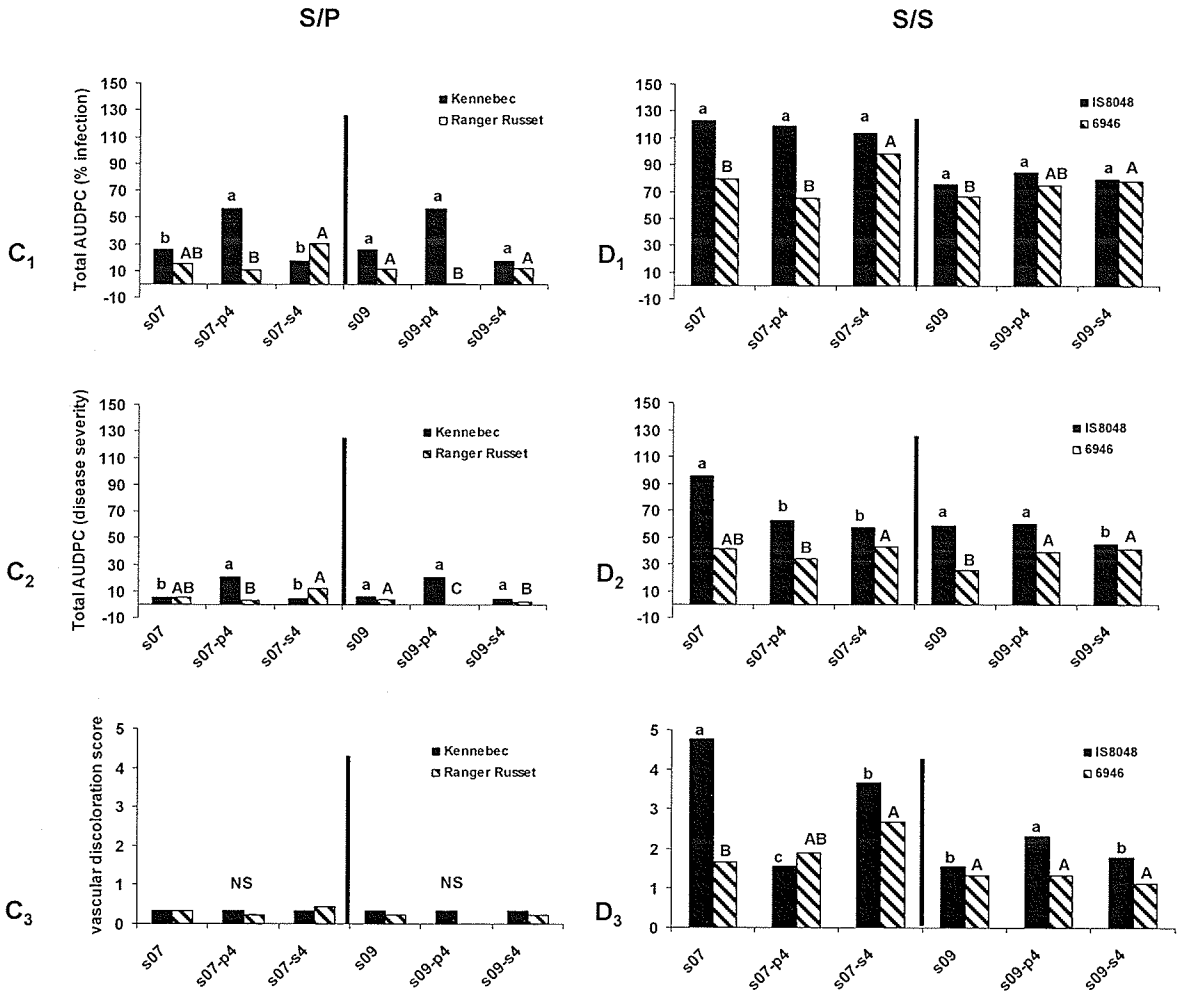

Figure 4.2. Percent infection total AUDPC (C_1,D_1), disease severity total AUDPC (C_2,D_2), and vascular discoloration of lower, middle and upper stem sections (C_3,D_3) of Kennebec, Ranger Russet potato cultivars (S/P) and IS8048, 6946 sunflower hybrids (S/S) infected by *V. dahliae* sunflower isolates, respectively. The isolates are represented by the initial sunflower isolates (s07, s09) and their fourth passage counterparts on either potato (s07-p4, s09-p4) or sunflower (s07-s4, s09-s4). Significant differences were recorded among cultivar reactions (lower *versus* upper case letters). To exclude any masking effect of the resistance of the tested cultivars/hybrids, isolates were compared separately for their pathogenicity either on susceptible cultivar/hybrid (lower case letters a, b...) or on moderately resistant cultivar/hybrid (upper case letters A, B...). Values with same letters, either in lower or upper case, are not significantly different according to LSD test at $P< 0.05$.

Two of the isolates recovered after the forth passage on potato cultivar Kennebec showed an increase in pathogenicity (higher Ipath) when inoculated on Kennebec, regardless of their host of origin. This increase was much more significant when they were passed through potato rather than sunflower. When Ipath indices were compared among isolates on susceptible sunflower hybrid IS8048, there was a noticeable increase in their pathogenicity over all, but this increase was comparable between isolates passed through potato or sunflower for four generations. The increase was to some extent lower than what was recorded on susceptible potato cultivar Kennebec.

In moderately resistant potato cultivar Ranger Russet, an overall only increase in Ipath of p09-p4 and p09-s4 was observed amongst the tested isolates with the exception of s09-p4, where a decrease was observed (Table 2). These increases were not as high as those observed with the isolates on susceptible cv. Kennebec. For instance, potato isolate p47 passed through susceptible sunflower hybrid IS8048, namely p47-s4, did not show any significant increase in pathogenicity on moderately resistant cv. Ranger Russet. Sunflower isolate s09, passed through susceptible potato cv. Kennebec, namely s09-p4, exhibited a reduced level of pathogenicity on Ranger Russet as compared to its original counterpart isolate s09 and that passed through sunflower s09-s4.

On the moderately resistant hybrid 6946, all the tested isolates showed a gain in pathogenicity, reflected in the higher Ipath indices (Table 2). These increases were comparable to the ones recorded on the susceptible hybrid IS8048 and to the ones recorded on moderately resistant potato cv. Ranger Russet but were lower than what was recorded on Kennebec.

Table 4.2. Relative pathogenicity indices (Ipath) of two potato (p47, p09) and two sunflower (s07, s09) isolates, their counterparts isolated from four consecutive passages on either potato cultivar Kennebec (p4) or sunflower hybrid IS8048 (s4). Isolates from the forth passage (-p4, -s4) were compared for their pathogenicity to the original ones on two potato cultivars (Kennebec, susceptible and Ranger Russet, moderately resistant) and two sunflower hybrids (IS8048, susceptible and 6946, moderately resistant).

		Hosts			
		Potato		Sunflower	
		Kennebec	Ranger Russet	IS8048	6946
Isolates	**p47**	1.0 b	1.0 a	1.0 a	**1.0 b**
	p47-p4	**2.2 a**	1.1 a	1.0 a	**1.5 a**
	p47-s4	**1.7 a**	0.3 a	1.0 a	1.4 a
	p09	1.0 b	1.0 b	1.0 b	1.0 b
	p09-p4	1.1 b	**1.6 a**	**1.4 a**	**1.4 a**
	p09-s4	**1.5 a**	**1.5 a**	1.2 ab	1.1 ab
	s07	1.0 b	1.0 a	1.0 a	1.0 b
	s07-p4	**2.3 a**	0.6 a	**0.6 b**	0.9 b
	s07-s4	0.8 b	1.8 a	**0.8 b**	**1.3 a**
	s09	1.0 a	1.0 a	1.0 a	1.0 b
	s09-p4	0.9 a	**0.2 b**	1.2 a	**1.2 a**
	s09-s4	2.0 a	0.9 a	1.0 a	**1.2 a**

Note: The Ipath values for each tested isolate and their counterparts from the fourth passage on either potato or sunflower are compared separately on each individual cultivar/hybrid, used to exclude variations due to the cultivar/hybrid level of resistance. Therefore, values within the same group of three (original isolate, its 4th generation isolates) having the same letter are not significantly different according to LSD test at P< 0.05.

4.5. Discussion

Understanding the mechanisms governing variations in the pathogenicity of *Verticillium dahliae*, when infecting consecutive hosts, is key to finding better ways to mitigate the impact of verticillium wilt. This is also applicable to most soilborne pathogens, because growers can choose which crop to grow in given fields, based on which pathogens are present the most current threat. Variations in pathogenicity of a given isolate may occur through gain or loss of virulence factors, which in turn could increase or decrease the level of aggressiveness (Wang et al. 2008a). In this study, two *V.*

dahliae isolates from potato (p47, p09) and two from sunflower (s07, s09) were selected to investigate the changes that may occur in their pathogenicity during several generations on their original host, in comparison to when they spent the same number of generations on an alternative host. To understand these changes, serial inoculations were used, where all selected isolates were passed four successive times through either the original or the alternative host (potato/sunflower). Comparing pathogenicity levels between original isolates and their relatives collected in the forth generation showed changes in their pathogenicity levels. For instance, sunflower isolates propagated for four generations on potato showed an enhanced level of aggressiveness on susceptible potato cv. Kennebec, while their levels of aggressiveness on sunflowers hybrids decreased, especially in IS8048. In contrast, potato isolates passed on sunflower for four generations did not show any signs of loss in pathogenicity, either towards potato or sunflower. These results showed the ability of *V. dahliae* isolates to quickly adapt to new hosts (sunflower to potato), possibly through the acquisition of new pathogenicity factors. Being a non-host-specific pathogen, *V. dahliae* is somewhat a "generalist" in terms of achieving its life cycle as opposed to host-specific pathogens (Agrios, 2005). It establishes in the hosts root system through passive, i.e., through wounds, or active mechanisms, i.e., using hydrolytic enzymes such as polygalacturonases, pectinases and cellulases (Pegg et al. 1976; Bidochka et al. 1999; Novo et al. 2006). It multiplies, then sporulates when enough host tissues have been colonized to support the hyphal biomass required to achieve this stage of development. Each of these key steps of the life cycle involves multiple interactions, between the invading pathogen and its host, resulting in disease or a blockage of the vascular system of the plant (Michelmore, 1995). From the host side,

these steps are controlled by several defenses mechanisms (Hammond-Kosack and Jones, 1997; Wang et al. 2006; 2008b) that rely on the up-regulation of defense-related genes and could vary in intensity and timing from one host to the other. However, these mechanisms are somewhat similar in their operating pathways between hosts and adapting to one host usually helps the pathogen acquire/transmit pathogenicity factors that could allow it overcome host's defenses. For instance, pathogens such as *Sclerotinia sclerotiorum* and *Sclerotium rolfsii*, known to infect over 200 plant species, are reported to secrete substantial amounts of oxalic acid during their invasion of plant tissues. This component is a pathogenicity factor that is a host-specific, and that triggers many mechanisms to overcome major plant defenses (Franceschi, 1989; Noyes and Hancock, 1981). From the pathogen side, *V. dahliae* adapts its genetic and metabolic processes, when changing hosts, to optimally achieve the three essential steps of its life cycle. These changes may involve acquired/transmitted abilities and temporal protoplasmic changes that do not lead to an alteration in gene complement (Christensen and DeJay, 1955) unless successive passages on the new host occur. This also implies that *V. dahliae*'s acquired and/or transmitted new pathogenicity factors could be reversibly lost if confronted with new hosts that are somewhat resistant. Nuclear changes in fungi are common and many of them, especially the ones linked to adaptation to new hosts, are likely the results of these genetic changes (Hirsch, 1949; Prasad, 1949; Zaffarano et al. 2008). The frequency, extent and range of these modifications and changes may be the results of confrontation to various substances while invading different hosts. However, it is usually difficult to assign such changes to solely pathogenicity gain or loss, unless the contribution of the host susceptibility is excluded. In the present study, we tested the

isolates recovered after four successive passages on the original and alternative hosts both on susceptible and moderately tolerant cultivars to be able to check whether there are gains or losses of pathogenicity factors regardless of the contribution of the tested hosts. Although it is not always easy to dissociate phenotypic variability from genetic variation in pathosystems involving *V. dahliae* as a pathogen, we denoted a range of variability in Verticillium wilt expression between original and alternative hosts and detect differences among the tested isolates in their aggressiveness levels.

Indicators of pathogenicity evolvement include the pathogen's reproductive rate (Parker and Gilbert, 2004; Park et al. 2002; Bull, 1994). In our study, sporulation rates of tested *V. dahliae* isolates did not show any significant changes among the initial set of isolates and their forth generation counterparts. This may be because too few generations were considered for the experiments.

Changes in pathogenicity among fungi are often gradual in their appearance, relatively stable and genetic in nature, although sudden and cyclic changes may also occur. In either case, those changes seem to be controlled at the nuclear dissociation or mutation levels at least in species exhibiting higher rates of heterokaryosis such as *V. dahliae*. Natural populations of pathogens especially the ones with broad host range often encompass distinct genetic variants associated with different host species (Skotnicki et al. 1996; Kofalvi et al. 1997; Mastari et al. 1998; Moury et al. 2001). For instance, five vegetative compatibility groups (VCGs) have been described in *V. dahliae* (Daayf et al. 1998; Joaquim and Rowe, 1990). Every one of these VCGs is adapted to a specific host (i.e., cotton, eggplant, mint, potato) even if they can cause reduced Verticillium wilt on alternative hosts. Pathogenic variability among *V. dahliae* isolates belonging to these

VCGs is often associated with factors such as plant cell-wall degrading enzymes i.e., cellulases, xylanases (Novo et al. 2006; Bidochka et al. 1999). Toxins are other pathogenic factors that are also often reported to explain pathogenic variability among *V. dahliae* isolates on a given host. These include protein-lipopolysaccharide complexes and glycoproteins (Buchner et al. 1982 ; Mansoori et al. 1995, Meyer et al. 1994; Palmer et al. 2005, Pu et al. 2007). In addition, Verticllium wilt often involves stunting, which can be the result of reduction in photosynthesis upon stomatal closure triggered by water stress (Flexas and Medrano, 2002). All these processes thought to be involved in pathogenicity of *V. dahliae* can either be gradually, cyclically or suddenly changed if the pathogen is passed through an alternative host rather than the host of origin. In the present study, a gain in pathogenicity by potato *V. dahliae* isolates after the forth passage was observed in susceptible potato and sunflower, confirming the occurrence of one or several changes that were transmitted to the isolates over time, regardless of the passage through the host of origin (potato) or an alternative host, for instance sunflower. This study shows also that sunflower isolates had gained pathogenicity on susceptible potato cv. Kennebec after four consecutive inoculations of this cultivar. It is likely that, if given more successive passages on Kennebec, sunflower isolates could reach their maximum fitness on this cultivar and exhibit a high and stable level of pathogenicity as described for other pathogens by Zhan et al. (2002). On both moderately resistant cultivars/hybrids, no such changes in pathogenicity were observed among the initial *V. dahliae* isolates or their forth passage counterparts. This may be due to the effectiveness of host-defense mechanisms (Fradin and Thomma, 2006; Alkher et al. 2009) put forward by these cultivar/hybrid towards these isolates over a short generation time period.

Based on the observed results and from an evolutionary point of view, fungi such as *V. dahliae* seem to be well equipped to stay ahead in the co-evolutionary race (Kaltz and Shykoff, 1998). Having relatively short generation times and usually high abundance compared to their hosts (Price, 1980; Imhoof and Schmid-Hempel, 1998), *V. dahliae* isolates are given the advantage to quickly overcome host defenses and gain pathogenicity and fitness that allow them to propagate on either their host of origin or alternative hosts. Ongoing studies using isolates with distinctive pathogenicity attributes and a set of differentially responding host plants in our research group are currently targeting the understanding of the pathogenicity determinism in *V. dahliae*, in order to detect weaknesses to be explored from the pathogen side, along with strengths from the host side to achieve a better control of Verticillium wilt in both potato and sunflower.

5.0 GENERAL DISCUSSION AND CONCLUSIONS

Potato and sunflower are important crops worldwide. Both are hosts to *Verticillium* spp. (i.e., *V. dahliae* and *V. albo-atrum*), which reduce their yield and quality. Although *V. dahliae* has a wide host range, it varies in its level of pathogenicity on different hosts (Bhat and Subbarao, 1999). Isolates from one host are able to cause disease on other plant species but symptoms are often more severe on the host of origin. However, it was not known whether sunflower isolates cause disease on potato, to what extent, and *vice-versa*. Several studies have focused on the evolution of pathogens and the relationship between pathogenicity and pathogen transmission (Wang et al, 2008a; Salvaudon et al, 2007; Frank, 1996; Bull, 1994). Serial passage has been attempted with several pathogens (Wang et al, 2008; Zhan et al., 2002). Fordyce and Green (1963) found that *V. albo-atrum* isolates from mint, which were nonpathogenic to tomato, became pathogenic after serial passages on this host. The purpose of this research was to evaluate the pathogenicity of *V. dahliae* that originated from potato and sunflower on the original and the alternative hosts, and to study the variation in *V. dahliae* pathogenicity after serial passages on potato and sunflower plants.

Two groups of isolates were isolated from infected potato and sunflower stems/tubers in Manitoba to study the cross-pathogenicity of *V. dahliae* using susceptible and moderately resistant potato and sunflower (Kennebec, Ranger Russet; IS8048, 6946), respectively, (chapter 1). For assessing Verticillium wilt on both plant species, evaluation scales ranging from 0 to 5 were developed in this study. The first scale was created to assess the external symptoms (chlorosis and necrosis), and the second scale was to assess the internal symptoms (the vascular discoloration). This step was useful to evaluate the

differences in the disease development caused by different groups of isolates and within each group on two different hosts. Moreover, the inclusion of a vascular discoloration scale was a helpful method to evaluate the disease because assessing on the external symptoms can lead to over estimation of disease severity as a result of the inclusion of similar symptoms caused by other biotic or abiotic stress on the plant.

Variability of pathogenicity of tested *V. dahliae* isolates was reported on potato cultivars and sunflower hybrids (chapter 1). Generally, each group of isolates caused more disease on the original host. However, *V. dahliae* isolates from potato were more aggressive than the ones from sunflower on both plant species. Significant high disease severity and vascular discoloration were induced on the susceptible potato cultivar and sunflower hybrid compared with the moderately resistant ones. These results support the finding of Bhat and Subbarao (1999). These results can be helpful for the producers since *V. dahliae* isolates coming from potato were more aggressive on sunflower. Thus, growing sunflower following a potato crop should be avoided. Rotation with these crops can not be the best management of Verticillium wilt since both groups of isolates caused disease on both potato and sunflower.

Studying the effect of serial passages of *V. dahliae* on potato and sunflower on the pathogenicity of the isolates on the original and the alternative hosts was the second goal in this study (chapter 2). To the best of our knowledge, this is the first study that describes a pathogenicity index (Ipath) to compare *V. dahliae* isolates among generations successively passed through either their original or alternative host. Two sunflower and two potato *V. dahliae* isolates were chosen for this study based on their low level of aggressiveness on both plant species (chapter 1). Observing changes in pathogenicity

between the initial isolate and its 4th generation counterpart on the susceptible plant will be more obvious than an isolate that is initially aggressive on the plant. The comparison was done among the initial isolates and their 4th passage counterparts both on potato and sunflower. The initial *V. dahliae* potato isolates and their 4th passage counterparts were more aggressive on both potato and sunflower. More gain in pathogenicity of the pathogen was noticed on Kennebec, the susceptible potato cultivar, inoculated with the 4th passage potato isolates. These isolates showed significant increase in percent infection and disease severity, which could be explained by the involvement of potential pathogenicity factors of potato isolates compared to the sunflower ones. In addition, colonization and sporulation in infected plant tissues are positively associated with the change in pathogenicity. In the case of the 4th passage isolates of *V. dahliae* sunflower isolates, no such changes were observed on either plant species. Even though variation between the isolates and their 4th passage counterparts was reported, more significant changes can probably be observed through more than four passages.

Variation among *V. dahliae* isolates on the host of origin and in the cross infection was also investigated in this thesis. This variation could be due to the pathogenicity factors involved in the infection. Further investigations are ongoing to identify some of these pathogenicity factors and study their functions during the pathogenesis. For instance, fungi such as *V. dahliae* subjected to various hosts and possessing parasexual abilities i.e., heterocaryosis, where the presence of two or more genetically different nuclei in the same spore or hyphae, could easily acquire/transmit new traits that could give rise to new pathotypes with various levels of pathogenicity on either the host of origin or the alternative host. Since heterocaryosis is a parasexual phenomenon that

involves exchange of genetic material through mechanisms such as anastomosis and fusion between germinating spores or mycelia, any mutation that occurs in the monocaryons (mycelia or spores) could be transmitted and carried on within the heterocaryon. In *Botrytis cinerea*, a pathogen that exhibit multinucleate hyphal cells and spores, studies by Hansan and Smith (1932) showed that the single-spore isolations resulted in cultures that resembled the original type as well as variants and intermediate types. This phenomenon is also commonly observed *in vitro* within *V. dahliae* colonies (Daayf et al., 1998; Uppal et al., 2007; El Hadrami et al., unpublished data). These artificial variations among isolates of the same fungus, especially the ones with parasexuality, are also commonly observed in living hosts, yet clearly demonstrated cases are in a restricted number. Using a single-spore isolate of *H. gramineum*, Christensen and Graham (1934) were able to clearly demonstrate that this heterocaryotic fungus was able to dissociate into 10 separate pathotypes after only one single passage through barley. Likewise, we presently show that isolates of *V. dahliae* originally recovered from one host were able to adapt to an alternative host and this adaptation occurred in both ways, hence suggesting gains and losses of pathogenicity factors by the pathogen. Given the small number of generations tested, the occurrence of this adaptation seemed to be more apparent on susceptible cultivars/hybrids than on the moderately resistant counterparts. Similar results were reported, where no increase in virulence on resistant varieties when pure races of *Tilletia foetida* were used, while the pathogenicity of a hybrid population greatly increased with repeated passage through certain varieties. This suggests that the extent to which pathogenicity varies in *V. dahliae* or *T. foetida* isolates depends on their genetic make-up but may also depend on the host degree of resistance. For instance,

Vielwerth (1938) reported on a gradual decrease in pathogenicity among a bunt population continuously propagated on moderately susceptible wheat variety. The impact of the host level of resistance/tolerance in the selection of new pathotypes has been also reported in many pathosystems including the ones involving smuts (Nicolaisen, 1931; 1934; Reed, 1927). These authors among others (Sampson and Western, 1938) reported on the increase in virulence of several races of loose and covered smuts, caused by *U. avenae* and *U. kolleri*, respectively, after serial passages through resistant oat cultivars. All these observations and reports from different pathosystems indicate that fungal pathogenicity can be altered during the passage through living host tissues which may in return lead to the selection of new pathotypes with various degrees of aggressiveness.

The nature of selective pressure exerted by the host toward invading pathogens depends on the hosts own degree of susceptibility/resistance. Resistant varieties or hosts are believed to exert much higher pressure than susceptible ones. However, there are likely no convincing reasons preventing susceptible varieties from selecting new pathotypes with high levels of pathogenicity if they are produced in a relatively equal proportion as the commonly found pathotypes. The effect of the host on the mycelial growth and sporulation of an invading pathogen seems to play a key role in altering pathogenicity in fungi, hence suggesting potential differences among fungal species exclusively adapted to one host and those having a wide host range. The host range of a pathogen is thought to dictate the evolution of its pathogenicity (Frank, 1996; Combes, 1997). Fungal pathogens able to infect multiple host species (i.e., *V. dahliae*) are somewhat adopting a so-called "generalist" strategy, that facilitates their successful

transmission, development and survival in presence of an original or alternative hosts (Anderson et al., 1982; Bull, 1994; Frank, 1996).

Verticillium dahliae has the ability to adapt to a new host from the same species (vertical adaptation) as well as to another host from a different plant species (horizontal adaptation), as we noticed in this study among susceptible and resistant cultivars/hybrids, and among potato and sunflower plants. From this point, we conclude that *Verticillium* spp. have plasticity and flexibility characteristics. They can infect and cause damage in plants among the original host species, and it can switch from that host and infect, colonize and adapt to other plants, which may or may not be related species. However, the adaptation to a new host doesn't mean that there is always increase in pathogenicity. The transmission of the pathogen over time either in the alternative or the original host could cause a gain or loss of aggressiveness. The question is in how many generations the pathogen reaches the maximum level of pathogenicity. Knowing the answer for this question would be useful.

In conclusion, *V. dahliae* plasticity is not surprising, but this study has provided information about the risks of cross-pathogenicity between potatoes and sunflowers.

6.0 REFERANCES

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