AN EPIDEMIOLOGICAL STUDY OF VERTICILLIUM WILT OF POTATOES: HOST-PATHOGEN INTERACTIONS OF VARIOUS SPECIES IN COMBINATION AND SINGLE INOCULATION STUDIES

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ABSTRACT

Verticillium wilt is caused by a soilborne fungal pathogen that infects the vascular system of a variety of plant species, including potatoes (Dobinson 1995; Mahuku et al. 1999). Verticillium wilt is found in most potato production areas all over the world and is an economically important disease that causes reduced tuber yields and produces diseased seed tubers (Mahuku et al 1999). The most effective control methods are chemical treatments that are costly and harmful to the environment and non-target organisms (Jeger et al. 1996). Other efficient and less harmful methods of control should be explored due to growing environmental concerns (Dobinson 1995; Keinath et al. 1991). One of these methods may include the use of a biological control agent (Davis et al. 2000; Fravel 1996). However, before selecting a biological control it is essential to know the epidemiology of the disease to be controlled (Keinath et al. 1991).

On Prince Edward Island, Verticillium wilt is caused primarily by two aggressive pathogens; *Verticillium albo-atrum* 'group 1'and *V. dahliae* (Mahuku et al 1999). Also present in the soil, are two less aggressive species, *V. albo-atrum* 'group 2' and *V. tricorpus* (Heinz and Platt 2000). Little is known about these less aggressive species and their role in the Verticillium wilt disease complex (Mahuku et al 2002; Mahuku et al 1999). Further studies will help to better define their role as it has been suggested that they may compete with the more aggressive Verticillium species and may be used as a biological control (Davis et al. 2000, Heinz and Platt 2000).

In a series of greenhouse and field studies, potato plants of the c.v. Superior were inoculated (approximately 12 x 10^6 spores/mL) with one of the four species in single inoculation studies, or a combination of an aggressive and a weak species in combined inoculation studies. In combined inoculation studies, plants were inoculated with two species at the same time or with a weak species followed by an aggressive species 4 days later. Polymerase chain reaction (PCR) techniques were used to determine relative population levels (RPLs) of each pathogen within soil and various plant parts. These results, along with visual wilt symptoms, were analysed to obtain a picture of host-pathogen interactions and pathogen-pathogen interactions. The four species, singly and in combination, were compared for similarities and differences in pathogenicity.

In single inoculation studies *V. albo-atrum* 'group 1' maintained the highest RPLs and foliar disease incidence in the greenhouse followed by *V dahliae*, *V. albo-atrum* 'group 2' and *V. tricorpus*. The aggressive species progressed throughout the plant quickly and were detected in plant tissues as early as 24 hours after inoculation. High RPLs were detected as early as 28 days after inoculation whereas visual wilt symptoms were not seen until later in the growing period. In the field experiments, *V. dahliae* was the most aggressive species and was present at high RPLs, which was probably due to the naturally occurring populations of *V. dahliae* in the soil. Single inoculation studies also revealed that the less aggressive species were able to colonize all portions of actively growing plants. *Verticillium albo-atrum* 'group2' and *V. tricorpus* were still considered weak pathogens as RPLs and foliar disease symptoms were generally low.

In combination studies where plants were inoculated with two species at the same time, the aggressive species were able to maintain higher RPLs than the less aggressive

species. However, foliar disease symptoms were lower in combination studies than in the single inoculation studies where an aggressive species was used for inoculation. Although *V. tricorpus and V. albo-atrum* 'group 2' were unable to suppress *V. albo-atrum* 'group 1' and *V. dahliae* RPLs, they appear to have some role in suppressing their pathogenicity. In combination studies where plants were inoculated with a weak pathogen followed by an aggressive pathogen 4 days later, the weak pathogens were able to establish themselves inside the plants and maintain RPLs that competed with the aggressive species. Weaker species in combination with *V. albo-atrum* 'group 1' had RPLs that were actually higher than the aggressive species. Even though *V. dahliae* RPLs were not significantly reduced, the less aggressive species did reduce visual wilt symptoms and maintained higher RPLs than in single inoculation studies. These studies show that *V. tricorpus* and *V. albo-atrum* 'group 2' have the ability to colonize all plant parts without causing significant damage to the plant, and they can also compete with the aggressive pathogens for space within the plant, which is an indication of biological control.

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Last but certainly not least, I would like to thank my parents, Brian and Martha Robinson for their unconditional love and encouragement, and for reminding me that I can do anything I put my mind to. This is dedicated to you both.

TABLE OF CONTENTS

LIST OF TABLES

TABLE OF FIGURES

1 CHAPTER ONE

1.1 Literature Review

Verticillium is a soil and tuber-borne fungal pathogen from the class Deuteromycetes, which is characterized by the lack of a sexual stage (Dobinson 1995; Heinz and Platt 2000a). It is a hyphomycete fungus which is characterized by verticillately branched conidiophores with phialides producing conidia terminally in mucilaginous heads (Berlanger and Powelson 2000; Heale 1988) (Figure 1.1). *Verticillium* species infect the vascular system of a variety of plant species causing premature plant death and are found in most agricultural areas around the world (Dan et al. 2001; Fravel 1996; Nachmias and Krikun 1984; Rowe et al. 1987). Over 300 agriculturally important crops including cotton, tomato, alfalfa, hops, olive and potato, are susceptible to Verticillium wilt (Nazar et al. 1991).

1.1.1 Economic Importance of Potatoes

Verticillium wilt of potatoes is a major disease in many potato production areas and has been ranked as one of the most important diseases of both seed and commercial potato crops (Lynch et al. 1997). It is also a major constraint on tuber yields in North America and in particular, the Maritime region (Celetti and Platt 1987; Kimpinski et al. 1998; Lynch et al. 1997).

The potato (*Solanum tuberosum* L.) is grown on all continents, except for Antarctica, as a valuable food staple. Besides being a source of nutrition, potato starch is used in the production of paper, adhesives, textiles, edible binding agents, as well as a variety of other uses (Hughes 1991). In Canada, the potato is the most valuable vegetable

(picture by M. Powelson)

Figure 1.1 Verticillately branched conidiophores of Verticillium showing phialides carrying a mass of conidia (Berlanger and Powelson 2000).

crop accounting for $952 million in 2002. The Atlantic area is the leading region in Canada with 45% of the national production. Over 50% of the potatoes are processed, mostly into French fries, and in 2002 $738 million worth of French fries were exported to the U.S. (Gagnon 2003). Tuber yield losses from Verticillium wilt have been reported to be as high as 30% to 50% (Mahuku et al. 1999; McKeen and Thorpe 1981; Nachmias et al. 1993). These yield losses make Verticillium wilt an economically important disease in need of control (Davis et al. 1994; Davis et al. 1996; Fravel 1996; Leben et al. 1987).

1.1.2 Lifecycle

1.1.2.1 Dormant stage

The life cycle of *Verticillium* species can be divided into three stages: dormant, parasitic and saprophytic (Berlanger and Powelson 2000) (Figure 1.2). During the dormant stage, the pathogen is able to survive adverse environmental conditions in soils by forming resting structures. Resting dark mycelium and melanized microsclerotia are the resting structures produced by Verticillium. Dark mycelium usually survives between 9 to 10 months whereas microsclerotia can survive more than 10 years. Dormancy of propagules, in the absence of growing plants, appears to be maintained in soils by fungistasis and microbial antagonism (Schnathorst 1981). Resting structures of *Verticillium* species are stimulated to germinate in the soil by root exudates. Such stimulatory effects appear to have little specificity, since resting structures will germinate in the root exudates or in the rhizosphere of non-host plants (Schreiber and Green 1963; Subbarao et al. 1995).

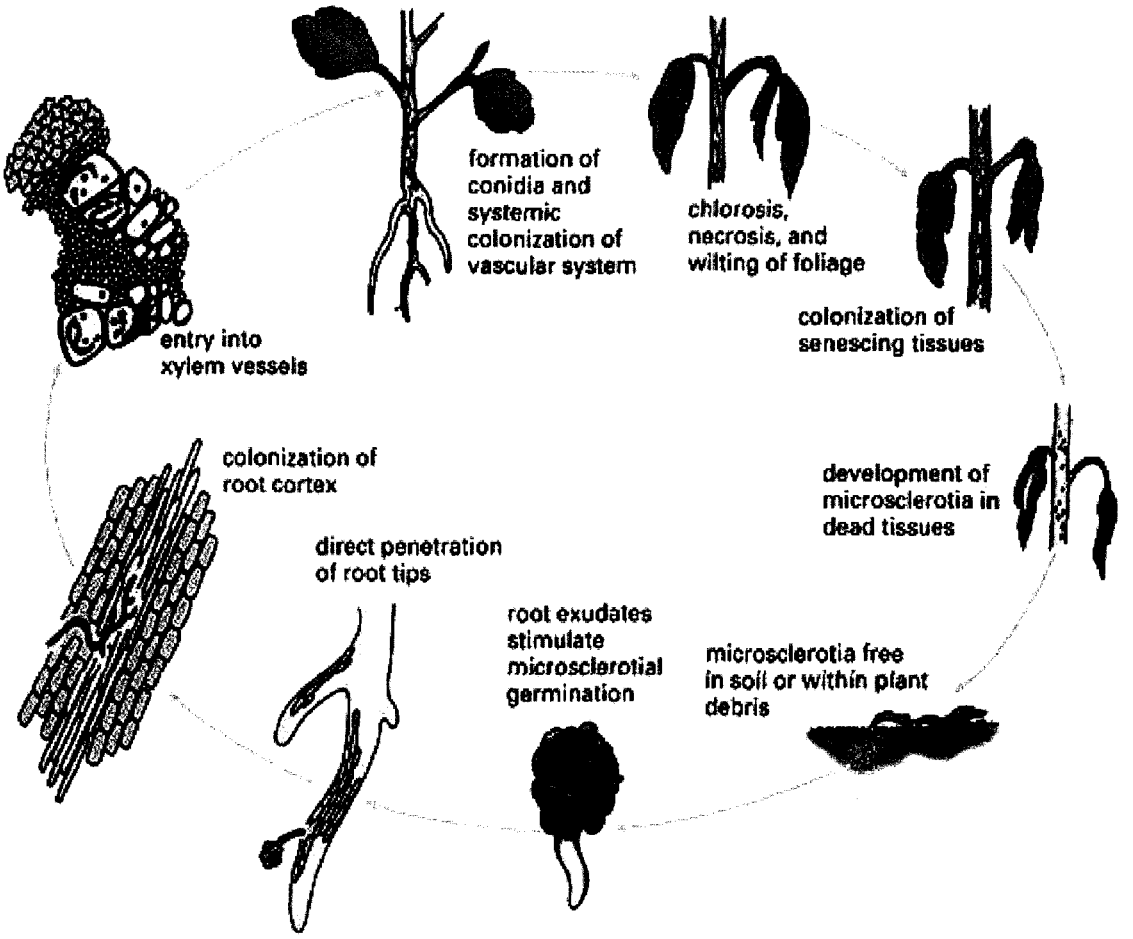

Figure 1.2 Lifecycle of Verticillium (drawing by Vickie Brewster, colored by Jesse Ewing, as found in Berlanger and Powelson 2000).

1.1.2.2 Parasitic stage

In the parasitic phase, the pathogen gains host entry by directly penetrating the cells that give rise to root hairs, or directly through the epidermal cells in the region of elongation in unwounded plants (Schnathorst 1981). Direct entry into the vascular system occurs through damaged root systems caused artificially or through natural wounds caused by rock particles, soil microfauna, or by dead tissue resulting from adverse environmental conditions. Once entry is gained, the pathogen mycelium colonizes the epidermis, penetrates the cortex and enters the xylem. Conidia are formed in the xylem shortly after hyphae penetration and contribute to the rapid growth of *Verticillium* species (Hu 1994). Some species have the ability to reach the top of the stem and produce large mycelial growths that cause blockages in the vascular system. These blockages cause the foliar disease symptoms characteristic of Verticillium wilt (Schnathorst 1981). These symptoms include wilting, chlorosis and premature death. Leaves become flaccid, and yellowing and dying proceed from the base of the plant upward. Stems lose their turgor, which is especially noticeable on hot days (Hu 1994). These symptoms will sometimes only occur on one side of the plant or on individual leaves (Powelson and Rowe 1993). Stem interiors of infected plants are always discoloured, and can be seen from the base of the plant and well into the plant top. Tubers may show brown discoloration of the vascular ring although other pathogens such as late blight may also cause this symptom (Berlanger and Powelson 2000; O'Brien and Rich 1979).

1.1.2.3 Saprophytic stage

The saprophytic stage occurs when infected plants scenesce and die. Resting structures begin to form at the end of the parasitic stage, but not in living tissue when the fungus is actively parasitic. Dark mycelium and melanized microsclerotia are capable of surviving adverse environmental conditions and, for the most part, are returned to the soil where the disease cycle can begin again (Schnathorst 1981).

1.1.3 Verticillium Pathogens

1.1.3.1 *Verticillium albo-atrum* 'group 1'

Four pathogenic species of *Verticillium* have been recognized to cause vascular wilt: *V. albo-atrum* 'group 1' Reinke & Berth., *V. dahliae* Kleb., *V. tricorpus* Isaac and *V. albo-atrum* 'group 2'. Most agricultural losses are caused by *V. albo-atrum* 'group 1' (VA1) and *V. dahliae* (VD) (Hu 1994; Schnathorst 1981). The optimal growing temperatures for VA1 range between 18°C to 24°C (Platt 1986). Since Maritime weather is generally cool and damp it tends to favour VA1, which is why it is considered the more aggressive pathogen (Mahuku et al. 1999). *Verticillium albo-atrum* 'group 1' rarely over winters and inoculum appears to be transmitted on diseased seed tubers (McKeen and Thorpe 1981; Platt 1986). It develops septate, resting dark mycelium in the host and in culture, and conidia of VA1 (6-12 x 2.5-3 μm) are larger in size than VD (3-5.5 x 1.5-2 μm) (Hooker 1981).

1.1.3.2 *Verticillium dahliae*

Verticillium dahliae is a strong soil-borne pathogen that is favoured by temperatures of 24°C to 28°C and forms microsclerotia that can survive between 10 to 30 years (Platt 1986; Tjamos and Fravel 1995). *Verticillium dahliae* vegetative mycelium is hyaline, septate and multinucleate. Conidia are ovoid or ellipsoid and usually single cell, and they are borne on phialides that are whorled around the conidiophore (Berlanger & Powelson 2000).

1.1.3.3 *Verticillium tricorpus*

Verticillium tricorpus (VT) resting structures are microsclerotia, resting mycelium and chlamydospores (Goud et al. 2003). Although VT is readily detected in soil samples, low recovery from stem tissues suggests a difference in host colonization and aggressiveness in potato. Evidence suggests that VT plays a saprophytic role and is unable to colonize potato stems like the more pathogenic species, and it proliferates in the soil or colonizes only severely infected plants. The role of VT in the Verticillium wilt complex is unclear, and it is not known whether VT is unable to infect and colonize actively growing plants (Mahuku et al. 1999).

1.1.3.4 *Verticillium albo-atrum* 'group 2'

Verticillium albo-atrum 'group 2' (VA2) is a *Verticillium* species that has recently been discovered (Robb et al. 1993). While it was originally thought to be a typical *V. albo-atrum* species, the failure to achieve DNA amplification by the polymerase chain reaction (PCR) using species specific primers led to further investigation. *V. albo-atrum*

'group 2' is morphologically similar to *V. albo-atrum*, which is now designated as 'group 1', except for the dark hyphae which form singly in 'group 1' and in bundles in 'group 2' (Robb et al. 1993). Differences in the internal transcribed spacer region (ITS) of sequenced rRNA confirmed VA2 as a new species (Robb et al. 1993) (Figure 1.3). In fact, it is more distantly related to VA1 than VD or VT (Morton et al. 1995). It is not known if VA2 isolates have always been present but could not be distinguished from 'group 1' isolates because of inadequate discrimination techniques (Mahuku and Platt 2002). Robb et al. (1993) reported that 'group 2' isolates were more genetically similar to the mainly saprophytic VT. However, Mahuku and Platt (2002) reported that 'group 2' is no more similar to VT than it is to 'group 1'. Further epidemiological studies need to be conducted with VA2 to elucidate its role in the Verticillium wilt disease complex (Mahuku et al. 1999).

1.1.4 Methods of Control

There are several management strategies available for control of Verticillium wilt including soil fumigation, crop rotation, host resistance, planting disease free seed and soil solarization. Although pathogen populations in the soil will decline following rotation to a non-host crop, crop rotation has not been regarded as a practical tactic for management of VD microsclerotia (Mol et al. 1996a; Rowe and Powelson 2002). For fields new to potato production, planting disease free seed would be a useful control method. However, a recent study found that seed tubers collected from commercial potato seed lots across North America showed that VD was successfully isolated from 30% of the lots tested, and seed tubers are thus thought to be primary sources of pathogen

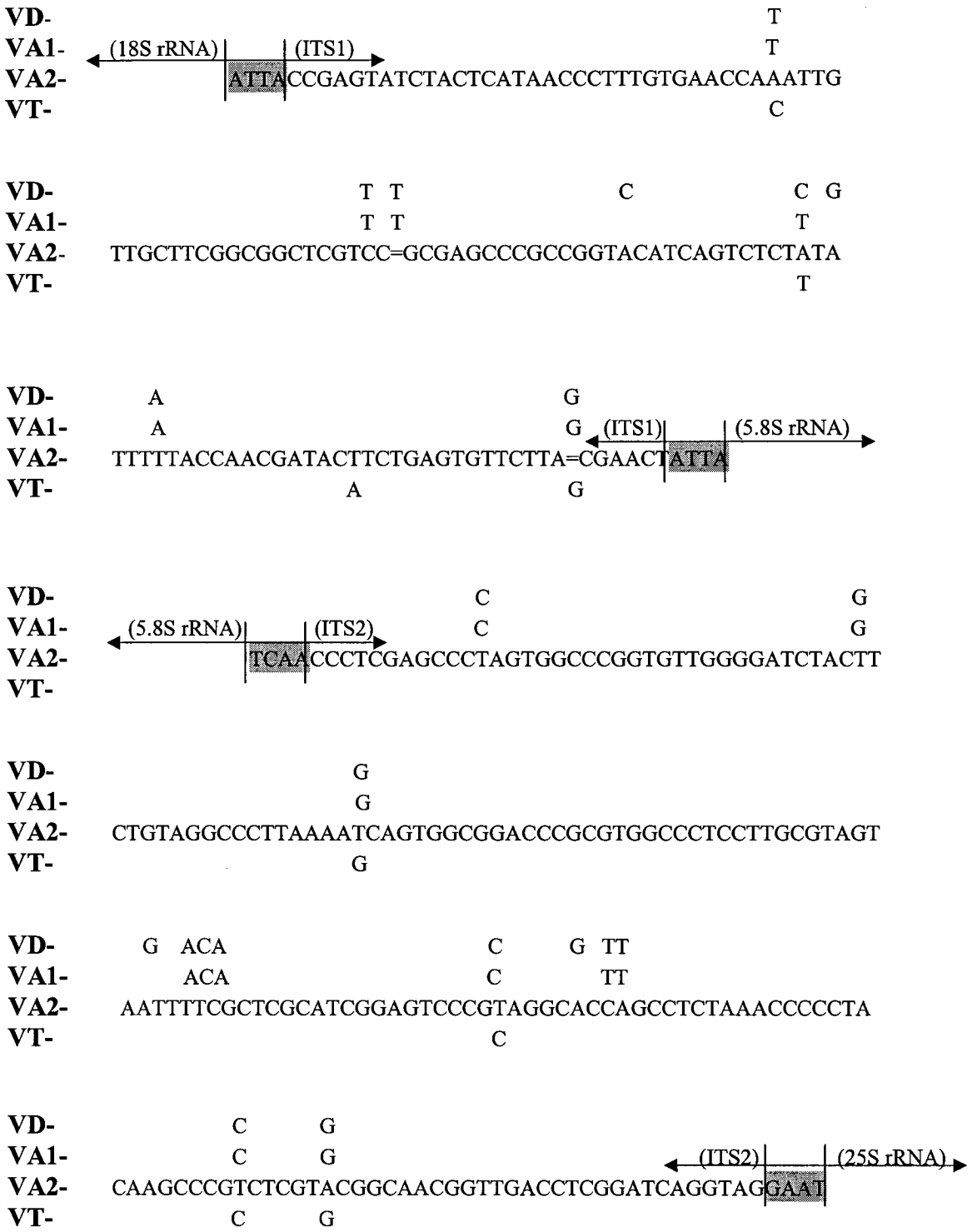

Figure 1.3 Sequence differences within two internal transcribed spacer (ITS) sequences of rRNA from the four *Verticillium* species.

inoculum (Omer et al. 2000). Despite considerable effort in developing resistant cultivars, only one (Russet Nugget) has effective resistance to Verticillium (Rowe and Powelson 2002). While varieties carrying different levels of resistance have been identified, cultivars react differently to varying environmental conditions and even the highest levels of resistance break down with high inoculum pressure (Hooker 1981). Also, some resistant cultivars produce tubers that are not preferred by growers (Lazarovits et al. 2001). Soil solarization is only effective in regions with a warm climate and can take long periods of time to be effective (Jeger et al. 1996). Although no single practice will provide complete control of Verticillium wilt (Davis 1985; Powelson and Rowe 1993), chemical treatments tend to be the most effective and widely used (Davis et al. 1996; Dobinson 1995). While soil fumigation produces effective results in an acceptable time period, high costs and adverse environmental concerns have prompted other efficient and less harmful methods of control to be explored (Davis et al. 1996; Keinath 1991; Powelson and Rowe 1993).

1.1.5 Biological Control Agents

Biological control techniques for managing Verticillium wilt have been tested with varied success (Davis et al. 1996; Powelson and Rowe 1993). Although research has not yet led to commercially available products (Jeger et al. 1996), it is thought that biological control techniques may provide an environmentally safe and economical approach to controlling Verticillium wilt (Davis 1985; Keinath et al. 1991). Lucus (1998) defines a biological control as "the reduction in attack of a crop species by a pathogen achieved using another living organism, or organisms.". To be effective against

a pathogen, a biological control agent should be able to colonize the niche where the pathogen is present, and achieve sufficient numbers to hinder the survival of the pathogen. A biological control agent might suppress a pathogen by physical exclusion, competition for nutrients or by direct antagonism. It is also possible for plant-pathogenic fungi to infect closely related species as these agents have diverse host-pathogen relationships. Therefore, instead of selecting a random agent, the best place to look for an antagonist would be the area where the pathogen itself is found (Lucus 1998). A biological control agent used to manage a particular *Verticillium* species may be another *Verticillium* species.

In potato, VT has been described as a weak pathogen with intermediate saprophytic ability (Isaac 1953; Mahuku et al. 1999) and has been recommended for biological control against VD (Davis et al. 2000). *Verticillium dahliae* and VA1 are the primary species that cause Verticillium wilt and they are often found in association with VT. Studies on the population dynamics of these species in combination may reveal more information about the competitiveness and possible moderating effects of VT on more aggressive species such as VD and VA1 (Heinz and Platt 2000a). A new Verticillium isolate, VA2 described by Robb et al. (1993), is thought to be similar to VT in that it is also a weak potato pathogen (Mahuku et al. 1999). It is possible that VA2 may also have some moderating effects on more aggressive *Verticillium* species. Because of concerns about potential adverse environmental effects of fumigants, biological control agents may be useful for management of Verticillium wilt and may in time provide a lasting approach for the suppression of aggressive species of *Verticillium* (Davis 1985).

Before selecting a biological control agent it is important to know the epidemiology of the disease to be controlled in order to obtain a potential antagonist (Keinath et al. 1991). Studies on the population dynamics of *Verticillium* species and competitiveness with other species may help to elucidate possible moderating effects of VT and VA2 on more pathogenic species (Heinz and Platt 2000a). Recent PCR detection and quantification methods for *Verticillium* species are more reliable and faster than traditional plating techniques. In addition, pathogens can be studied under a wider range of variables. These methods are useful when studying epidemiological aspects of *Verticillium* species and their importance in the Verticillium wilt disease complex (Mahuku et al. 1999; Mol et al. 1996b).

1.1.6 PCR techniques

Adequate disease management requires that population density estimates and correct pathogen identifications are made. Therefore, a reliable detection method must be used (Heinz and Platt 2000b; Mahuku et al. 1999; Mpofu and Hall 2003). Traditional detection methods require time consuming plating of plant parts and soil on selective media and incubation before identifications can be made (Platt and Bollen 1995; Robb et al. 1993). Even after incubation, positive identifications are difficult as other pathogens can mask the presence of *Verticillium* species and it is difficult to discriminate between colonies of VA2 and VA1 (Mahuku et al.1999) as well as VT and VD (Heinz and Platt 2000b). Monitoring symptom expression in the field is unreliable as symptoms are difficult to distinguish from some physiological disorders and, in other cases, plants may be extensively colonized but develops few symptoms (Dan et al. 2001).

Molecular detection with the PCR is accurate and dependable for diagnosis of *Verticillium* species (Hu et al. 1993; Morton et al. 1995; Moukhamedov et al. 1994; Nazar et al. 1991; Robb et al. 1993). Using species specific primers, Verticillium rDNA is amplified at the ITS region (Robb et al. 1993). The specific Verticillium primer sets for the four pathogens in this study were obtained from Robb et al. (1993) and are as follows:

VA1: primer 1: 5' CCGGTACATCAGTCTCTTTA 3'

primer 2: 5' ACTCCGATGCGAGCTGTAAT 3'

VD: primer 1: 5' CCGGTCCATCAGTCTCTCTG 3'

primer 2: 5' ACTCCGATGCGAGCTGTAAC 3'

VT: primer 1: 5' CGCCGGTACATCAGTCTCTT 3'

primer 2: 5' ACTCCGATGCGAGCGAA 3'

VA2: primer 1: 5' CCGGTACATCAGTCTCTATA 3'

primer 2: 5' CAACCGTTGCCGTACGAGA 3'

PCR can effectively be used to monitor the development of fungal colonization during the disease process or the spread of a pathogen in epidemiological studies. Ultimately, PCR has resulted in new opportunities to increase our understanding of plant-pathogen relationships and to improve the monitoring and control of plant diseases (Hu et al. 1993).

1.2 Objectives

The first objective of this study was to examine the epidemiology of two aggressive pathogens, VA and VD, as well as two less aggressive pathogens, VA2 and VT, in both greenhouse and field studies. The pathogens were identified at particular locations and at various time intervals within various soil and plant tissues of potato plants, using a DNA-based, PCR detection system. PCR was also used to estimate a relative population level (RPL) for each pathogen by visually comparing the DNA results to a series of samples with known concentrations and ranking them according to a devised scale. RPLs were also plotted over time to generate relative area under pathogen population curve (RAUPPC) values. Visual wilt symptoms were recorded on a weekly basis and consisted both of disease incidence as well as the percentage of each individual plant that was wilted. These values created a depiction of the disease and how pathogen population levels change throughout the plants over the growing period. All information was combined to obtain a clear picture of individual host-pathogen relationships, and compared and contrasted among the four pathogens for similarities and differences in pathogenicity. Characterizing weaker species in comparison to aggressive species is important as differences in pathogenicity may lead to better methods of control.

The second objective of this study was to examine how combinations of various pathogens affect host development and the effects that one species has on the colonization of another. In greenhouse and field studies, potato plants were inoculated with two pathogens at the same time in one of the following combinations: VA1+VA2, VA1+VT, VD+VA2 or VD+VT. Another group of potato plants was inoculated with a weak pathogen first, followed by an aggressive pathogen 4 days later. The combinations

were as follows: VA2+4dVA1, VA2+4dVD, VT+4dVA1 or VT+4dVD. Both pathogens were identified at particular locations and at different time intervals within various soil and potato tissues using a DNA based, PCR detection system. An estimation of RPLs for each pathogen was determined by visually comparing the DNA results to a series of samples with known concentrations and ranking them according to a devised scale. Visual wilt ratings, which consist of disease incidence and individual plant wilt assessments, were recorded on a regular basis. All information was combined to obtain a clear picture of how different combinations of pathogens behave within the host and how these pathogens affect each other. Time delayed experiments were compared to same day inoculations to see if the time of inoculation had any effect on disease development. All pathogens in the combinations were examined individually and compared to the single inoculation studies, which serve as a baseline, for differences in population levels. Any significant population differences were viewed as competitive behaviour between species in combination and may be an indication of a possible biological control agent.

2 CHAPTER TWO

2.1 Comparative disease development of *Verticillium albo-atrum* and *V. dahliae*: causal agents of verticillium wilt of potato

2.1.1 Introduction

Verticillium species are soil-borne and tuber-borne fungal pathogens that infect the vascular system of a variety of plant species, including potatoes (Heinz and Platt 2000; Mahuku and Platt 2002). Verticillium wilt is found in most potato production areas around the world and is an economically important disease that causes reduced tuber yields, premature plant death and produces diseased seed tubers (Mahuku et al. 1999). Tuber yield losses have been reported to be as high as 30 to 50% (Mahuku et al. 1999; McKeen and Thorpe 1981; Nachmias et al. 1993). Although soil fumigation with fungicides is the most effective way of controlling Verticillium populations, due to expense and environmental concerns other methods of control are needed (Dobinson 1995; Simko et al. 2004). While a biological control agent may be a useful alternative, it is necessary to understand disease development before new methods of control can be developed (Keinath et al. 1991).

Two major Verticillium pathogens on Prince Edward Island are *V. albo-atrum* 'group 1' Reinke & Berth and *V. dahliae* Kleb. *Verticillium albo-atrum* 'group 1' (VA1) favours cool temperatures of 18°C to 24°C (Platt 1986). Since Maritime weather is generally cool and damp, it tends to favour VA1, which is why it is considered the more aggressive pathogen (Mahuku et al. 1999; Platt and Sanderson 1987). *Verticillium albo-atrum* 'group 1' rarely over-winters and inoculum appears to enter the area on diseased seed tubers (McKeen and Thorpe 1981; Platt 1986; Sampson 1980). *Verticillium dahliae*

(VD) is a strong soil-borne pathogen that favours warmer temperatures of 24°C to 28°C and forms microsclorotia that can survive between 10 to 30 years (Platt 1986; Tjamos and Fravel 1995). Although infectious propagules can germinate and produce conidia within hours (Hooker 1981), wilt symptoms are usually not observed until later in the growing season (Platt 1986).

Verticillium resting structures in the soil germinate when stimulated by roots exudates. The pathogen then penetrates the roots through breaks or wounds and moves into the xylem. Some factors that can increase disease and affect symptom expression are heavy soils and cold, wet spring weather followed by hot, dry summer conditions (Schnathorst 1981).

In greenhouse and field studies reported here, VA1 and VD were studied using polymerase chain reaction (PCR) techniques to monitor relative population levels (RPLs) of the pathogens, disease incidence, and locations of the pathogens as they progressed within the soil, plant and tubers during a growing season. The purpose of these studies was to obtain a better understanding of how these pathogens interact within the host in both controlled and field environments.

2.1.2 Materials and Methods

2.1.2.1 Greenhouse experiment

A greenhouse experiment was conducted in 2003 at the Crops and Livestock Research Center in Charlottetown, Prince Edward Island. The susceptible potato c.v. Superior was used. Seed tubers (Elite 3) were planted into pots, 15 cm in diameter, containing autoclaved soil so that background pathogens were eliminated. In a

temperature controlled greenhouse set to approximately 24°C, the pots were placed into three rows and each row was divided into four replicates with five plants per replicate. A factorial experiment was conducted using a Randomized Complete Block Design. The plants were watered on a regular basis and fertilized with all purpose fertilizer (20-20-20). Approximately 30 days after planting, pots in each row were randomly selected and inoculated with one of two *Verticillium* species, VA1 or VD, or with water for the control group. On predetermined destructive sampling dates, one plant per replicate was sampled. The sampling dates were recorded as the number of days post inoculation. For the greenhouse experiment, sampling dates occurred 1, 3, 10, 20 and 37 days post inoculation.

2.1.2.2 Field experiments

Field experiments were conducted at the Harrington Research Farm in Prince Edward Island in 2002 and 2003, and established in the same manner as the greenhouse experiments except that the sample size was three times larger and non-sterile soil was used. The susceptible c.v. Superior was used again. Seed tubers (Elite 3) were planted into three rows and divided into four replicates with fifteen plants per replicate following a Randomized Complete Block Design. On predetermined destructive sampling dates, three plants per replicate were sampled. For the field experiment of 2002, sampling dates occurred 7, 14, 21, 28 and 65 days post inoculation. For the field experiment of 2003, sampling dates occurred 1, 6, 15, 28 and 63 days post inoculation.

2.1.2.3 Inoculum and inoculation

The inoculum was prepared from fungal cultures grown on plates containing potato dextrose agar (PDA). Each pathogen inoculum suspension consisted of a combination of three Prince Edward Island isolates of that species to ensure that the pathogenicity of the inoculum was optimal. Plates were emptied into a blender and the agar/culture mixture was blended until smooth. Spore counts were performed and the mixture was diluted, using distilled water, to a concentration of approximately 12 x 10^6 spores/mL. Inoculation via the soil involved digging a small hole, 7 cm deep, on either side of the plant so that the roots were disturbed/damaged. A volume of 50 mL of inoculum was divided among the two holes and the holes were refilled. The control group was inoculated with 50 mL of water only.

2.1.2.4 Destructive sampling

On destructive sampling dates, one potted plant from each replicate for the greenhouse experiment and three field grown plants from each replicate for the field experiments were randomly selected and separated into six parts: roots, lower stem, mid stem, top stem, tuber stem-end and tuber eye-end. Soil samples were taken from the hole that the plant was pulled out of and care was taken to avoid collecting any visible plant material. Leaves were removed from the stems and discarded. Soil, root and stem samples were stored separately in sealed, plastic bags. Tubers were stored in paper bags. The stem tissue and roots were kept at -20°C and the tubers and soils were kept at 4°C to preserve sample tissues until DNA extractions were performed.

2.1.2.5 DNA extractions

0.75 g frozen cross section samples from the lower, mid, and top stem areas were placed into three separate 1.5 mL tubes with a tungsten carbide beads. 0.75 g samples from tubers were collected using a #3 cork borer and placed in 1.5 mL tubes with beads. Roots were washed, chopped and 0.20 g was also placed in 1.5 mL tubes with beads. The tubes were then placed in an Applied Biosystems (Toronto, Ont.) Mixer Mill MM 300 at 25 Hz for 10 min to disrupt the plant tissues. QIAGEN (Mississauga, Ont.) DNeasy Plant Mini Extraction Kits were used to extract 50 μL samples of DNA from stem tissues, roots and tuber samples. Mo Bio (Solana Beach, Calif.) UltraClean Soil DNA Isolation Kits were used to extract 100 μL samples of DNA from 0.25 g soil samples. All extracted DNA was stored at -20°C.

2.1.2.6 Polymerase Chain Reaction

Using the system for evaluating Verticillium biomass in colonized plants described by Robb et al. (1993) as a guideline, PCR was performed using Verticillium primers that are specific to each species. The primer sequences for VA1 and VD are given in a report by Nazar et al. (1991). Each sample was examined for the species that it was inoculated with and the control group was examined for both species. DNA amplification with species specific primers was performed on all samples in 25.0 μL reactions. PCR reactions contained 10.9 μL PCR H_2O, 2.5 μL BSA (bovine serum albumin), 2.5 μL 10X PCR buffer, 2.0 μL (25 mM) $MgCl_2$, 1.0 μL (1.25 mM) of each nucleotide, 1.0 μL (25 μM) of each primer, 0.1 μL (5 U/μL) AmpliTaq polymerase and 1.0 μL of template DNA. Reactions were placed in a heated top Applied Biosystems

(Toronto, Ont.) GeneAmp PCR System 2700 thermocycler and began with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation, amplification and extension at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min respectively. A final extension step occurred at 72°C for 10 min. Aliquots of 12.5 μL of the PCR product were run through 1.2% agarose gels containing ethidium bromide.

The resulting bands were examined and ranked according to visual intensity, which is related to the concentration of the pathogen DNA, using a series of standards with known concentration. Each sample was assigned a relative population level (RPL) value between 0 and 3 based on the standards. The PCR results from these experiments were used to determine if the pathogen was present in a particular part of the plant at a particular time. Band intensity, time and location data was combined to describe the host-pathogen relationship. The number of plants showing visible signs of foliar wilt in each plot was recorded on a weekly basis in all experiments. Statistical analysis consisted of ANOVA F-probability tests, with a least significant difference (LSD) value calculated at 5% for assessment of significant differences among the RPLs of each pathogen, relative area under pathogen population curves (RAUPPC) and correlations to percentage of foliar disease. Genstat version 7.1 and Minitab version 11.0 were the statistical software packages used for these analyses.

2.1.2.7 DNA band intensity assessments based on quantified standards

Due to the large number of samples to be processed, a quick method was needed for assigning a quantitative value to band intensity. Because DNA band intensity is related to product concentration, comparing sample intensities to standards of known

concentrations is less subjective than visual assessments alone, and more cost and time effective than traditional quantification methods.

A competitive PCR-based system for quantifying fungal DNA (Hu et al. 1993; Mahuku et al. 1995) was used to quantify Verticillium DNA samples of high concentration. A serial dilution of these samples was created to generate high, medium and low intensity DNA bands on a 1.2% agarose gel. The three standards were tested repeatedly and their band intensities consistently remained within three different ranges of concentration. These samples became the standards against which all other samples would be compared. PCR was performed on all samples and every time a sample set was prepared for the thermocycler, a series of standards was included. Three standards, each of a different concentration and a negative control were used to devise a ratings system based on band intensity. The numbers (0-3) are referred to as RPLs (relative to the standards). This rating system is quick, reliable and if needed, an estimated concentration could be assigned to the VA1 and VD inoculated samples. Table A.1.1 shows the estimated concentration ranges of the different band intensities and the corresponding RPL and, Figure A.1.1 is a gel photo showing the DNA band intensities of the standards.

2.1.3 Results

Verticillium albo-atrum 'group 1' inoculated plants in the greenhouse showed signs of wilt before VD inoculated plants and the symptoms were more severe. In most cases, VA1 caused entire plant death. In the field experiments, VD produced more wilted plants but VA1 inoculated plant wilt symptoms were again more severe. The first signs of VA1 and VD foliar wilt symptoms were recorded approximately 20 days after

inoculation, whereas PCR was able to detect the pathogens as early as 24 hours after inoculation in the lower stems for both pathogens. *Verticillium dahliae* generally reached higher RPLs before VA1, which showed more gradual colonization trends.

2.1.3.1 RPLs of *Verticillium albo-atrum* 'group 1'

The RPLs of VA1 within seven different sample types in three separate experiments are shown in Table 2.1. Each RPL value is an average of four replicates. The five RPL values for each sample type are from the five destructive sampling dates. The percentage of diseased plants showing visible foliar wilt symptoms were also recorded for each of the sampling dates.

In the greenhouse VA1 maintained medium RPLs (refer to Table A.1.1) in soil samples with no significant increase or decrease detected on any of the five sampling dates throughout the plants life-cycle. Once the pathogen entered the roots, it was able to colonize the host up to the mid stem at high RPLs by the last sampling date with a significant increase over time. In the top of the stem, RPLs reached medium-high levels by sampling date 5, however, this increase was not significant (Table 2.1). Tubers were only collected on the last two sampling dates and did not reveal any detectable signs of VA1. In all soil and plant parts RPLs of VA1 increased as time increased. RPL values decreased as the pathogen moved up the plant. This trend is better represented in Table 2.2 by RAUPPC values.

In the 2002 field experiment, RPLs significantly increased from the first sampling date to the last date in all sample types. As the pathogen moved from the soil throughout

Table 2.1 Mean relative population levels over five sampling dates, within *Verticillium albo-atrum* inoculated soil and various plant parts, in relation to foliar disease.

Sample Type	SD	Greenhouse 2003		Field 2002		Field 2003	
		% FD	RPL	% FD	RPL	% FD	RPL
soil	1	0 c	2.2 a	0 c	0.8 c	0 b	2.5 bc
	2	0 c	1.5 a	0 c	2.0 b	0 b	2.2 cd
	3	0 c	2.2 a	0 c	3.0 a	0 b	2.0 d
	4	10 b	2.0 a	75 b	3.0 a	0 b	2.8 ab
	5	80 a	2.0 a	100 a	3.0 a	62 a	3.0 a
FProb./LSD(P<0.05)		**0.001/0.55**	**0.51/NS**	**0.001/0.46**	**0.001/0.74**	**0.001/0.62**	**0.03/0.45**
roots	1	0 c	2.2 b	0 c	0.0 d	0 b	0.0 b
	2	0 c	1.8 c	0 c	0.8 c	0 b	0.8 a
	3	0 c	3.0 a	0 c	2.0 b	0 b	1.5 a
	4	10 b	3.0 a	75 b	3.0 a	0 b	0.0 b
	5	80 a	3.0 a	100 a	3.0 a	62 a	1.5 a
FProb./LSD(P<0.05)		**0.001/0.55**	**0.001/0.35**	**0.001/0.46**	**0.001/0.47**	**0.001/0.62**	**0.01/0.74**
lower stem	1	0 c	0.8 b	0 c	0.0 d	0 b	0.0 c
	2	0 c	1.2 b	0 c	1.2 c	0 b	0.8 bc
	3	0 c	1.2 b	0 c	2.2 b	0 b	1.2 b
	4	10 b	2.8 a	75 b	3.0 a	0 b	2.8 a
	5	80 a	3.0 a	100 a	3.0 a	62 a	3.0 a
FProb./LSD(P<0.05)		**0.001/0.55**	**0.01/0.95**	**0.001/0.46**	**0.001/0.54**	**0.001/0.62**	**0.001/0.92**
mid stem	1	0 c	1.2 bc	0 c	0.0 c	0 b	0.0 d
	2	0 c	1.2 bc	0 c	0.5 c	0 b	0.5 c
	3	0 c	0.5 cd	0 c	1.8 b	0 b	0.2 cd
	4	10 b	1.8 b	75 b	2.2 b	0 b	2.0 b
	5	80 a	3.0 a	100 a	3.0 a	62 a	3.0 a
FProb./LSD(P<0.05)		**0.001/0.55**	**0.01/0.87**	**0.001/0.46**	**0.001/0.55**	**0.001/0.62**	**0.001/0.38**
top stem	1	0 c	0.8 a	0 c	0.0 b	0 b	0.0 c
	2	0 c	0.8 a	0 c	0.2 b	0 b	0.2 c
	3	0 c	0.8 a	0 c	1.8 a	0 b	0.0 c
	4	10 b	1.2 a	75 b	1.8 a	0 b	0.8 b
	5	80 a	2.5 a	100 a	2.0 a	62 a	2.0 a
FProb./LSD(P<0.05)		**0.001/0.55**	**0.06/NS**	**0.001/0.46**	**0.001/0.48**	**0.001/0.62**	**0.001/0.54**
tuber stem-end	1	0 c	*	0 c	*	0 b	*
	2	0 c	*	0 c	*	0 b	*
	3	0 c	*	0 c	*	0 b	*
	4	10 b	0.0	75 b	0.0 b	0 b	0.5 b
	5	80 a	0.0	100 a	2.5 a	62 a	1.5 a
FProb./LSD(P<0.05)		**0.001/0.55**	**NA**	**0.001/0.46**	**0.001/0.50**	**0.001/0.62**	**0.05/0.71**

cont' next page

Sample Type	SD	Greenhouse 2003		Field 2002		Field 2003	
		% FD	RPL	% FD	RPL	% FD	RPL
tuber eye-end	1	0 c	*	0 c	*	0 b	*
	2	0 c	*	0 c	*	0 b	*
	3	0 c	*	0 c	*	0 b	*
	4	10 b	0.0	75 b	0.0 b	0 b	0.0 a
	5	80 a	0.0	100 a	1.5 a	62 a	0.2 a
FProb./LSD(P<0.05)		**0.001/0.55**	**NA**	**0.001/0.46**	**0.001/0.50**	**0.001/0.62**	**0.36/NS**

Note: SD = sample date, which is the number of days post-inoculation for the greenhouse (1, 3, 10, 20, 37) and field (1, 6, 15, 28, 63) studies; % FD = percent foliar disease; RPL = relative population level; * = no tubers; NA = not available; NS = not significant; Fprob. = F probability value from AVOVA tests. Means were separated using a least significant difference (LSD) at P<0.05.

Table 2.2 Relative area under pathogen population curve values and their correlation to foliar disease.

	Sample Type	Greenhouse 2003		Field 2002		Field 2003	
		RAUPPC	R^2	RAUPPC	R^2	RAUPPC	R^2
Verticillium albo-atrum 'group 1'	soil	72.1	0.88	208.9	0.86	162.5	0.98
	roots	101.6	0.90	188.2	0.86	48.0	0.93
	lower stem	79.6	0.97	192.9	0.86	137.5	0.97
	mid stem	60.2	0.99	159.8	0.85	106.8	0.99
	top stem	48.6	0.99	122.1	0.87	54.7	1.00
	tuber stem-end	0.0	NA	58.8	NA	35.0	NA
	tuber eye-end	0.0	NA	35.2	NA	4.4	NA
Verticillium dahliae	soil	71.8	0.84	218.8	0.98	178.9	0.98
	roots	106.9	0.85	211.7	0.98	109.4	0.84
	lower stem	40.4	-0.22	201.0	0.98	175.6	0.97
	mid stem	34.9	-0.03	168.9	0.98	133.0	0.98
	top stem	34.4	0.62	139.6	0.98	121.2	0.99
	tuber stem-end	0.0	NA	51.7	NA	43.8	NA
	tuber eye-end	0.0	NA	58.8	NA	26.2	NA

Note: RAUPPC = relative area under pathogen population curve. RAUPPC values are determined from RPLs plotted over time. The resulting area under the curve provides a value which is representative of the pathogen levels over the entire growing season and can be correlated to foliar disease; R^2 = correlation; NA = not available. No correlations were calculated for the tubers, which were collected on the last two sampling dates, because only one data point was generated by the RAUPPC.

the host, RPLs decreased. RPLs were the highest in the soil and VA1 managed to maintain high RPLs up to the stem top where levels decreased to medium. Tubers were sampled on the last two sampling dates in which no VA1 was detected on sampling date 4. On the last sampling date, VA1 was present at medium-high levels in the tuber stem-end and at medium-low levels in the tuber eye-end (Table 2.1).

In the 2003 field experiment, RPLs significantly increased over time in all sample types except for tuber eye-ends. RPLs were highest in the soil and decreased in the roots. However, the pathogen managed to colonize the lower stems at low levels. *Verticillium albo-atrum* 'group 1' reached high RPLs in the lower and mid stem portions by the last sampling date and reached moderate levels in the top stem. *Verticillium albo-atrum* 'group 1' was present at low RPLs in the tuber stem-end on sampling date 4 and at medium-low levels on the last sampling date. In the tuber eye-end, VA1 was present at low RPLs on the last sampling date only (Table 2.1).

2.1.3.2 RPLs of *Verticillium dahliae*

Verticillium dahliae data was prepared and examined as previously described for VA1.

In the greenhouse, VD RPLs in the soil were generally moderate with no significant differences between sampling dates. RPLs in the roots were all high with no significant differences between sampling dates and RPLs in the lower, mid and top stem samples all increased to medium-high levels by the second sampling date and then significantly decreased to low levels by the last sampling date (Table 2.3). No VD

Table 2.3 Mean relative population levels over five sampling dates, within *Verticillium dahliae* inoculated soil and various plant parts, in relation to foliar disease.

Sample Type	SD	Greenhouse		Field 2002		Field 2003	
		% FD	RPL	% FD	RPL	% FD	RPL
soil	1	0 b	2.2 a	0 b	3.0 a	0 c	2.2 a
	2	0 b	2.0 a	0 b	2.5 a	0 c	2.2 a
	3	0 b	1.5 a	0 b	3.0 a	0 c	3.0 a
	4	0 b	2.8 a	0 b	3.0 a	10 b	3.0 a
	5	32 a	1.2 a	100 a	3.0 a	94 a	3.0 a
Fprob./LSD(P<0.05)		**0.001/0.86**	**0.16/NS**	**0.001/0.66**	**0.44/NS**	**0.001/0.68**	**0.30/NS**
roots	1	0 b	3.0 a	0 b	2.0 b	0 c	0.2 c
	2	0 b	2.8 a	0 b	2.2 b	0 c	2.8 a
	3	0 b	3.0 a	0 b	2.8 a	0 c	3.0 a
	4	0 b	3.0 a	0 b	3.0 a	10 b	1.5 b
	5	32 a	3.0 a	100 a	3.0 a	94 a	1.5 b
Fprob./LSD(P<0.05)		**0.001/0.86**	**0.44/NS**	**0.001/0.66**	**0.001/0.28**	**0.001/0.68**	**0.001/0.54**
lower stem	1	0 b	2.2 b	0 b	1.0 d	0 c	0.2 b
	2	0 b	3.0 a	0 b	1.5 c	0 c	2.5 a
	3	0 b	1.8 b	0 b	2.5 b	0 c	3.0 a
	4	0 b	0.2 c	0 b	3.0 a	10 b	3.0 a
	5	32 a	0.8 c	100 a	3.0 a	94 a	3.0 a
Fprob./LSD(P<0.05)		**0.001/0.86**	**0.001/0.76**	**0.001/0.66**	**0.001/0.39**	**0.001/0.68**	**0.001/0.55**
mid stem	1	0 b	1.2 b	0 b	0.8 c	0 c	0.0 c
	2	0 b	2.8 a	0 b	1.0 c	0 c	1.5 b
	3	0 b	1.5 b	0 b	2.0 b	0 c	2.0 b
	4	0 b	0.0 c	0 b	2.2 b	10 b	2.0 b
	5	32 a	1.0 b	100 a	3.0 a	94 a	3.0 a
Fprob./LSD(P<0.05)		**0.001/0.86**	**0.001/0.73**	**0.001/0.66**	**0.001/0.32**	**0.001/0.68**	**0.001/0.78**
top stem	1	0 b	0.8 bc	0 b	0.0 d	0 c	0.0 d
	2	0 b	2.0 a	0 b	0.5 c	0 c	1.0 c
	3	0 b	1.2 b	0 b	1.0 b	0 c	1.2 c
	4	0 b	0.2 c	0 b	2.2 b	10 b	2.0 b
	5	32 a	1.2 b	100 a	2.5 a	94 a	3.0 a
Fprob./LSD(P<0.05)		**0.001/0.86**	**0.03/0.76**	**0.001/0.66**	**0.001/0.40**	**0.001/0.68**	**0.001/0.52**
tuber stem-end	1	0 b	*	0 b	*	0 c	*
	2	0 b	*	0 b	*	0 c	*
	3	0 b	*	0 b	*	0 c	*
	4	0 b	0.0	0 b	0.0 b	10 b	0.8 b
	5	32 a	0.0	100 a	2.2 a	94 a	1.8 a
Fprob./LSD(P<0.05)		**0.001/0.86**	**NA**	**0.001/0.66**	**0.001/0.21**	**0.001/0.68**	**0.03/0.61**

Cont' next page

Sample Type	SD	Greenhouse 2003		Field 2002		Field 2003	
		% FD	RPL	% FD	RPL	% FD	RPL
tuber eye-end	1	0 b	*	0 b	*	0 c	*
	2	0 b	*	0 b	*	0 c	*
	3	0 b	*	0 b	*	0 c	*
	4	0 b	0.0	0 b	0.5 b	10 b	0.5 a
	5	32 a	0.0	100 a	2.0 a	94 a	1.0 a
FProb./LSD(P<0.05)		**0.001/0.86**	**NA**	**0.001/0.66**	**0.001/0.43**	**0.001/0.68**	**0.13/NS**

Note: SD = sample date, which is the number of days post-inoculation for the greenhouse (1, 3, 10, 20, 37) and field (1, 6, 15, 28, 63) studies; % FD = percent foliar disease; RPL = relative population level; * = no tubers; NA = not available; NS = not significant; Fprob. = F probability value from AVOVA tests. Means were separated using a least significant difference (LSD) at P<0.05.

was detected in tuber samples. As the pathogen continued to move up the plant, RPLs decreased.

In the field experiment of 2002, VD maintained high RPLs in the soil with no significant changes throughout the growing season. In all other plant parts, RPLs significantly increased as time increased. The roots, lower stem and mid stem all reached high RPLs and the top stem reached medium-high levels by the last sampling date (Table 2.3). Both the tuber stem-end and the tuber eye-end had RPLs in the medium range. As the pathogen moved up the plant, the RPLs decreased.

In the field experiment of 2003, VD maintained medium-high RPLs in the soil on all sampling dates with no significant changes. RPLs in the roots significantly increased to high by the third sampling date and then significantly decreased to medium-low levels. The pathogen managed to enter the lower stems at low RPLs and significantly increase over time in all remaining plant parts except for the tuber eye-end which increased but not significantly (Table 2.3). In this experiment, RPLs in all sample types increased as time increased, except for the roots, and decreased as the pathogen moved up the plant.

2.1.3.3 Field experiment summary

Graphical representations of both VA1 and VD RPLs in the soil, roots and stems, from the 2002 and 2003 field experiments, are presented in Figure 1.4. Figure 1.4 shows a steady increase in RPLs at all sample dates, however, RPLs did decrease in the roots for part of the sampling period.

The percentage of foliar disease increased during the study period for both VA1 and VD in the combined field experiments (Figure 1.5). More plants inoculated with VA1 (37.5%) showed foliar disease than VD (5%) inoculated plants by sampling date 4. By the last sampling date, 97% of plants inoculated with VD showed foliar disease symptoms, which was higher than the 81% with VA1.

2.1.3.4 Correlation between foliar disease and RAUPPC

Correlations were computed to discover if there is a relationship between visual ratings of foliar disease symptoms and pathogen population assessments (RAUPPC) obtained from PCR. For plant disease development assessments, visual ratings of foliar disease symptoms were recorded. RPL values were plotted over number of days post inoculation, to produce RAUPPC for each of the three experiments and seven sample types. The RAUPPC produced values that are representative of pathogen population changes over the entire growing season. Each of the experiments had a set of different sampling times and RAUPPC take this into consideration. The RAUPPC values for the greenhouse and field experiments are displayed in Table 2.2 along with correlation values between RAUPPC and foliar disease.

Visual signs of wilt caused by VA1, in the greenhouse experiment, were not recorded until many days after inoculation and by 20 days after inoculation (sampling date 4) 10% of the plants inoculated with VA1 showed foliar disease symptoms. On this same date, PCR detected medium-high levels of the pathogen in various sample types. On the last sampling date, 80% of the plants showed foliar disease symptoms. Correlations between RAUPPC and foliar disease were all over 0.88.

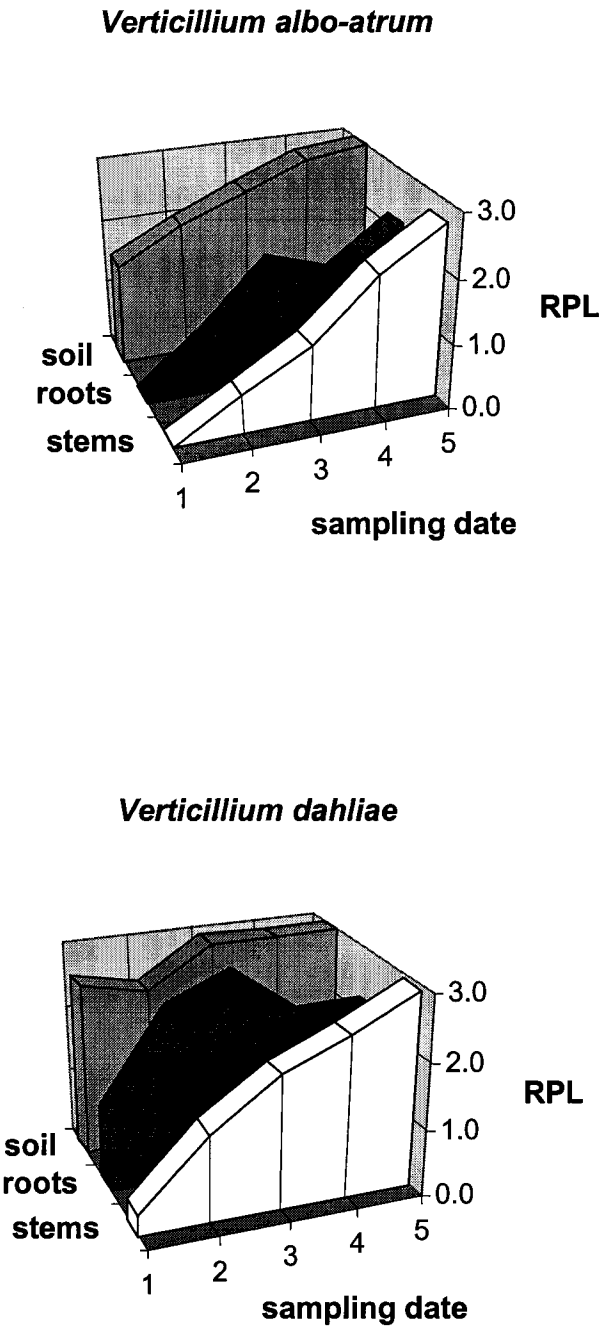

Figure 1.4 *Verticillium albo-atrum* and *Verticillium dahliae* RPLs over time for combined field experiments 2002 and 2003.

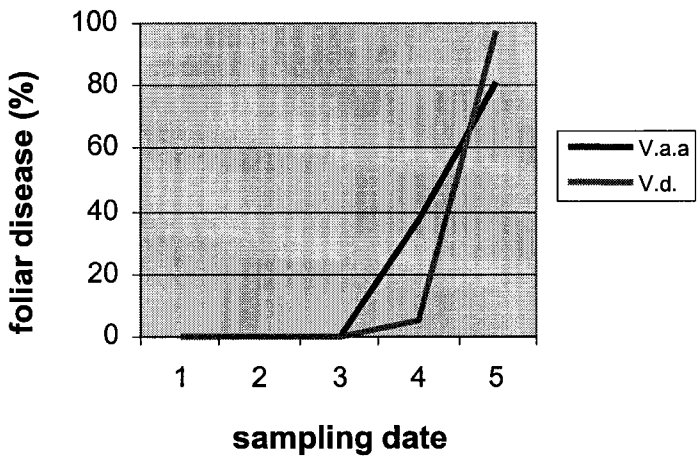

Figure 1.5 *Verticillium albo-atrum* and *Verticillium dahliae* foliar disease (%) assessments in combined field experiments over time.

In the field, as in the greenhouse, VA1 foliar disease symptoms were not seen until many days after the pathogen was detected with PCR. By sampling date 4, in the field experiment of 2002, 75% of the plants inoculated with VA1 showed foliar disease symptoms, and 100% of the plants were affected by the last sampling date. In the field experiment of 2002, correlations were all over 0.85. In the field experiment of 2003, on the last sampling date (63 days after inoculation), 62% of the plants showed foliar disease symptoms and correlation values were all over 0.93.

Verticillium dahliae, in the greenhouse experiment, caused foliar disease symptoms, on the last sampling date, on only 32% of the plants. Correlation values did not show a strong relationship between foliar disease and RAUPPC values (Table 2.2).

In the field experiment of 2002, 100% of the plants inoculated with VD showed foliar disease symptoms on the last sampling date and correlations were greater than 0.85. In the field experiment of 2003, 10% of plants showed foliar disease by sampling date 4 and by the last sampling date, 94% of the plants were wilted. Correlation values were all over 0.84.

For both species, the field experiments had higher RAUPPC values than the greenhouse experiment and there was also a strong correlation between these values and foliar disease symptoms. *Verticillium dahliae* RAUPPC values were less than VA1 values in the greenhouse and greater than VA1 values in the field experiments. Correlations for the tuber samples could not be calculated because there was only one data point generated by the RAUPPC.

2.1.4 Discussion

In order to obtain a better understanding of how VA1 and VD interact within the host, RPLs of each pathogen and the locations of the pathogens, as they progressed throughout potato plant tissues during the course of disease development, were monitored using PCR technology. *Verticillium albo-atrum* 'group 1', which is typically known as an aggressive plant pathogen and the most problematic pathogen of the *Verticillium* species in most potato growing areas (McKeen and Thorpe 1981), maintained high RPLs and caused a higher incidence of foliar disease than VD in the greenhouse. However, VD which is recognized as a strong soil-borne pathogen (Platt 1986), maintained higher RPLs than VA1 in both field experiments conducted in 2002 and 2003 (Table 2.2). *Verticillium dahliae* also caused a higher incidence of foliar disease than VA1 in the field experiment of 2003. Although the incidence of foliar disease caused by VD was lower than VA1 in the field experiment of 2002, the difference was not significant. Tables A.1.2 – A.1.4 are the ANOVA tables for all statistical analysis of variance tests conducted for this thesis.

2.1.4.1 Comparison of *Verticillium albo-atrum* 'group 1' inoculated experiments

The field experiments in 2002 and 2003 yielded pathogen RPLs that were very similar in all sample types and only differed in the roots. The RPLs in the roots of field 2003 were lower than in field 2002. These experiments were conducted in two different fields with different crop rotation history. Therefore, there may have been different microorganisms in the field soil in 2003 that kept VA1 from colonizing the roots to the extent that it normally would. Leben et al. (1987) stated that some bacterial strains of *Psedomonas*, *Cellulomonas* and *Streptomyces* species had antagonistic effects on VD and

were able to colonize significantly more roots of potato plants. They also stated that there are more than 150 bacterial isolates from potato roots that are antagonistic to *Verticillium* species. In the field experiment of 2002, VA1 RPLs increased dramatically (0 to 75%) in only a week. Weather data revealed cool (2.5°C), wet (103 mm) spring conditions followed by high (25°C) summer temperatures. Clearly, weather conditions are an important factor on the growth of Verticillium and the devastation it can cause in the field. Disease severity increases as temperatures increase to optimal growing levels and wilt symptoms are enhanced by periods of low soil moisture late in the growing season (Platt 1986; Powelson and Rowe 1993).

In the greenhouse experiment, the soil was autoclaved and free of contaminants, therefore, VA1 was able to establish itself inside the roots at high RPLs with no interference. Because of the high RPLs in the roots and moderate levels in the soil, it would be expected that the tubers have some disease. The absence of the pathogen in the tubers may be due to the fact that the plants in the greenhouse were inoculated before stolons had developed, which means that they were not damaged/disturbed the way that the roots were.

Hoyos et al. (1991) studied the relationship between colony forming units per mL and percentage of foliar wilt, and found that there were high correlations between the two. For all experiments in this study, correlations between pathogen population assessments obtained from PCR (RAUPPC) and foliar wilt were high (>0.85). RPLs increased steadily over time and by the last sampling date, almost all soil and plant tissues had high RPLs of VA1.

2.1.4.2 Comparison of *Verticillium dahliae* inoculated experiments

The greenhouse experiment had lower RPLs in all sample types, except the roots, than both of the field experiments. The RPLs of root samples in the greenhouse were higher than the field experiment of 2003. *Verticillium dahliae* is known for being a strong soil pathogen (Schnathorst 1981) and in this case VD did maintain high RPLs in the soil. Above the soil, RPLs peaked early and then died off. Perhaps the greenhouse environment was not optimal for VD growth in that the temperature was a moderate 24°C. The optimal growing temperature range for VD is 24°C to 28°C (Platt 1986). Foliar disease symptoms were compared to RAUPPC and although high correlations were found for both soil and root samples, this would be expected as there were no significant changes in the RPLs over time and the percentage of plants showing foliar disease was also low. No major fluctuations in disease for both measurements would result in a high correlation. However, this is misleading because even though the RPLs did not fluctuate, they were all medium (soil) or high (roots) whereas the percentage of foliar disease was low. Correlations for stem samples were poor because of the sudden increase in RPLs by sampling date 2, followed by a significant decrease in RPLs.

In the field, VD behaved more like VA1, reaching medium-high levels quickly and maintaining these levels for the rest of the growing season. This may have been due to the presence of naturally occurring populations of VD in the soil and diseased seed tubers, as well as the occurrence of favourable weather conditions for this pathogen. The 2002 and 2003 growing seasons were warm (25°C), dry and perfect growing conditions for VD. Because VD can survive in the soil for long periods of time (microsclerotia), soil samples from the control group were reviewed (Table 2.4). They showed high RPLs of

Table 2.4 Mean relative population levels of *V. dahliae* in uninoculated potato plants from the field experiment in 2002.

		Field 2002
Sample Type	SD	RPL
soil	1	3.0
	2	3.0
	3	3.0
	4	3.0
	5	3.0
roots	1	2.2
	2	2.8
	3	3.0
	4	3.0
	5	3.0
lower stem	1	1.5
	2	2.5
	3	3.0
	4	3.0
	5	3.0
mid stem	1	0.2
	2	1.8
	3	2.5
	4	3.0
	5	3.0
top stem	1	0.0
	2	0.5
	3	0.5
	4	2.2
	5	3.0
tuber stem-end	1	*
	2	*
	3	*
	4	0.0
	5	1.5
tuber eye-end	1	*
	2	*
	3	*
	4	0.0
	5	0.5

Note: SD=sampling date; RPL=relative population level.

VD in the soil and almost identical RPLs were found in the plants on all sampling dates when compared to the inoculated group. This indicates that the naturally occurring populations in the soil played a role in contributing to the RPLs found in the plants. Greenhouse soil was sterile when the seed tubers were planted and therefore only the inoculum added thirty days after planting and perhaps some inoculum from the seed tubers would be the cause of any disease. A study conducted by Omer et al. (2000) found that commercial certified seed lots throughout North America are commonly infected with VD and may serve as a primary source of inoculum. Almost all seed tubers tested for VD and VA1 before planting in the greenhouse showed moderate RPLs of VD.

From these experiments it was found that both VA1 and VD entered the plants quickly and move to all plant parts, eventually ending up in the tubers and becoming a source of inoculum for another season and visual foliar disease ratings are not sufficient warning signs for treatment. Symptoms did not show up until late in the growing season whereas the pathogen colonized the plant within 24 hours. By the time the effects of the disease on the foliage are seen, high levels of the pathogen have become a source of inoculum for neighbouring plants and the next growing season.

For future studies it is important to obtain disease free seed tubers to assess the effects of this source of inoculum on the subsequent growing season. The seed tubers used in all experiments were from the same seed source and contained moderate level of VD but only a few samples contained low levels of VA1. These studies should also be repeated in a field free of VD as naturally occurring population were thought to be a major contributor to RPLs seen in these field experiments. However, for most potato growers, it is not reasonable to leave a field out of potato production for 10 years.

Therefore, the control groups in the field experiments are a realistic representation of population levels found in the soil after a typical three year crop rotation.

3 CHAPTER THREE

3.1 Epidemiological aspects of verticillium wilt of potato caused by *Verticillium tricorpus* and *V. albo-atrum* 'group 2'

3.1.1 Introduction

Verticillium wilt of potato, caused by *Verticillium albo-atrum* 'group 1' Reinke & Berth. and *Verticillium dahliae* Kleb., is a vascular disease that causes reduced tuber yields and diseased seed tubers (Lynch et al. 1997). Quantification of *Verticillium* species in the vascular tissue has been used as a measure of pathogen virulence and has revealed differences that were not detected by visual disease assessments (Hoyos et al. 1991). Two other species associated with the Verticillium wilt disease complex are *V. tricorpus* Isaac and *V. albo-atrum* 'group 2' (Mahuku et al. 1999).

Verticillium tricorpus (VT) resting structures are microsclerotia, resting mycelium and chlamydospores (Goud et al. 2003). Because VT is generally harmless and VD is strongly pathogenic, VT has been recommended as a biological control agent against VD in potato (Davis et al. 2000). Further studies of the population dynamics of this species and its competitiveness with other *Verticillium* species will help clarify the role of VT in the Verticillium wilt disease complex and reveal possible moderating effects of VT on more pathogenic species such as VD and VA1 (Heinz and Platt 2000). Although VT is readily detected in soil samples, low recovery from stem tissues suggests a difference in host colonization and aggressiveness in potato. Evidence suggests that VT plays a saprophytic role and is unable to colonize potato stems like the more pathogenic species and it proliferates in the soil or colonizes only severely infected plants. The role of VT in

the Verticillium wilt complex is unclear and it is not known whether VT is able to infect and colonize actively growing plants (Mahuku et al. 1999).

Verticillium albo-atrum 'group 2' (VA2) is a new *Verticillium* species recently discovered (Robb et al. 1993). It was originally thought to be a typical VA1 species, however, failure to amplify using species specific primers led to further investigation. *Verticillium albo-atrum* 'group 2' is morphologically similar to VA1 except for the dark hyphae which forms singly in 'group 1' and in bundles in 'group 2' (Robb et al. 1993). Differences in the internal transcribed spacer region of sequenced rRNA confirmed VA2 as a new species. In fact, it is more distantly related to VA1 than VD or VT (Morton et al. 1995). It is not known if VA2 isolates have always been present but they could not be distinguished from 'group 1' isolates because of inadequate discrimination techniques (Mahuku and Platt 2002). Robb et al. (1993) reported that 'group 2' isolates were more genetically similar to the mainly saprophytic VT. However, Mahuku and Platt (2002) reported that 'group 2' is no more similar to VT than it is to 'group 1'. Further epidemiological studies need to be conducted with VA2 to elucidate its role in the Verticillium wilt disease complex (Mahuku et al. 1999).

The purpose of this study was to obtain a better understanding of how VT and VA2 react in inoculated soils and potato hosts. Host-pathogen interactions were studied in both greenhouse and field experiments using PCR techniques to monitor relative population levels (RPLs), disease incidence and locations of the pathogens as they progressed within the soil, plant and tubers throughout a growing season.

3.1.2 Materials and Methods

3.1.2.1 Greenhouse experiment

A greenhouse experiment was conducted in 2003 at the Crops and Livestock Research Center in Charlottetown, Prince Edward Island. Seed tubers (Elite 3), of the susceptible c.v. Superior, were planted into pots, 15 cm in diameter, containing autoclaved soil so that background pathogens were eliminated. In a temperature controlled greenhouse (approximately 24°C), a Randomized Complete Block Design was followed. Pots were placed into three rows and each row was divided into four replicates with five plants per replicate. The plants were watered on a regular basis and fertilized with all purpose fertilizer (20-20-20). Approximately 30 days after planting, pots in each row were randomly selected and inoculated with one of two *Verticillium* species, VT or VA2, or with water for the control group. One plant per replicate was sampled on predetermined destructive sampling dates. The sampling dates were recorded as the number of days post inoculation. For the greenhouse experiment, sampling dates occurred 1, 3, 10, 20 and 37 days post inoculation.

3.1.2.2 Field experiment

A field experiment was conducted at the Harrington Research Farm in Prince Edward Island in 2003. It was established in the same manner as the greenhouse experiment except that the sample size was three times larger and non-sterile soil was used. The susceptible c.v. Superior was used again. Seed tubers (Elite 3) were planted into three rows and divided into four replicates with fifteen plants per replicate following a Randomized Complete Block Design. Three plants per replicate were sampled on

predetermined destructive sampling dates. For the field experiment, sampling dates occurred 1, 6, 15, 28 and 63 days post inoculation.

3.1.2.3 Inoculum preparation and inoculation

The inoculum was prepared from fungal cultures grown on potato dextrose agar (PDA). Each inoculum suspension consisted of a combination of three Prince Edward Island isolates of a particular species to ensure that the pathogenicity of the inoculum was optimal. Petri plates were emptied into a blender and the agar/culture mixture was blended until smooth. Spore counts were performed and the mixture was diluted, using distilled water, to a concentration of approximately 12 x 10^6 spores/mL. Soil inoculations involved digging a small hole, 7 cm deep, on either side of the plant so that the roots were disturbed/damaged, and dividing a volume of 50 mL of inoculum among the two holes which were refilled afterward. The control group was inoculated with 50 mL of water only.

3.1.2.4 Sample preparation

On destructive sampling dates, one potted plant from each replicate for the greenhouse experiment and three field grown plants from each replicate for the field experiment was randomly selected and separated into six parts: roots, lower stem, mid stem, top stem, tuber stem-end and tuber eye-end. Soil samples were collected from the hole that the plant was pulled out of, taking care to avoid collecting any visible plant material. Leaves were removed from the stems and discarded. Soil, root and stem samples were stored separately in sealed, plastic bags. Tubers were stored in paper bags.

The stem tissue and roots were kept at -20°C and the tubers and soils were kept at 4°C until DNA extractions were performed.

0.75 g frozen cross section samples from the lower, mid, and top stem areas were placed into 1.5 mL tubes with tungsten carbide beads. 0.75 g samples from tubers were collected using a cork borer (#3) and placed into 1.5 mL tubes with beads. Roots were washed, chopped and 0.20 g were also placed in 1.5 mL tubes with beads. The tubes were then placed in an Applied Biosystems (Toronto, Ont.) Mixer Mill MM 300 at 25 Hz for 10 min to disrupt the plant tissues. QIAGEN (Mississauga, Ont.) DNeasy Plant Mini Extraction Kits were used to extract 50 μL samples of DNA from stem tissues, roots and tuber samples. Mo Bio (Solana Beach, Calif.) UltraClean Soil DNA Isolation Kits were used to extract 100 μL samples of DNA from 0.25 g soil samples. All extracted DNA samples were stored at -20°C.

3.1.2.5 PCR techniques

Using the system for evaluating Verticillium biomass in colonized plants described by Robb et al. (1993) as a guideline, PCR was performed using Verticillium primers that are specific to each species. Each sample was examined for the species that it was inoculated with and the control group was examined for both species. DNA amplification with species specific primers (Robb et al. 1993) was performed on all samples in 25.0 μL reactions. PCR reactions contained 10.9 μL PCR H_2O, 2.5 μL BSA (bovine serum albumin), 2.5 μL 10X PCR buffer, 2.0 μL (25 mM) $MgCl_2$, 1.0 μL (1.25 mM) of each nucleotide, 1.0 μL (25 μM) of each primer, 0.1 μL (5 U/μL) AmpliTaq polymerase and 1.0 μL of template DNA. Reactions were placed in a heated top Applied

Biosystems (Toronto, Ont.) GeneAmp PCR System 2700 thermocycler and began with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation, amplification and extension at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min respectively. A final extension step occurred at 72°C for 10 min. Aliquots of 12.5 μL of the PCR product were run through 1.2% agarose gels containing ethidium bromide.

The resulting bands were examined and ranked according to visual intensity, which is related to the concentration of the pathogen DNA, using a series of standards with known concentration. Each sample was assigned a relative population level (RPL) value between 0 and 3 based on the standards. The PCR results from these experiments indicate if the pathogen is present in the soil or a particular part of the plant at a particular time. Band intensity, time and location data was combined to describe the host-pathogen relationship. Visual ratings of both experiments were recorded on a weekly basis. Visual ratings are the recorded number of plants showing visible signs of foliar wilt in each plot. Statistical analysis consisted of ANOVA F-probability tests, with a least significant difference (LSD) value at 5% for assessment of significant differences among the RPLs of each pathogen, relative area under pathogen population curves (RAUPPC) and correlations to percentage of foliar disease. Statistical software packages, Genstat 7.1 and Minitab11.0 were used for these analyses.

3.1.2.6 DNA band intensity assessments based on quantified standards

Due to the large number of sample to be processed, a quick and reliable method was needed for assigning a quantitative value to band intensity. Because DNA band intensity is related to sample concentration, comparing sample intensities to standards of

known concentration is less subjective than visual assessments alone, and more cost and time effective than traditional quantification methods. A competitive PCR-based system for quantifying fungal DNA (Hu et al. 1993; Mahuku et al. 1995) was used to quantify Verticillium DNA samples of high concentration. A serial dilution of these samples was created to generate high, medium and low intensity DNA bands on a 1.2% agarose gel. For every sample tested, the three band intensities consistently remained within three different ranges of concentration. These samples became the standards against which all other samples would be compared. Traditional PCR was performed on all samples and every time a sample set was prepared for the thermocycler, three standards, each of a different concentration and a negative control were included. A ratings system, based on band intensity, was devised using these standards. This rating system is quick, reliable, and if needed, an estimated concentration could be assigned to the VT and VA2 inoculated samples. Table A.1.1 shows the concentration ranges of the different band intensities and the corresponding RPL and, Figure A.1.1 is a gel photo showing the DNA band intensities of the standards.

3.1.3 Results

All plants inoculated with VT or VA2 had few symptoms of foliar disease. Wilt symptoms were restricted to the lower half of the plants and at most, affected only 25% of the plant. *Verticillium tricorpus* RPLs were generally low, in both greenhouse and field studies, while VA2 RPLs were medium in the lower portions of the plants. Correlations between RAUPPC and foliar disease could not be calculated for the greenhouse experiment because no foliar disease was observed. For the field experiment,

correlations were generally close to 1.00. Tubers were collected only on the last two sampling dates.

3.1.3.1 *Verticillium tricorpus* RPLs and foliar disease

In the greenhouse experiment, conducted in 2003, RPLs of VT inoculated plants were medium in the soils with no significant increase or decrease over time (Table 3.1). In the roots, RPLs significantly increased from medium to high by sampling date 2 (48 hours) and then significantly decreased to medium levels by the last sampling date. The pathogen entered all parts of the stems by sampling date 2 at low levels and remained in these parts until the last sampling date with no significant increase. The pathogen entered the tubers at low RPLs and did not significantly increase over time. No foliar disease was observed in the greenhouse.

In the field experiment, conducted in 2003, RPLs of VT in the soils were medium and did not significantly increase or decrease over time (Table 3.1). The pathogen entered the roots at medium-low RPLs and significantly increased to high levels by sampling date 3. RPLs of the pathogen significantly decreased in the roots and became undetectable after sampling date 3. In all stem parts, RPLs significantly increased from not detectable to low levels between sampling dates 1 and 2. RPLs remained low for the rest of the growing season with a significant decrease in the top stems over time. In the tubers, RPLs were low with no significant changes. Only 7% of inoculated plants showed foliar disease.

Table 3.1 Mean relative population levels over five sampling dates, within *Verticillium tricorpus* inoculated soil and various plant parts, in relation to foliar disease.

		Greenhouse 2003		Field 2003	
Sample Type	SD	% FD	RPL	% FD	RPL
soil	1	0	2.8	0	2.0
	2	0	2.0	0	1.5
	3	0	1.8	0	1.8
	4	0	2.8	0	2.2
	5	0	1.8	7	2.5
FProb./LSD(P<0.05)		**NA**	**0.41/NS**	**0.33/NS**	**0.44/NS**
root	1	0	2.0 bc	0	1.8 b
	2	0	2.8 ab	0	1.8 b
	3	0	2.5 b	0	3.0 a
	4	0	1.5 c	0	0.0 c
	5	0	2.0 bc	7	0.0 c
FProb./LSD(P<0.05)		**NA**	**0.05/0.62**	**0.33/NS**	**0.001/0.35**
lower stem	1	0	0.8	0	0.0 b
	2	0	0.8	0	1.0 a
	3	0	1.0	0	1.2 a
	4	0	0.5	0	1.2 a
	5	0	1.0	7	1.8 a
FProb./LSD(P<0.05)		**NA**	**0.93/NS**	**0.33/NS**	**0.05/0.82**
mid stem	1	0	0.8	0	0.0 b
	2	0	0.2	0	1.0 a
	3	0	0.2	0	0.5 ab
	4	0	0.2	0	0.8 a
	5	0	0.8	7	1.0 a
FProb./LSD(P<0.05)		**NA**	**0.76/NS**	**0.33/NS**	**0.001/0.61**
top stem	1	0	0.0	0	0.0 d
	2	0	0.2	0	1.0 a
	3	0	0.8	0	0.8 ab
	4	0	0.0	0	0.5 bc
	5	0	0.8	7	0.2 cd
FProb./LSD(P<0.05)		**NA**	**0.14/NS**	**0.33/NS**	**0.03/0.45**
tuber stem-end	1	0	*	0	*
	2	0	*	0	*
	3	0	*	0	*
	4	0	0.5	0	0.5
	5	0	1.0	7	0.8
FProb./LSD(P<0.05)		**NA**	**0.67/NS**	**0.33/NS**	**0.54/NS**

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Sample Type	SD	Greenhouse 2003		Field 2003	
		% FD	RPL	% FD	RPL
tuber eye-end	1	0	*	0	*
	2	0	*	0	*
	3	0	*	0	*
	4	0	0.2	0	0.2
	5	0	0.2	7	0.0
Fprob./LSD(P<0.05)		**NA**	**1.00/NS**	**0.33/NS**	**0.36/NS**

Note: SD = sample date, which is the number of days post-inoculation for the greenhouse (1, 3, 10, 20, 37) and field (1, 6, 15, 28, 63) studies; FD = percent foliar disease; RPL = relative population level; * = no tubers; NA = not available; NS = not significant; FProb. = F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at P<0.05.

3.1.3.2 *Verticillium tricorpus* RAUPPC and correlations to foliar disease

Correlations were computed to discover if there is a relationship between visual ratings of foliar disease symptoms and pathogen population assessments (RAUPPC) obtained from PCR. For plant disease development assessments, visual ratings of foliar disease symptoms were recorded. RPL values were plotted over number of days post inoculation, to produce RAUPPC for each of the three experiments and seven sample types. The RAUPPC produced values that are representative of pathogen population changes over the entire growing season. Each of the experiments had a set of different sampling times and RAUPPC take this into consideration. The RAUPPC values for the greenhouse and field experiments are displayed in Table 3.2 along with correlation values between RAUPPC and foliar disease.

In the greenhouse experiment, correlations were not calculated because no visual disease symptoms were seen (Table 3.2).

In the field experiment, correlation values for soils, lower stems, and mid stem samples were all over 0.97. The roots had a correlation of -0.83 and the top stems correlation value was 0.80. No correlations were calculated for the tubers because only one data point was generated by the RAUPPC.

Table 3.2 Relative area under pathogen population curve values and their correlation to foliar disease for *V. tricorpus* and *V. albo-atrum* 'group 2' inoculated potato plants.

		Greenhouse 2003		Field 2003	
	Sample Type	RAUDPC	R^2	RAUDPC	R^2
V. tricorpus	soil	78.6	NA	121.5	0.99
	root	72.9	NA	49.6	-0.83
	lower stem	27.9	NA	81.4	0.97
	middle stem	13.8	NA	48.0	0.98
	top stem	13.9	NA	31.6	0.80
	tuber stem-end	12.8	NA	21.9	NA
	tuber eye-end	4.2	NA	4.4	NA
V. albo-atrum 'group 2'	soil	75.2	NA	86.8	0.98
	roots	96.9	NA	49.7	0.06
	lower stem	50.0	NA	159.1	0.96
	middle stem	21.6	NA	88.4	0.94
	top stem	11.8	NA	40.8	0.97
	tuber stem-end	4.2	NA	13.1	NA
	tuber eye-end	0.0	NA	0.0	NA

Note: RAUPPC = relative area under pathogen population curve. RAUPPC values are determined from RPLs plotted over time. The resulting area under the curve provides a value which is representative of the pathogen levels over the entire growing season and can be correlated to foliar disease; R^2 = correlation; NA = not available. No correlations were calculated for the tubers, which were collected on the last two sampling dates, because only one data point was generated by the RAUPPC.

3.1.3.3 *Verticillium albo-atrum* 'group 2' RPLs and foliar disease

In the greenhouse experiment of 2003, RPLs of VA2 in the soils were medium with no significant increase or decrease over time (Table 3.3). In the roots, RPLs significantly increased to high by the last sampling date. The pathogen entered all stem parts at low RPLs and did not significantly increase or decrease over time. RPLs in the tuber stem-ends were low with no changes over time and the pathogen was not detected in the tuber eye-ends. No foliar disease was observed in this experiment.

In the field experiment of 2003, RPLs of VA2 in the soils were medium to low with no significant changes over time (Table 3.3). In the roots, RPLs significantly increased to medium by sampling date 3 and then significantly decreased to undetectable levels by the last sampling date. RPLs significantly increased from undetectable to medium levels in all stem parts by sampling date 2. In the lower stems, RPLs remained medium to high for the remainder of the growing season and in the mid stems, RPLs remained medium to low. In the top stems, the pathogen was undetectable on sampling date 3 and then showed up at low levels by sampling date 4. *Verticillium albo-atrum* 'group 2' was present in the tuber stem-ends at low RPLs with no changes over time and it was not detected in the tuber eye-ends. The amount of foliar disease observed in the field was 10%.

Table 3.3 Mean relative population levels over five sampling dates, within *Verticillium albo-atrum* 'group 2' inoculated soil and various plant parts, in relation to foliar disease.

Sample Type	SD	Greenhouse 2003		Field 2003	
		% FD	RPL	% FD	RPL
soil	1	0	2.2	0	0.8
	2	0	2.0	0	1.5
	3	0	2.2	0	1.2
	4	0	1.8	0	1.2
	5	0	2.5	10	1.8
Fprob./LSD(P<0.05)		**NA**	**0.78/NS**	**0.34/NS**	**0.15/NS**
roots	1	0	3.0 a	0	0.0 c
	2	0	1.0 b	0	1.0 b
	3	0	2.8 a	0	2.2 a
	4	0	3.0 a	0	0.8 b
	5	0	3.0 a	10	0.0 c
Fprob./LSD(P<0.05)		**NA**	**0.001/0.47**	**0.34/NS**	**0.001/0.78**
lower stem	1	0	0.8	0	0.0 b
	2	0	2.0	0	2.5 a
	3	0	1.0	0	2.5 a
	4	0	1.2	0	2.8 a
	5	0	1.8	10	2.8 a
Fprob./LSD(P<0.05)		**NA**	**0.15/NS**	**0.34/NS**	**0.001/0.67**
mid stem	1	0	1.0	0	0.0 b
	2	0	1.0	0	2.0 a
	3	0	0.5	0	1.5 a
	4	0	0.2	0	1.5 a
	5	0	1.0	10	1.2 a
Fprob./LSD(P<0.05)		**NA**	**0.45/NS**	**0.34/NS**	**0.02/0.85**
top stem	1	0	1.0	0	0.0 c
	2	0	0.5	0	2.0 a
	3	0	0.5	0	0.0 c
	4	0	0.0	0	0.8 b
	5	0	0.5	10	0.5 bc
Fprob./LSD(P<0.05)		**NA**	**0.25/NS**	**0.34/NS**	**0.001/0.69**
tuber stem-end	1	0	*	0	*
	2	0	*	0	*
	3	0	*	0	*
	4	0	0.2	0	0.2
	5	0	0.2	10	0.5
Fprob./LSD(P<0.05)		**NA**	**1.00/NS**	**0.34/NS**	**0.54/NS**

Cont' next page

Sample Type	SD	Greenhouse 2003		Field 2003	
		% FD	RPL	% FD	RPL
tuber eye-end	1	0	*	0	*
	2	0	*	0	*
	3	0	*	0	*
	4	0	0.0	0	0.0
	5	0	0.0	10	0.0
FProb./LSD(P<0.05)		**NA**	**NS**	**0.34/NS**	**NS**

Note: SD = sample date, which is the number of days post-inoculation for the greenhouse (1, 3, 10, 20, 37) and field (1, 6, 15, 28, 63) studies; FD = percent foliar disease; RPL = relative population level; * = no tubers; NA = not available; NS = not significant; FProb. = F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at $P<0.05$.

3.1.3.4 *Verticillium albo-atrum* 'group 2' RAUPPC and correlations to foliar disease

In the greenhouse experiment, RAUPPC values show that RPLs were highest in the roots. No correlations were calculated for this experiment, due to the lack of foliar wilt (Table 3.2).

In the field experiment, RAUPPC values showed that the highest RPLs are found in the lower stems and as the pathogen progressed throughout the rest of the plants, the values decreased (Table 3.2). Strong correlations of more than 0.94 were calculated for the soils and stem samples. For the root samples, a weak correlation of 0.06 was found and no correlations were calculated for the tuber samples.

3.1.4 Discussion

3.1.4.1 *Verticillium tricorpus*

Verticillium tricorpus RPLs were generally medium (refer to Table A.1.1) in soil and root samples and low in stem samples in both greenhouse and field experiments. In the greenhouse and field, the RPLs in the roots increased significantly by the second and third sampling dates respectively. This increase was followed by a significant decrease in both cases as seen in Table 3.1. This trend supports other evidence that VT is a weak plant pathogen and proliferates in the soil as a saprophyte (Mahuku et al. 1999). In the field experiment, the pathogen was undetectable in the roots towards the latter half of the growing season. Perhaps VT RPLs were reduced because of a possible antagonistic effect. Leben et al. (1987) stated that some bacterial strains have an antagonistic effect on *Verticillium* species in the roots of potato plants. Also in the field experiment, the

results show that RAUPPC values decreased by more than half from the soils to the roots as seen in Table 3.2. This was due to the decrease in RPLs in the roots on sampling date 4. The correlation value (-0.83) in the roots was examined closer because the negative correlation suggests that there is a negative relationship between foliar disease and RPL or as foliar disease symptoms increased, RPLs decreased. This does not follow the trend set by the other soil and plant parts which all had positive correlations. Because RPLs were generally low and VT is known to be a weak pathogen (Heinz and Platt 2000), limited foliar disease symptoms perhaps should be expected and in fact none were seen in the greenhouse, and only 7% of the plants in the field showed wilt symptoms which covered approximately 25% of the plant.

From these experiments it can be concluded that VT can infect and colonize actively growing plants. Although all sample types contained some amount of the pathogen, RPLs and foliar disease symptoms were generally low, supporting the evidence stated by Mahuku et al (1999) that VT is a weak pathogen of potato and proliferates in the soil.

3.1.4.2 *Verticillium albo-atrum 'group 2'*

Verticillium albo-atrum 'group 2' RPLs were medium to high (refer to Table A.1.1) in the soil and root samples in the greenhouse experiment and medium to low in the field experiment. Stem and tuber samples in both experiments generally had low RPLs, except for the lower stems in the field experiment which had medium to high RPLs as seen in Table 3.3. In the greenhouse experiment, RPLs in the roots significantly

decreased from high to low levels on sampling date 2. Although greenhouse soils were autoclaved and free of micro-organisms, an antagonistic relationship may still have occurred. The seed tubers may have carried traces of bacteria or other contaminants that briefly affected the growth of VA2. Tjamos et al. (2004) showed that seed tubers dusted with bacteria show a significant reduction in symptom development in heavily Verticillium infested potato fields. By sampling date 3, RPLs had increased to high levels again. In the field experiment RPLs in the roots significantly decreased after sampling date 2. This situation is similar to that seen in VT inoculated experiments and may have happened because of a similar antagonistic relationship. In the greenhouse, RAUPPC values are highest in the roots. In the field, RAUPPC values are highest in the lower stems as seen in Table 3.2. Correlation values in the field show a strong relationship between foliar disease and RAUPPC in all sample types except for the roots. The poor correlation value for the roots was caused by a significant increase in RPLs by sampling date 3, followed by a significant decrease. RPLs in the greenhouse experiment were higher than in the field experiment. Perhaps other micro-organisms in the soil were out-competing VA2 for space, causing the pathogen to have trouble establishing itself in the plants.

Similar to the findings revealed by Mahuku et al. (1999), VA2 was readily detected in soil samples using PCR detection techniques. However, the pathogen was also detected in all stem samples, which was not the case in the study conducted by Mahuku et al. (1999). Robb et al. (1993) found that VA2 was as virulent as VA1. The results from the VA2 experiments were compared with results from a similar study

conducted with strong pathogens VD and VA1 (see Chapter 1). RPLs of VA2 did not differ significantly from 'group 1' in all sample types (refer to Tables A.1.2 and A.1.4). Even though RPLs were generally similar, foliar disease symptoms were significantly more severe in 'group 1' inoculated plants than in 'group 2' inoculated plants. In conclusion, VA2 may be able to establish itself in potato plant at similar RPLs as VA1, however, 'group 2' does not appear to be as virulent as 'group 1'.

3.1.4.3 Comparison of *Verticillium tricorpus* and *Verticillium albo-atrum* 'group 2'

In the greenhouse experiment, RPLs did not differ significantly between VA2 and VT (Table A.1.2). In the field experiment, RPLs differed only in the soils between the two pathogens. RPLs were significantly higher in VT inoculated soils than in VA2 inoculated soils (Table A.1.4). *Verticillium tricorpus* is known to proliferate in the soil as a saprophyte (Mahuku et al 1999), which may explain why RPLs of this pathogen were higher than VA2. Foliar disease symptoms also did not differ significantly between the two pathogens.

Additional studies involving various strains of VA2 are needed to clarify discrepancies concerning the pathogen's virulence. Studies conducted under various environmental conditional would also be necessary as the effects of such conditions, on the growth of these species, are unknown (Heinz and Platt 2000). *Verticillium tricorpus* has been recommended as a biological control agent against VD because it is weakly pathogenic (Davis et al. 2000). Further studies involving VT and VA2, which also proved to be a weak pathogen in this case, should be conducted along with VD and VA1

to determine if any suppressions in RPLs of these virulent strains occur. It is possible that biological control agents may reduce or eliminate the need for fumigants and become a lasting approach for suppression of Verticillium wilt of potato (Davis 1985).

4 CHAPTER FOUR

4.1 The effects of combined inoculations of various *Verticillium* species on verticillium wilt disease development in potato

4.1.1 Introduction

Verticillium wilt affects several economically important crops, including potato, cotton and tomato (Dobinson 1995; Jeger et al. 1996; Schnathorst 1981). In potato production areas, declining tuber yields and diseased tubers are major problems caused by soilborne *Verticillium* species. Therefore, management strategies are aimed at reducing populations of these pathogens in the soil (Powelson and Rowe 1993). There are several management strategies available including soil fumigation, crop rotation, host resistance, planting disease free seed and soil solarization. Although no single practice will provide complete control of Verticillium wilt (Davis 1985; Powelson and Rowe 1993), chemical treatments tend to be the most effective and widely used (Davis 1996; Dobinson 1995). Due to high costs and adverse environmental concerns, other efficient and less harmful methods of control should be further explored (Keinath 1991; Powelson and Rowe 1993). The effects of green manures as a biological control for Verticillium wilt of potato has been studied and shows significant results in pathogen reduction (Davis et al. 1996).

Biological control techniques for managing Verticillium wilt have been tested with varied success (Powelson and Rowe 1993). Although research has not yet led to commercially available products (Jeger et al. 1996), it is thought that biological control techniques may provide an environmentally safe and economical approach to controlling Verticillium wilt disease (Davis 1985; Keinath et al. 1991). A biological control agent

might suppress a pathogen by physical exclusion, competition for nutrients or by direct antagonism. It is also possible for plant-pathogenic fungi to infect closely related species as these agents have diverse host-pathogen relationships (Lucus 1998). Therefore, it is possible that weaker *Verticillium species* may be able to control the more aggressive *Verticillium species*. *Verticillium tricorpus* (VT) has been recommended for biological control against *V. dahliae* (VD) (Davis et al. 2000). In potato, VT has been described as a weak pathogen with intermediate saprophytic ability (Isaac 1953; Mahuku et al. 1999). *Verticillium dahliae* and *V. albo-atrum* 'group 1' (VA1) are the primary species that cause Verticillium wilt and they are often found in association with VT. Studies on the population dynamics of these species, in combination, may reveal more information about the competitiveness and possible moderating effects of VT on more aggressive species such as VD and VA1 (Heinz and Platt 2000). A new Verticillium isolate, *Verticillium albo-atrum* 'group 2' (VA2) described by Robb et al. (1993), is thought to be similar to VT in that it is also a weak potato pathogen (Mahuku et al. 1999). A previous study revealed that VA2 was able to colonize all plant parts without causing significant foliar wilt (see Chapter 3). It is possible that VA2 may also have some moderating effects on more aggressive *Verticillium* species.

In this study, potato plants were inoculated with various combinations of an aggressive pathogen and a weaker pathogen. Polymerase chain reaction (PCR) techniques were used to assess relative population levels (RPLs) to determine if the weaker pathogens had any affect on populations of aggressive pathogens.

4.1.2 Materials and Methods

4.1.2.1 Greenhouse experiment

A greenhouse experiment was conducted in 2003 at the Crops and Livestock Research Center in Charlottetown, Prince Edward Island. The susceptible potato c.v. Superior was used. Seed tubers (Elite 3) were planted into pots, 15 cm in diameter, containing autoclaved soil so that background pathogens were eliminated. In a temperature controlled greenhouse set to approximately 24°C, the pots were placed into five rows and each row was divided into four replicates with five plants per replicate. A Randomized Complete Block Design was followed. The plants were watered on a regular basis and fertilized with all purpose fertilizer (20-20-20). Approximately 30 days after planting, pots in each row were randomly selected and inoculated with one of the following pathogen combinations: VA1+VT, VA1+VA2, VD+VT, VD+VA2. The control group was inoculated with water. On predetermined destructive sampling dates, one plant per replicate was sampled. The sampling dates were recorded as the number of days post inoculation. For the greenhouse experiment, sampling dates occurred 1, 3, 10, 20 and 37 days post inoculation.

4.1.2.2 Field experiment

A field experiment was conducted at the Harrington Research Farm in Prince Edward Island in 2003 and established in the same manner as the greenhouse experiment except that the sample size was three times larger and non-sterile soil was used. Using the susceptible c.v. Superior, seed tubers (Elite 3) were planted into nine rows and divided into four replicates with fifteen plants per replicate following a Randomized

Complete Block Design. Approximately 30 days after planting, four out of the nine rows were inoculated with one of the following pathogen combinations: VA1+VT, VA1+VA2, VD+VT, VD+VA2. Another four rows were inoculated with a weak pathogen followed by an aggressive pathogen four days later. The combinations were as follows: VA2+4dVA1, VT+4dVA1, VA2+4dVD, VT+4dVD. The last row, the control group, was inoculated with water. On predetermined destructive sampling dates, three plants per replicate were sampled. For the field experiment, sampling dates occurred 1, 6, 15, 28 and 63 days post inoculation.

4.1.2.3 Inoculum and inoculation

The inoculum was prepared from fungal cultures grown on plates containing potato dextrose agar (PDA). Each pathogen inoculum suspension consisted of a combination of three Prince Edward Island isolates of that species to ensure that the pathogenicity of the inoculum was optimal. Plates were emptied into a blender and the agar/culture mixture was blended with distilled water until smooth. Spore counts were performed and the mixture was diluted, using distilled water, to a concentration of approximately 12 x 10^6 spores/mL. Inoculation via the soil involved digging a small hole with a trowel, 7 cm deep, on either side of the plant so that the roots were disturbed/damaged. For same day co-inoculations, a volume of 25 mL of one pathogen inoculum was divided among the two holes followed by another 25 mL of a different pathogen inoculum, and the holes were refilled. For inoculations that were delayed four days, the weaker pathogen was added in the same manner as above and four days later,

the holes were dug again so that the aggressive pathogen could be added. The control group was inoculated with 50 mL of water only.

4.1.2.4 Sample preparation

On destructive sampling dates, one potted plant from each replicate for the greenhouse experiment and three field grown plants from each replicate for the field experiment were randomly selected and separated into six parts: roots, lower stem, mid stem, top stem, tuber stem-end and tuber eye-end. Soil samples were taken from the hole that the plant was pulled out of and care was taken to avoid collecting any visible plant material. Leaves were removed from the stems and discarded. Soil, root and stem samples were stored separately in sealed, plastic bags. Tubers were stored in paper bags. The stem tissue and roots were kept at -20°C and the tubers and soils were kept at 4°C until DNA extractions were performed.

0.75 g frozen cross section samples from the lower, mid, and top stem areas were placed into 1.5 mL tubes with tungsten carbide beads. 0.75 g samples from tubers were collected using a cork borer (#3) and placed into 1.5 mL tubes with beads. Roots were washed, chopped and 0.20 g were also placed in 1.5 mL tubes with beads. The tubes were then placed in an Applied Biosystems (Toronto, Ont.) Mixer Mill MM 300 at 25 Hz for 10 min to disrupt the plant tissues. QIAGEN (Mississauga, Ont.) DNeasy Plant Mini Extraction Kits were used to extract 50 μL samples of DNA from stem tissues, roots and tuber samples. Mo Bio (Solana Beach, Calif.) UltraClean Soil DNA Isolation Kits were used to extract 100 μL samples of DNA from 0.25 g soil samples. All extracted DNA was stored at -20°C.

4.1.2.5 PCR techniques

Using the system for evaluating Verticillium biomass in colonized plants described by Robb et al. (1993) as a guideline, PCR was performed using Verticillium primers that are specific to each species. The primer sequences for the four species are given in a report by Robb et al. (1993). Each sample was examined for the species that it was inoculated with and the control group was examined for both species. DNA amplification with species specific primers was performed on all samples in 25.0 μL reactions. PCR reactions contained 10.9 μL PCR H_2O, 2.5 μL BSA (bovine serum albumin), 2.5 μL 10X PCR buffer, 2.0 μL (25 mM) $MgCl_2$, 1.0 μL (1.25 mM) of each nucleotide, 1.0 μL (25 μM) of each primer, 0.1 μL (5 U/μL) AmpliTaq polymerase and 1.0 μL of template DNA. Reactions were placed in a heated top Applied Biosystems (Toronto, Ont.) GeneAmp PCR System 2700 thermocycler and began with an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation, amplification and extension at 94°C for 1 min, 60°C for 1 min and 72°C for 2 min respectively. A final extension step occurred at 72°C for 10 min. Aliquots of 12.5 μL of the PCR product were run through 1.2% agarose gels containing ethidium bromide.

The resulting bands were examined and ranked according to visual intensity, which is related to the concentration of the pathogen DNA, using a series of standards with known concentration. Each sample was assigned a relative population level (RPL) value between 0 and 3 based on the standards. The PCR results from these experiments were used to determine if the pathogen was present in a particular part of the plant at a particular time. Band intensity, time and location data was combined to describe the

host-pathogen relationship. The number of plants showing visible signs of foliar wilt in each plot was recorded on a weekly basis in all experiments.

4.1.2.6 Data analysis

Statistical analysis consisted of ANOVA F-probability tests, with a least significant difference (LSD) value calculated at $P<0.05$ for assessment of significant differences among the RPLs of each pathogen and foliar disease incidence. Genstat version 7.1 was the statistical software package used for these analyses. Tables A.1.2 – A.1.4 are the ANOVA tables for all statistical analysis of variance tests conducted for this thesis.

4.1.2.7 DNA band intensity assessments based on quantified standards

Due to the large number of samples to be processed, a quick method was needed for assigning a quantitative value to band intensity. Since DNA band intensity is related to product concentration, comparing sample intensities to standards of known concentrations is less subjective than visual assessments alone, and more cost and time effective than traditional quantification methods. A competitive PCR-based system for quantifying fungal DNA (Hu et al. 1993; Mahuku et al. 1995, Mahuku and Platt 2002) was used to quantify Verticillium DNA samples of high concentration. A serial dilution of these samples was created to generate high, medium and low intensity DNA bands on a 1.2% agarose gel. For every sample tested, the three band intensities consistently remained within three different ranges of concentration. These samples became the standards against which all other samples would be compared. PCR was performed on

all samples and every time a sample set was prepared for the thermocycler, a series of standards was included. Three standards, each of a different concentration and a negative control were used to devise a ratings system based on band intensity. This rating system is quick, reliable and if needed, an estimated concentration could be assigned to the Verticillium inoculated samples. Table A.1.1 shows the estimated concentration ranges of the different band intensities and the corresponding RPL and, Figure A.1.1 is a gel photo showing the DNA band intensities of the standards.

4.1.3 Results

4.1.3.1 Pathogen interactions from same day inoculations

For plants inoculated with the combination VA1+VA2, visual wilt symptoms in the field were recorded at 77% while 37 % of the greenhouse plants showed visual signs of wilt (Table 4.1). In general, VA1 RPLs increased as the sampling dates increased while VA2 RPLs decreased over time and as it progressed throughout the plants. In the greenhouse, the results showed that VA2 RPLs were significantly lower than VA1 RPLs in the roots and all areas of the stems (Table 4.2). In all plant parts, VA1 RPLs increased as time increased while in the soil, both VA1 and VA2 RPLs decreased from high levels on sampling date 1 to medium-low levels on the last sampling date. In the field study, VA2 RPLs were slightly lower than VA1 populations in the soil and mid stem. In all other plant parts, VA1 was present at low levels except for the lower stem where VA1 reached medium levels on the last two sampling dates.

As shown in Table 4.1, for the combination VA1+VT, 87% of the plants in the field and 50% of the plants in the greenhouse had visual wilt symptoms. In both the

Table 4.1 Foliar disease symptoms expressed following inoculation of potato plants with various combinations of *Verticillium species.*

		% Foliar Disease		
		Greenhouse	Field	Field
Pathogen Interactions		**VA1+VA2**	**VA1+VA2**	**VA2+4dVA1**
SD	1	0a	0a	0a
	2	0a	0a	0a
	3	0a	0a	0a
	4	0a	0a	0a
	5	37b	77b	35b
(FProb./LSD)		(0.001/0.51)	(0.001/0.76)	(0.001/0.89)
Pathogen Interactions		**VA1+VT**	**VA1+VT**	**VT+4dVA1**
SD	1	0a	0a	0a
	2	0a	0a	0a
	3	0a	0a	0a
	4	0a	0a	0a
	5	50b	87b	26b
(FProb./LSD)		(0.001/0.87)	(0.001/0.63)	(0.001/0.72)
Pathogen Interactions		**VD+VA2**	**VD+VA2**	**VA2+4dVD**
SD	1	0	0a	0a
	2	0	0a	0a
	3	0	0a	0a
	4	0	0a	0a
	5	7	52b	48b
(FProb./LSD)		(0.23/NS)	(0.001/0.78)	(0.001/0.93)
Pathogen Interactions		**VD+VT**	**VD+VT**	**VT+4dVD**
SD	1	0	0a	0a
	2	0	0a	0a
	3	0	0a	0a
	4	0	0a	0a
	5	7	50b	45b
(FProb./LSD)		(0.34/NS)	(0.001/0.76)	(0.001/0.83)

Note: SD = sampling date; % FD = percent foliar disease; FProb.= F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at $P<0.05$.

Table 4.2 Mean relative population levels of *V. albo-atrum* 'group 1' (VA1) and *V. albo-atrum* 'group 2' (VA2) in soil and plant tissue samples over five sampling dates, following inoculation with the pathogens in various combinations.

		Pathogen Combination Inoculations					
		VA1+VA2				VA2+4dVA1	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VA1	VA2	VA1	VA2	VA1	VA2
	1	3.0	3.0	0.5ab	0.0a	2.0	1.2
	2	2.2	2.5	1.5cd	0.0a	2.2	1.2
soil	3	2.0	1.8	2.2de	1.0bc	1.8	2.2
	4	1.2	2.0	3.0f	2.0d	3.0	2.2
	5	1.5	1.5	3.0f	2.5def	2.8	2.2
(Mean RPL)		(1.98)	(2.16)	(2.04)	(1.10)	(2.36)	(1.80)
FProb./LSD		**0.69/NS**		**0.007/0.72**		**0.21/NS**	
	1	2.2cd	2.0c	0.0	0.0	0.0	0.0
	2	1.2b	0.5a	0.0	0.0	1.0	1.5
roots	3	2.8de	1.8bc	1.2	0.2	1.0	1.5
	4	2.8de	2.0c	0.5	2.0	0.2	1.0
	5	3.0e	2.0c	1.0	0.2	1.0	1.8
(Mean RPL)		(2.40)	(1.66)	(0.54)	(0.48)	(0.64)	(1.16)
FProb./LSD		**0.02/0.62**		**0.31/NS**		**0.14/NS**	
	1	2.0cd	1.5b	0.0	0.0	0.0a	0.0a
	2	1.0ab	1.0ab	0.5	0.8	0.0a	3.0b
lower stem	3	0.5a	0.8ab	0.8	0.5	0.5ab	3.0b
	4	2.2d	0.8ab	2.0	0.8	1.5c	3.0b
	5	3.0e	0.5a	2.5	1.0	1.2bc	3.0b
(Mean RPL)		(1.74)	(0.92)	(1.16)	(0.62)	(0.64)	(2.40)
FProb./LSD		**0.008/0.62**		**0.09/NS**		**0.001/0.74**	
	1	1.5c	1.5c	0.0a	0.0a	0.0a	0.0a
	2	1.0bc	1.2c	0.0a	0.0a	0.0a	1.2bc
mid stem	3	0.0a	0.0a	0.8b	0.5ab	0.0a	0.8b
	4	2.8d	0.5ab	1.8c	0.0a	1.2bc	1.8c
	5	3.0d	0.5ab	2.2c	0.5ab	0.8b	3.0d
(Mean RPL)		(1.66)	(0.74)	(0.96)	(0.20)	(0.40)	(1.36)
FProb./LSD		**0.01/0.67**		**0.009/0.55**		**0.003/0.60**	
	1	1.0bc	1.2c	0.0	0.0	0.0a	0.0a
	2	1.0bc	0.5ab	0.0	0.0	0.0a	1.8d
top stem	3	0.5ab	0.0a	0.0	0.5	0.0a	1.0bc
	4	1.2c	0.2a	0.8	0.2	1.5cd	1.8d
	5	2.8d	0.2a	0.2	0.2	0.5ab	2.8e
(Mean RPL)		(2.20)	(0.42)	(0.20)	(0.18)	(0.40)	(1.48)
FProb./LSD		**0.003/0.54**		**0.15/NS**		**0.001/0.58**	

Cont' next page

		VA1+VA2				VA2+4dVA1	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VA1	VA2	VA1	VA2	VA1	VA2
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber stem-end	3	*	*	*	*	*	*
	4	0.0	0.0	0.2	0.2	0.2	0.5
	5	0.0	0.0	0.5	0.0	0.8	0.8
(Mean RPL)		(0.00)	(0.00)	(0.35)	(0.10)	(0.50)	(0.65)
FProb./LSD		**NA**		**0.28/NS**		**0.70/NS**	
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber eye-end	3	*	*	*	*	*	*
	4	0.2	1.0	0.2	0.0	0.0	0.2
	5	1.2	1.2	0.5	0.0	0.0	0.2
(Mean RPL)		(0.70)	(1.10)	(0.35)	(0.00)	(0.00)	(0.20)
FProb./LSD		**0.08/NS**		**0.06/NS**		**0.15/NS**	

Note: SD = sample date, which is the number of days post inoculation for the greenhouse (1, 3, 10, 20 and 37) and field (1, 6, 15, 28 and 63) studies; RPL = relative population level; VA1+VA2 = coinoculation of the pathogens; VA2+4dVA1 = inoculation with VA1 four days after VA2 inoculation; * = no tubers; NA = not available; NS = not significant; FProb. = F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at $P<0.05$. The F probability values indicate significant differences between the two pathogens.

greenhouse and the field, RPLs of both pathogens increased as the sampling dates increased and decreased as each pathogen progressed throughout the plants. In the greenhouse, RPLs of VT in the combination VA1+VT were significantly lower than VA1 levels in the roots, stems and tuber stem-end (Table 4.3). High RPLs were reached by VA1 in the soil, roots and stems by the last sampling date. Although VT maintained high levels in the soil, RPLs in the stems decreased over time until the pathogen was not detected. In the field, RPLs of both pathogens did not differ significantly except for in the roots where VT levels were lower than VA1.

In the combination VD+VA2, 52% of the plants in the field study had visual wilt symptoms while only 7% of the plants in the greenhouse were wilted (Table 4.1). In both studies, RPLs of both pathogens decreased as the pathogens progressed throughout the plants. In the field study, RPLs of VD and VA2 increased over time where as RPLs in the greenhouse were irregular with no discernable pattern. Table 4.4 shows that plants inoculated with the combination VD+VA2 in the greenhouse, had RPLs of VA2 that were significantly lower than VD levels in all stem parts. In the stems, VD levels increased to medium levels by the sampling date 2 and then decreased to low levels by the last sampling date. Levels of VA2 in the stems remained low throughout the entire sampling period. In the field, RPLs of VA2 were significantly lower in all plant parts except for the mid and top stem, which were lower but not significantly. In the mid and top stem, VD RPLs, like VA2, were medium-low for the majority of the sampling period. On the last sampling date, VD reached high RPLs in all sample types, except for the tubers.

Table 4.3 Mean relative population levels of *V. albo-atrum* 'group 1' (VA1) and *V. tricorpus* (VT) in soil and plant tissue samples over five sampling dates, following inoculation with the pathogens in various combinations.

		Pathogen Combination Inoculations					
		VA1+VT				VT+4dVA1	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VA1	VT	VA1	VT	VA1	VT
	1	3.0	2.5	0.0	0.2	1.2ab	2.2de
	2	3.0	2.8	0.2	2.2	1.2ab	1.5bc
soil	3	2.8	2.8	1.8	2.2	1.8cd	2.5e
	4	2.2	2.2	2.0	3.0	0.8a	2.0d
	5	3.0	3.0	2.2	2.2	2.0d	3.0f
(Mean RPL)		(2.80)	(2.66)	(1.24)	(1.96)	(1.40)	(2.24)
FProb./LSD		**0.51/NS**		**0.30/NS**		**0.001/0.42**	
	1	2.8c	1.0a	0.0a	0.0a	0.0a	1.5bc
	2	1.8b	1.5ab	0.8b	0.0a	0.5a	1.2b
roots	3	2.5c	1.5ab	2.0d	1.2bc	1.5bc	0.5a
	4	2.8c	1.5ab	2.8e	1.5cd	0.0a	0.5a
	5	3.0c	1.0a	3.0e	1.5cd	0.2a	1.8c
(Mean RPL)		(2.58)	(1.30)	(1.72)	(0.84)	(0.44)	(1.10)
FProb./LSD		**0.001/0.50**		**0.02/0.69**		**0.02/0.52**	
	1	1.8cd	1.8cd	0.0	0.0	0.0a	0.0a
	2	2.2d	0.8b	1.5	1.2	0.0a	1.5c
lower stem	3	1.5c	0.2a	0.5	0.8	0.0a	1.0b
	4	3.0e	0.0a	2.2	1.2	0.2a	1.8cd
	5	3.0e	0.0a	3.0	2.2	0.2a	2.0d
(Mean RPL)		(2.30)	(0.56)	(1.44)	(1.08)	(0.08)	(1.26)
FProb./LSD		**0.001/0.56**		**0.34/NS**		**0.001/0.48**	
	1	1.5b	1.2b	0.0	0.0	0.0a	0.0a
	2	2.8d	1.0b	1.2	0.8	0.0a	0.8b
mid stem	3	1.2b	0.0a	0.2	0.8	0.0a	0.0a
	4	2.2c	0.0a	1.8	0.8	0.0a	1.2c
	5	3.0d	0.0a	2.8	2.5	0.2a	1.5c
(Mean RPL)		(2.14)	(0.44)	(1.20)	(0.98)	(0.04)	(0.70)
FProb./LSD		**0.001/0.52**		**0.45/NS**		**0.001/0.38**	
	1	1.2b	1.0b	0.0	0.0	0.0a	0.0a
	2	1.2b	1.0b	1.0	1.0	0.0a	1.2b
top stem	3	0.8b	0.0a	0.0	1.0	0.0a	0.0a
	4	2.5c	0.0a	1.2	0.8	0.0a	1.0b
	5	3.0c	0.0a	2.8	2.8	0.0a	1.0b
(Mean RPL)		(1.74)	(0.40)	(1.00)	(1.12)	(0.00)	(0.64)
FProb./LSD		**0.001/0.57**		**0.76/NS**		**0.001/0.30**	

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		VA1+VT				VT+4dVA1	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VA1	VT	VA1	VT	VA1	VT
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber stem-end	3	*	*	*	*	*	*
	4	1.0b	0.0a	0.5	0.2	0.0	0.2
	5	1.2b	0.2a	1.5	0.5	0.5	1.0
(Mean RPL)		(1.10)	(0.10)	(1.00)	(0.35)	(0.25)	(0.60)
FProb./LSD		**0.002/0.35**		**0.07/NS**		**0.25/NS**	
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber eye-end	3	*	*	*	*	*	*
	4	0.0	0.0	0.2	0.0	0.0	0.2
	5	0.8	0.5	0.8	0.2	0.2	0.5
(Mean RPL)		(0.40)	(0.25)	(0.50)	(0.10)	(0.10)	(0.35)
FProb./LSD		**0.62/NS**		**0.12/NS**		**0.28/NS**	

Note: SD = sample date, which is the number of days post inoculation for the greenhouse (1, 3, 10, 20 and 37) and field (1, 6, 15, 28 and 63) studies; RPL = relative population level; VA1+VT = coinoculation of the pathogens; VT+4dVA1 = inoculation with VA1 four days after VT inoculation; * = no tubers; NA = not available; NS = not significant; FProb. = F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at $P<0.05$. The F probability values indicate significant differences between the two pathogens.

Table 4.4 Mean relative population levels of *V. dahliae* (VD) and *V. albo-atrum* 'group 2' (VA2) in soil and plant tissue samples over five sampling dates, following inoculation with the pathogens in various combinations.

		Pathogen Combination Inoculations					
		VD+VA2				VA2+4dVD	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VD	VA2	VD	VA2	VD	VA2
	1	1.2	2.2	1.8a	2.5b	1.5ab	1.2a
	2	3.0	1.8	2.8b	1.8a	3.0d	1.5ab
soil	3	1.5	1.8	3.0b	2.5b	2.5cd	1.2a
	4	2.2	1.8	3.0b	1.8a	3.0d	1.5ab
	5	1.2	2.2	3.0b	1.8a	2.2bc	2.5cd
(Mean RPL)		(1.82)	(1.96)	(2.72)	(2.08)	(2.44)	(1.58)
FProb./LSD		**0.80/NS**		**0.02/0.54**		**0.03/0.76**	
	1	3.0	3.0	0.5a	0.0a	0.8b	0.0a
	2	2.8	3.0	2.5c	0.0a	1.5cd	0.8b
roots	3	2.5	3.0	1.8b	0.0a	2.0d	1.0bc
	4	2.5	2.5	2.8c	0.2a	1.0bc	0.5ab
	5	2.8	3.0	3.0c	0.5a	1.8d	1.5cd
(Mean RPL)		(2.72)	(2.90)	(2.12)	(0.14)	(1.42)	(0.76)
FProb./LSD		**0.18/NS**		**0.001/0.51**		**0.02/0.53**	
	1	1.5cd	0.8ab	0.0a	0.0a	0.0	0.0
	2	2.2e	1.0abc	2.5de	1.2b	1.0	0.5
lower stem	3	2.0de	1.5cd	3.0e	1.5bc	0.5	1.8
	4	0.5a	0.5a	2.2cd	1.2b	1.8	2.8
	5	1.8d	1.2bc	3.0e	2.2cd	2.5	3.0
(Mean RPL)		(1.60)	(1.00)	(2.14)	(1.22)	(1.16)	(1.62)
FProb./LSD		**0.02/0.50**		**0.02/0.76**		**0.27/NS**	
	1	1.2c	0.0a	0.0	0.0	0.0	0.0
	2	1.8d	0.2ab	1.5	0.5	0.2	1.0
mid stem	3	0.5b	0.5b	2.2	1.2	0.8	0.2
	4	0.2ab	0.0a	1.8	2.0	1.2	1.0
	5	1.0c	0.2ab	3.0	1.5	2.2	2.0
(Mean RPL)		(0.94)	(0.18)	(1.70)	(1.04)	(0.88)	(0.84)
FProb./LSD		**0.001/0.42**		**0.06/NS**		**0.87/NS**	
	1	0.5b	0.2ab	0.0	0.0	0.0	0.0
	2	2.0c	0.2ab	1.5	0.2	0.2	1.0
top stem	3	0.2ab	0.0a	1.8	1.0	0.0	0.0
	4	0.2ab	0.0a	1.2	1.5	0.8	0.5
	5	0.5b	0.0a	3.0	2.0	2.0	1.5
(Mean RPL)		(0.68)	(0.08)	(1.50)	(0.94)	(0.60)	(0.60)
FProb./LSD		**0.003/0.39**		**0.11/NS**		**1.00/NS**	

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Sample Type	SD	VD+VA2				VA2+4dVD	
		Greenhouse		Field		Field	
		RPL		RPL		RPL	
		VD	VA2	VD	VA2	VD	VA2
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber stem-end	3	*	*	*	*	*	*
	4	0.0	0.0	0.8bc	0.2a	0.5	0.2
	5	0.5	1.0	1.0c	0.5ab	0.2	0.0
(Mean RPL)		(0.25)	(0.50)	(0.90)	(0.35)	(0.35)	(0.10)
FProb./LSD		**0.44/NS**		**0.04/0.30**		**0.28/NS**	
	1						
	2						
tuber eye-end	3						
	4	0.0	0.0	0.5b	0.0a	0.0	0.0
	5	0.0	0.0	0.8c	0.0a	0.0	0.0
(Mean RPL)		(0.00)	(0.00)	(0.65)	(0.00)	(0.00)	(0.00)
FProb./LSD		**NA**		**0.004/0.25**		**NA**	

Note: SD = sample date, which is the number of days post inoculation for the greenhouse (1, 3, 10, 20 and 37) and field (1, 6, 15, 28 and 63) studies; RPL = relative population level; VD+VA2 = coinoculation of the pathogens; VA2+4dVD = inoculation with VD four days after VA2 inoculation; * = no tubers; NA = not available; NS = not significant; FProb. = F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at $P<0.05$. The F probability values indicate significant differences between the two pathogens.

For plants inoculated with the combination VD+VT, visual wilt symptoms showed that only 7% of the plants in the greenhouse and 50% of the plants in the field were wilted (Table 4.1). In general, RPLs of both pathogens decreased as they progressed throughout the plants in both studies and in the greenhouse, RPLs of both pathogens decreased over time which was not the case in the field study. The greenhouse study also revealed significantly lower RPLs of VT in the roots and stems (Table 4.5). On the last two sampling dates, VT was not detected in the stems. However, RPLs of VT in the soil were significantly higher than VD levels. In the field, almost all sample types had significantly lower levels of VT.

4.1.3.2 Pathogen interactions from inoculations with a time delay

Plants inoculated with the combination VA2+4dVA1 showed 35% foliar wilt as compared to the other field combination, VA1+VA2, which affected 77% of the plants (Table 4.1). Both pathogens increased as time increased and decreased as they progressed throughout the plants. In this time delayed combination, VA1 RPLs were significantly lower in all stem parts and lower in roots and tubers, although not significantly (Table 4.2). In all stem parts, VA2 reached high RPLs by the last sampling date whereas VA1 was present at low levels only. In the soil, RPLs of VA1 were higher than VA2, but not significantly.

In the combination VT+4dVA1, only 26% of the plants showed foliar wilt symptoms (Table 4.1). RPLs decreased as both pathogens moved throughout the plants and increased as the sampling date increased. In Table 4.3, the RPLs of VA1 in the combination VT+4dVA1 were significantly lower than VT in all sample types except for

Table 4.5 Mean relative population levels of *V. dahliae* (VD) and *V. tricorpus* (VT) in soil and plant tissue samples over five sampling dates, following inoculation with the pathogens in various combinations.

		Pathogen Combination Inoculations					
		VD+VT				VT+4dVD	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VD	VT	VD	VT	VD	VT
	1	1.5ab	2.5cd	2.2b	0.0a	3.0d	1.0a
	2	2.0bc	1.8bc	3.0c	2.0b	2.5bc	2.5bc
soil	3	2.2bcd	2.8d	3.0c	2.0b	2.8cd	3.0d
	4	0.8a	2.8d	3.0c	2.0b	3.0d	2.2b
	5	1.5ab	2.5cd	3.0c	2.0b	3.0d	2.8cd
(Mean RPL)		(1.60)	(2.48)	(2.84)	(1.60)	(2.86)	(2.30)
FProb./LSD		**0.03/0.77**		**0.001/0.56**		**0.02/0.45**	
	1	3.0e	2.0c	0.5a	0.5a	0.2	2.0
	2	2.5d	1.2a	2.5cd	1.2b	2.0	0.5
roots	3	2.8de	1.5ab	2.8d	0.2a	2.2	2.2
	4	2.8de	1.8bc	2.5cd	2.0c	1.5	0.0
	5	3.0e	1.2a	3.0d	2.5cd	2.2	0.8
(Mean RPL)		(2.82)	(1.54)	(2.26)	(1.28)	(1.62)	(1.10)
FProb./LSD		**0.001/0.39**		**0.008/0.69**		**0.13/NS**	
	1	2.5g	0.2ab	0.0a	0.0a	0.8	0.0
	2	2.2fg	0.8cd	1.0cd	1.0cd	1.8	2.0
lower stem	3	2.0f	0.5bc	2.0e	0.0a	2.0	1.8
	4	1.5e	0.0a	1.5de	0.2ab	2.5	1.8
	5	1.2de	0.0a	2.8f	0.8bc	3.0	2.0
(Mean RPL)		(1.88)	(0.30)	(1.46)	(0.40)	(2.02)	(1.52)
FProb./LSD		**0.001/0.49**		**0.002/0.64**		**0.16/NS**	
	1	2.0d	0.0a	0.0a	0.0a	0.2	0.0
	2	2.0d	0.2ab	0.0a	0.5ab	0.0	0.2
mid stem	3	1.2c	0.2ab	0.8b	0.0a	0.8	0.5
	4	0.5b	0.0a	1.8c	0.5ab	1.8	2.0
	5	1.2c	0.0a	2.5d	0.5ab	2.5	1.8
(Mean RPL)		(1.38)	(0.08)	(1.02)	(0.30)	(1.06)	(0.90)
FProb./LSD		**0.001/0.47**		**0.01/0.55**		**0.67/NS**	
	1	1.5d	0.0a	0.0a	0.0a	0.0	0.0
	2	2.2e	0.5b	0.2a	0.8b	0.2	1.0
top stem	3	1.0c	0.2ab	0.0a	0.0a	0.5	0.8
	4	0.2ab	0.0a	1.2b	0.0a	0.2	1.5
	5	0.0a	0.0a	1.8c	0.2a	1.2	0.5
(Mean RPL)		(0.98)	(1.14)	(0.64)	(0.20)	(0.42)	(0.76)
FProb./LSD		**0.001/0.47**		**0.03/0.50**		**0.16/NS**	

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		VD+VT				VT+4dVD	
		Greenhouse		Field		Field	
Sample Type	SD	RPL		RPL		RPL	
		VD	VT	VD	VT	VD	VT
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber stem-end	3	*	*	*	*	*	*
	4	0.0	0.0	0.5b	0.2ab	0.2	0.2
	5	0.2	0.0	1.5c	0.0a	0.8	1.2
(Mean RPL)		(0.10)	(0.00)	(1.00)	(0.10)	(0.50)	(0.70)
FProb./LSD		**0.33/NS**		**0.01/0.40**		**0.44/NS**	
	1	*	*	*	*	*	*
	2	*	*	*	*	*	*
tuber eye-end	3	*	*	*	*	*	*
	4	0.0	0.0	0.0	0.0	0.0	0.2
	5	0.0	0.5	0.5	0.0	0.2	0.5
(Mean RPL)		(0.00)	(0.25)	(0.25)	(0.00)	(0.10)	(0.35)
FProb./LSD		**0.15/NS**		**0.15/NS**		**0.28/NS**	

Note: SD = sample date, which is the number of days post inoculation for the greenhouse (1, 3, 10, 20 and 37) and field (1, 6, 15, 28 and 63) studies; RPL = relative population level; VD+VT = coinoculation of the pathogens; VT+4dVD = inoculation with VD four days after VT inoculation; * = no tubers; NA = not available; NS = not significant; FProb. = F probability value from ANOVA tests. Means were separated using a least significant difference (LSD) at $P<0.05$. The F probability values indicate significant differences between the two pathogens.

the tubers, which had lower levels but not significantly. In all plant parts, RPLs of VT were generally medium to low. In the roots and stems, VA1 RPLs were low and the pathogen was not detected in the top stem.

Plants inoculated with the combination VA2+4dVD had visual wilt symptoms (48%) that differed by only 4% from the other field combination VD+VA2 (52%) (Table 4.1). For both pathogens, RPLs increased as time increased and decreased as the pathogens progressed throughout the plants. When compared to VA2, RPLs of VD were significantly higher in the soil and roots (Table 4.4). In all other sample types, RPLs of VD were higher than VA2 levels but not significantly.

Visual wilt symptoms for the combination VT+4dVD (45%) differed only by 5% from the other field combination VD+VT (50%) (Table 4.1). Overall, both pathogen RPLs decreased as they progressed throughout the plants and increased as time increased. In this delayed combination, RPLs of VD were significantly higher in the soil only (Table 4.5). RPLs of VD were higher, although not significantly, than VT in the roots, lower and mid stem samples. In the top stem and tuber samples, VD RPLs were lower than VT levels, but again not significantly.

4.1.4 Discussion

4.1.4.1 Pathogen interactions from same day inoculations

In both the greenhouse and field experiment, the results from potato plants, inoculated with two different pathogens at the same time, showed that the aggressive pathogens were able to maintain higher RPLs than the weaker species. The aggressive pathogen VA1 maintained higher RPLs than VA2 and VT in both the greenhouse and the

field experiment and visual wilt symptoms showed that VA1 was still able to cause a significant amount of disease (Table 4.1). Although VA1 RPLs were higher than the weaker species in most sample types, the RPLs of VA2 and VT were not always significantly lower. *Verticillium tricorpus* and VA2, have both been described as weak pathogens of potatoes (Mahuku et al 1999). *Verticillium tricorpus* is frequently found in association with the more aggressive species VA1 and VD (Heinz and Platt 2000), however, it is thought that VT does not colonize stems and instead it proliferates in the soil as a strong saprophyte (Mahuku et al 1999). The results from this study showed that VT was able to colonize plant stems and in fact did so at levels not significantly different from VA1 in the field (Table 4.3). The results from a previous study (see Chapter 2), where potato plants were inoculated with VA1 only, were reviewed and overall the RPLs were similar to the RPLs from this combination study (Table A.1.4). However, the foliar disease symptoms in the greenhouse combination study (44%) affected half as many plants as the single inoculation study (80%). Although VT and VA2 were able to colonize all sample types in the presence of an aggressive pathogen, they were unable to significantly suppress VA1 RPLs.

The aggressive pathogen VD was able to maintain higher RPLs than VA2 and VT in both the greenhouse and field experiment. Even though RPLs were higher than the weaker pathogens, VD levels in the greenhouse were generally medium, decreasing to low by the last sampling date (Tables 4.4 and 4.5). This explains the low percentage of plants with visual wilt symptoms in the greenhouse (7%). Due to the low percentage of visual wilt, these results were compared to the previous study (see Chapter 2) where plants were inoculated with VD only. While RPLs from both greenhouse experiments

were generally similar, the percentage of foliar wilt from the previous study (32%) was significantly higher than this combination study. The same situation was seen in VA1 inoculated plants and this may indicate that although VT and VA2 were unable to suppress VD or VA1 RPLs, they appear to have some role in suppressing VD and VA1 pathogenicity. The weaker pathogens may have caused the reduced pathogenicity of the aggressive species through competition for space and nutrients within the plant, a process known as hyphal interference (Lucus 1998). In the field, VD RPLs and percentage of visual wilt symptoms were higher than in the greenhouse and showed no suppression by VA2 or VT. This may have been due to the naturally occurring VD populations which have been known to survive in the soil for more than 10 years (Easton et al 1992; Keinath 1991; Tjamos and Fravel 1995). These naturally occurring VD populations have been shown to affect uninoculated plants to an extent that is similar to that of inoculated plants (see Chapter 2).

4.1.4.2 Pathogen interactions from inoculations with a time delay

Results for plants inoculated with a weak pathogen followed by an aggressive pathogen four days later showed that the weak pathogens were able to establish themselves inside the plant and maintain RPLs that competed with the aggressive pathogens for space. When plants were inoculated with VA2 followed by VA1, VA2 reached high RPLs in the stems and VA1 was suppressed to low levels until it was undetectable in the stems on most sampling dates (Table 4.2). Mahuku et al. (1999) described VA2 as a weak pathogen because it was not readily detected in the stems of potato plants, and Robb et al. (1993) reported that VA2 was as aggressive as VA1,

causing severe wilt symptoms. The results from this study showed that VA2 was readily isolated from all plant parts at RPLs as high as or higher than VA1. However, visual wilt symptoms were lower than the other field combination VA1+VA2. From this study and from Chapter 3, where low levels of VA2 foliar disease (10%) were observed, it can be concluded that VA2 may be able to colonize the plant at levels similar to VA1, but it is not as pathogenic. Even though VT was present at medium-low RPLs throughout the plant, it was still able to maintain significantly higher levels than VA1 and actually suppressed it to undetectable levels in the stems (Table 4.3). *Verticillium tricorpus* is thought to proliferate in the soil as a saprophyte (Mahuku et al. 1999) and in fact RPLs in the soil from this study were significantly higher than VA1 levels and VT prevented VA1 from entering the plant at the soil level. From single inoculation studies previously reported (see Chapter 2), foliar disease in the field caused by VA1 went from 81% to 26% when inoculated four days after VT.

In combinations where plants were inoculated with a weak pathogen followed by VD four days later, the weaker pathogens were able to colonize all sample types at medium levels. Even though there were no significant differences between the levels of VA2 and VD in the stems, VD RPLs were significantly higher than VA2 in the soil and roots. This may again be due to natural population levels of VD in the soil. Due to naturally occurring population levels of VD, inoculation with VA2 prior to VD would not be as effective in suppressing the aggressive pathogen. However, the RPLs from the VD single inoculation study were higher than the VD RPLs in delayed combination with VA2. When present in combination with VT, VD again maintained higher RPLs although not significantly in most sample types (Table 4.5). Without naturally occurring

populations of VD, it is possible that VA2 and VT may have moderating effects on VD. Compared to single inoculation studies, all combination studies had fewer foliar wilt symptoms and reduced RPLs of VA1 and VD.

Visual wilt symptoms from the combinations VA2+4dVA1 and VT+4dVA1, were lower than the other field combinations VA1+VA2 and VA1+VT. This shows that the weaker species may have some moderating effect on VA1. Visual wilt symptoms from the combinations VA2+4dVD and VT+4dVD were not much different from the other field combinations and may be due to naturally occurring VD soil populations (Table 4.1). It is thought that VT may be used as a biological control agent against VD because of its ability to proliferate in the soil and its weak pathogenic nature (Davis et al. 2000; Heinz and Platt 2000). Therefore, initial soil populations of VD may not have given the weaker species a chance to colonize the plant without interference. Further delayed combination studies in a greenhouse environment will help to clarify the role of natural Verticillium soil populations. Also, additional studies involving different concentration levels of weak and aggressive species may help to support the idea of using weaker species as biological control agents.

5 CHAPTER FIVE

5.1 General Discussion

Verticillium wilt is a serious disease that infects potato as well as a variety of other plant species (Dan et al. 2001; Fravel 1996; Isaac 1967; Mpofu and Hall 2003). *Verticillium* species are hard to eradicate as they are introduced into new areas by contaminated seed tubers, and resting structures such as microsclerotia can remain dormant in the soil for many years and can even survive on non-host species until conditions are again conducive to growth (Platt and Mahuku 2000; Mol et al. 1996). Population levels of Verticillium can be suppressed by a number of different control methods but no method is completely successful in eradicating this disease. Because chemical control methods for plant diseases are expensive and/or environmentally damaging, studies involving biological control agents are becoming increasingly popular (Dobinson 1995; Davis 1996; Keinath et al. 1991; Lucus 1998). Adequate disease management requires epidemiological studies which are lacking in the area of *Verticillium* species and their interactions with host potato plant and other pathogens (Mol et al. 1996; Mpofu and Hall 2003).

5.1.1 Single Inoculation Studies

To obtain a better understanding of how VA1, VD, VA2, and VT interact within the host, RPLs of each pathogen and the locations of the pathogens as they progressed throughout potato plant tissues during the course of disease development, were monitored using PCR technology. Verticillium disease development was seen as RPLs increased as time progressed and decreased as the pathogen traveled up the plant.

5.1.1.1 *Verticillium albo-atrum* 'group 1'

Verticillium albo-atrum 'group 1', which is typically known as an aggressive plant pathogen and the most problematic pathogen of the *Verticillium* species in most potato growing areas (McKeen and Thorpe 1981), maintained high RPLs and caused the highest incidence of foliar disease (80%) in the greenhouse. In the field experiments conducted in 2002 and 2003, RPLs were again high and disease incidence in the field also remained high at more than 62%. *Verticillium albo-atrum* 'group1' progressed throughout the plant quickly and was detected in plant tissues as early as 24 hours after inoculation in the greenhouse and 3 days after inoculation in the field experiments. High RPLs were detected in some plant tissues as early as 28 days after inoculation and by the last sampling date almost all soil and plant tissues had high RPLs of VA1. The field experiment conducted in 2003 was performed in a different field from the experiment in 2002. Both fields had different crop rotation histories and weather conditions. It was found that different microorganisms in the soil and weather conditions affected the growth of Verticillium. The RPLs in the roots of the field experiment in 2003 were lower than in the field experiment of 2002. It was suspected that there may have been different microorganisms in the field soil in 2003 that kept VA1 from colonizing the roots to the extent that it normally would. There are more than 150 bacterial isolates that survive on potato roots and that are antagonistic to *Verticillium* species (Leben et al. 1987). For all experiments inoculated with VA1, correlations between population assessments and foliar wilt were high (>0.85). These results are supported by another study where the relationship between colony forming units and percentage of foliar wilt was studied and high correlations between the two were found (Hoyos et al. 1991).

5.1.1.2 *Verticillium dahliae*

Verticillium dahliae is recognized as a strong soil-borne pathogen (Platt 1986) and in the field experiments conducted in 2002 and 2003, VD maintained the highest RPLs. In the field, VD reached medium-high RPLs quickly (approximately 15 days after inoculation) in most sample types and maintained these levels for the rest of the growing season. Disease incidence in the field was also high (>94%) but was not seen at these high levels until the last sampling date. Because VD can survive in the soil for long period of time (microsclerotia), soil samples from the control group were reviewed. Results showed that RPLs of VD in the inoculated soil and plants were almost identical to RPLs in the control group. This indicates that the naturally occurring populations in the soil played a role in contributing to the RPLs found in the plants. A recent study found that commercial certified seed lots throughout North America are commonly infected with VD and may serve as a primary source of inoculum (Omer et al. 2000).

The greenhouse experiment had lower RPLs in almost all sample types than both of the field experiments. *Verticillium dahliae* is known for being a strong soil pathogen (Schnathorst 1981) and in this case VD did maintain high RPLs in the soil. Above the soil, RPLs peaked early and then died off. Perhaps the greenhouse environment was not optimal for VD growth in that the temperature was a moderate 24°C. The optimal growing temperature range for VD is 24°C to 28°C (Platt 1986). Although high correlations were found between foliar disease symptoms and population level assessments in the soil and roots, this is misleading as RPLs were high with no significant changes over time, and the percentage of foliar wilt was low on all sample dates. No major fluctuations for both measurements would result in a high correlation. Correlations

for stem samples were poor due to the sudden increase in RPLs, followed by a significant decrease. This dying off of VD is another indication that the greenhouse environment may not have been optimal for VD growth.

5.1.1.3 *Verticillium tricorpus*

In both the greenhouse and field experiments, RPLs of VT were generally medium-low in all sample types. As the pathogen progressed into the roots, RPLs dropped from medium to low levels in all experiments before entering the stems. This trend supports other evidence that VT is a weak plant pathogen and proliferates in the soil as a saprophyte (Mahuku et al. 1999). In the field, VT was undetectable in the roots in the latter half of the growing season and it was thought that RPLs may have been reduced by a possible antagonistic effect. Bacterial strains of *Psedomonas*, *Cellulomonas* and *Streptomyces* are known to compete with Verticillium by colonizing significantly more roots of potato plants (Leben et al. 1987).

Almost all correlations between foliar disease and population assessments were high (>80%). Because RPLs were generally low and VT is known to be a weak plant pathogen (Heinz and Platt 2000a), limited foliar disease symptoms would be expected and in fact, none were seen in the greenhouse and only 7% of the plants in the field showed wilt symptoms which affected, on average, 25% of each individual plant. From these experiments it can be concluded that VT can infect and colonize actively growing plants. Although all sample types contained some amount of the pathogen, RPLs and foliar disease symptoms were generally low, supporting the evidence stated by Mahuku et al. (1999) that VT is a weak pathogen of potato and proliferates in the soil.

5.1.1.4 *Verticillium albo-atrum* 'group 2'

In the greenhouse experiment, RPLs of VA2 were generally medium-high in the soil and roots and low in the stems and tubers. In the roots, RPLs significantly decreased from high to low on the second sampling date. Even though greenhouse soil was autoclaved, it is still possible that an antagonistic relationship occurred and could have been the cause of this decrease. Seed tubers have been known to carry traces of bacteria and in a study conducted by Tjamos et al. (2004), seed tubers dusted with bacteria showed a significant reduction in symptom development in heavily Verticillium infested fields. In both field experiments, RPLs were generally medium-low in the soil and roots and low in the stems. Correlations between foliar disease and population assessments were generally high (>94%). The greenhouse experiment differed from the field experiment in the soil and root samples with RPLs being higher in the greenhouse experiment. Perhaps other microorganisms in the field soil were out-competing VA2 for space, causing the pathogen to have trouble establishing itself in the plants.

Similar to the findings by Mahuku et al. (1999), VA2 was readily detected in soil samples using PCR techniques. However, the pathogen in this study was also detected in all stem samples, which was not the case in the study by Mahuku et al (1999). From studies conducted by Robb et al. (1993), VA2 was found to be as virulent as VA1. From these single inoculation studies, RPLs of VA2 did not differ significantly from 'group 1' in all sample types except from soil samples in the field experiment. 'Group 1' had significantly higher RPLs in soil samples than 'group 2'. Even though RPLs were generally similar, foliar disease symptoms were significantly more severe in 'group 1' inoculated plants than in 'group 2' inoculated plants. Therefore, VA2 may be able to

establish itself in potato plants at similar RPLs as VA1, however, 'group 2' does not appear to be as virulent as 'group 1'.

5.1.2 Combination Inoculation Studies

5.1.2.1 Pathogen interactions from same day inoculations

In both the greenhouse and field experiment, the results from potato plants inoculated with two different pathogens at the same time showed that the aggressive pathogens were able to maintain higher RPLs than the weaker species. The aggressive pathogen VA1 maintained higher RPLs than VA2 and VT in both the greenhouse and the field experiment and visual wilt symptoms showed that VA1 was still able to cause a significant amount of disease. Although VA1 RPLs were higher than the weaker species in most sample types, the RPLs of VA2 and VT were not always significantly lower. The results from the single inoculation study, where potato plants were inoculated with VA1 only, were compared to the results from this combination study and overall, the RPLs were not significantly different. However, foliar disease symptoms in the greenhouse combination study (44%) affected half as many plants as the VA1 single inoculation study (80%). Although VT and VA2 were able to colonize all sample types in the presence of an aggressive pathogen, they were unable to significantly suppress VA1 RPLs.

The aggressive pathogen VD also maintained higher RPLs than VA2 and VT in both the greenhouse and field experiment. Even though RPLs were higher than the weaker pathogens, VD levels in the greenhouse were generally medium, decreasing to low by the last sampling date. This explains the low percentage of plants with visual wilt

symptoms (7%). Due to the low percentage of visual wilt, these results were compared to the single inoculation study, where plants were inoculated with VD only. There were no significant differences in RPLs between greenhouse studies. However, the percentage of foliar wilt from the single inoculation study (32%) was higher than the combination study. In the field, VD RPLs and percentage of visual wilt symptoms were higher than in the greenhouse and showed no suppression by VA2 or VT. This may have been due to the naturally occurring VD populations which have been know to survive in the soil for more than 10 years (Easton et al 1992; Keinath 1991; Tjamos and Fravel 1995). Although VT and VA2 were unable to suppress VD or VA1 RPLs, they appear to have some role in reducing VD and VA1 pathogenicity. The weaker pathogens may have caused the reduced pathogenicity of the aggressive species through competition of space and nutrients within the plant, a process known as hyphal interference (Lucus 1998).

5.1.2.2 Pathogen interactions from inoculations with a time delay

The results for plants inoculated with a weak pathogen followed by an aggressive pathogen four days later showed that the weak pathogens were able to establish themselves inside the plant and maintain RPLs that competed with the aggressive pathogens for space. When plants were inoculated with VA2 followed by VA1, VA2 reached high RPLs in the stems and VA1 was suppressed to low levels until it was undetectable in the stems on most sampling dates. *Verticillium albo-atrum* 'group 2' was readily isolated from all plant parts at RPLs as high as or higher than VA1 and visual wilt symptoms were significantly lower in the combination VA2+4dVA1 than in the other field combination VA1+VA2. Plants inoculated with VT followed by VA1, had

medium-low RPLs of VT throughout the plant. Even though VT levels were not significantly higher than the results from the single inoculation study, it was still able to maintain significantly higher levels than VA1 and actually suppressed VA1 to undetectable levels in the stems. From single inoculation studies, foliar disease in the field caused by VA1 went from 81% to 26% when inoculated four days after VT.

In combinations where plants were inoculated with a weak pathogen followed by VD four days later, the weaker pathogens were able to colonize all sample types at medium levels. In the soil and roots, RPLs of VD were significantly higher than VA2, but there were no significant differences between the levels of VA2 and VD in the stems. This may again be due to natural population levels of VD in the soil. Because of naturally occurring population levels of VD, inoculation with VA2 prior to VD would not be as effective in suppressing the aggressive pathogen. However, RPLs of VD in delayed combination with VA2 were significantly lower than VD RPLs in the single inoculation study. When in combination with VT, VD again maintained higher RPLs although not significantly in most sample types. Without naturally occurring populations of VD, it is possible that VA2 and VT may have moderating effects on VD. Compared to single inoculation studies, all combination studies had fewer foliar wilt symptoms and reduced RPLs of VA1 and VD.

5.1.3 Conclusions and Further Studies

From these experiments it was found that both VA1 and VD enter the plant quickly and moved to all plant parts, eventually ending up in the tubers and becoming a source of inoculum for another season. In a controlled environment, VA1 was the more

aggressive pathogen while in the field, VD was just as aggressive as VA1. This may have been due to the presence of naturally occurring populations of VD in the soil and the use of diseased seed tubers. Weather conditions were also believed to have influenced the development of these aggressive pathogens. Weather data reveal cool and wet spring conditions, followed by hot and dry weather in the summer. Disease severity increases as temperatures increase to optimal growing levels, and wilt symptoms are enhanced by periods of low soil moisture late in the growing season (Platt 1986; Powelson and Rowe 1993). It can also be concluded that visual disease ratings are not sufficient warning signs for treatment. Symptoms did not show up until late in the growing season whereas the pathogen colonized the plant within 24 hours. By the time the effects of the disease on the foliage are seen, high levels of the pathogen have become a source of inoculum for neighbouring plants and the next growing season.

Single inoculation experiments revealed new and interesting information about weak pathogens VA2 and VT. *Verticillium tricorpus* was thought to be a strong saprophyte that was unable to colonize plant stems (Mahuku et al. 1999). This study revealed that VT populations were highest in the soil. However, VT was able to colonize all areas of the plant stem and also the tubers. *Verticillium albo-atrum* 'group 2' experiments revealed that VA2 was also able to colonize all sample types and at levels similar to VA1. However, it was still found to be a weak pathogen as foliar disease symptoms were low.

From combination studies where a weak pathogen was inoculated with an aggressive pathogen, the weaker species provided indications of moderating abilities. Visual wilt symptoms from both VA2+4dVA1 and VT+4dVA1 combinations were

significantly lower than the other field combinations VA1+VA2 and VA1+VT, and RPLs of the weaker species were generally the same as or higher than the aggressive species. Visual wilt symptoms from the combinations VA2+4dVD and VT+4dVD were not significantly different from the other field combinations and may be due to naturally occurring VD soil populations. It is thought that VT may be used as a biological control agent against VD because of its ability to proliferate in the soil and its weak pathogenic nature (Davis et al. 2000; Heinz and Platt 2000a). Therefore, initial soil populations of VD may not have given the weaker species a chance to colonize the plant without interference.

For future studies it is important to obtain disease free seed tubers to assess the effects of this inoculum source on subsequent growing seasons. These studies should also be repeated in a field free of VD as naturally occurring populations were thought to be a major contributor to RPLs of this pathogen seen in the field experiments. Additional studies involving various strains of VA2 are needed to clarify discrepancies concerning the pathogen's virulence. Studies conducted under various environmental conditions would also be necessary as the effects of such conditions, on the growth of VA2 are unknown (Heinz and Platt 2000a). *Verticillium tricorpus* has been recommended as a biological control agent against VD because it is weakly pathogenic (Davis et al. 2000). Further studies involving VT and VA2, which also proved to be a weak pathogen in this case, should be conducted to elucidate possible moderating abilities (Heinz and Platt 2000a). Additional time delayed combination studies in a greenhouse environment will help to clarify the role of natural Verticillium soil populations. Also, studies involving

different concentration levels of weak and aggressive species may help to support the idea of using weaker species as biological control agents.

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7 APPENDIX

Table A.1.1 Quantification based on band intensity, as compared to standardized DNA samples, producing a relative population level (RPL).

Band Intensity	Estimated Concentration	RPL
no band	no DNA	0
weak	< 0.01μg·g^{-1}	1
medium	1.0 μg·g^{-1} - 0.01μg·g^{-1}	2
high	> 1.0 μg·g^{-1}	3

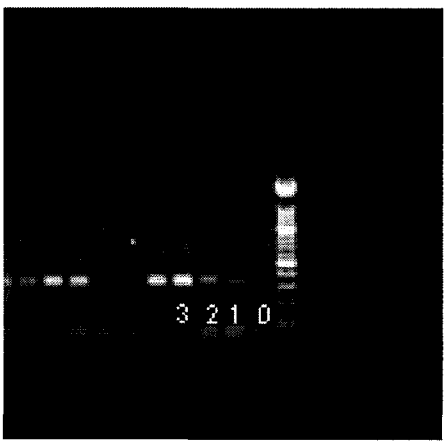

Figure A.1.1 Gel photo showing DNA bands of three standards from the three concentration ranges and a negative control.

Table A.1.2 Statistical program (Genstat) output data for analysis of variance in the greenhouse experiment.

Variate: soil

Rep stratum	**F.Prob**
Species	<.001
Sampledate	0.165
Species*Sampledate	0.607

Table of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	2.000	2.000	2.150	2.800	2.650

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	2.150	1.950	1.950	1.850	1.600

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	2.450	2.200	0.350	0.750	0.200

Species	water(Vt)
	0.400

Sampledate	1	2	3	4	5
	1.984	1.656	1.688	1.562	1.688

Species*Sampledate	1	2	3	4	5
Va1	2.250	1.500	2.250	2.000	2.000
Va1+Va2(Va1)	3.000	2.250	2.000	1.250	1.500
Va1+Va2(Va2)	3.000	2.500	1.750	2.000	1.500
Va1+Vt(Va1)	3.000	3.000	2.750	2.250	3.000
Va1+Vt(Vt)	2.500	2.750	2.750	2.250	3.000
Va2	2.250	2.000	2.250	1.750	2.500
Vd	2.250	2.000	1.500	2.750	1.250
Vd+Va2(Va2)	2.250	1.750	1.750	1.750	2.250
Vd+Va2(Vd)	1.250	3.000	1.500	2.250	1.250
Vd+Vt(Vd)	1.500	2.000	2.250	0.750	1.500
Vd+Vt(Vt)	2.500	1.750	2.750	2.750	2.500
Vt	2.750	2.000	1.750	2.750	1.750
water(Va)	0.250	0.000	0.500	0.250	0.750
water(Va2)	1.500	0.000	0.500	0.250	1.500
water(Vd)	0.250	0.000	0.500	0.000	0.250
water(Vt)	1.250	0.000	0.250	0.000	0.500

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.6179	0.3454	1.3817

Variate: roots

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species*Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	2.600	2.400	1.650	2.550	1.300

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	2.550	2.950	2.900	2.700	2.800

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	1.550	2.150	0.000	0.050	0.350

Species	water(Vt)
	0.600

Sampledate	1	2	3	4	5
	1.953	1.484	1.906	1.828	1.922

Species*Sampledate	1	2	3	4	5
Va1	2.250	1.750	3.000	3.000	3.000
Va1+Va2(Va1)	2.250	1.250	2.750	2.750	3.000
Va1+Va2(Va2)	2.000	0.500	1.750	2.000	2.000
Va1+Vt(Va1)	2.750	1.750	2.500	2.750	3.000
Va1+Vt(Vt)	1.000	1.500	1.500	1.500	1.000
Va2	3.000	1.000	2.750	3.000	3.000
Vd	3.000	2.750	3.000	3.000	3.000
Vd+Va2(Va2)	3.000	3.000	3.000	2.500	3.000
Vd+Va2(Vd)	3.000	2.750	2.500	2.500	2.750
Vd+Vt(Vd)	3.000	2.500	2.750	2.750	3.000
Vd+Vt(Vt)	2.000	1.250	1.500	1.750	1.250
Vt	2.000	2.750	2.500	1.500	2.000
water(Va)	0.000	0.000	0.000	0.000	0.000
water(Va2)	0.250	0.000	0.000	0.000	0.000
water(Vd)	1.000	0.000	0.750	0.000	0.000
water(Vt)	0.750	1.000	0.250	0.250	0.750

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3400	0.1901	0.7602

Variate: lower

Rep stratum	F.Prob
Species	<.001
Sampledate	0.083
Species*Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	1.800	1.750	0.900	2.300	0.550

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	1.150	1.600	1.000	1.600	1.900

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	0.300	0.800	0.050	0.000	0.350

Species	water(Vt)
	0.000

Sampledate	1	2	3	4	5
	1.062	1.141	0.906	0.875	1.094

Species*Sampledate	1	2	3	4	5
Va1	0.750	1.250	1.250	2.750	3.000
Va1+Va2(Va1)	2.000	1.000	0.500	2.250	3.000
Va1+Va2(Va2)	1.500	1.000	0.750	0.750	0.500
Va1+Vt(Va1)	1.750	2.250	1.500	3.000	3.000
Va1+Vt(Vt)	1.750	0.750	0.250	0.000	0.000
Va2	0.750	2.000	1.000	1.250	1.750
Vd	2.250	3.000	1.750	0.250	0.750
Vd+Va2(Va2)	0.750	1.000	1.500	0.500	1.250
Vd+Va2(Vd)	1.500	2.250	2.000	0.500	1.750
Vd+Vt(Vd)	2.500	2.250	2.000	1.500	1.250
Vd+Vt(Vt)	0.250	0.750	0.500	0.000	0.000
Vt	0.750	0.750	1.000	0.500	1.000
water(Va)	0.000	0.000	0.250	0.000	0.000
water(Va2)	0.000	0.000	0.000	0.000	0.000
water(Vd)	0.500	0.000	0.250	0.750	0.250
water(Vt)	0.000	0.000	0.000	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.4068	0.2274	0.9097

Variate: mid

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	1.550	1.750	0.800	1.900	0.500

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	0.750	1.300	0.150	0.850	1.400

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	0.050	0.450	0.150	0.000	0.100

Species	water(Vt)
	0.000

Sampledate	1	2	3	4	5
	0.859	0.906	0.437	0.531	0.922

Species*Sampledate	1	2	3	4	5
Va1	1.250	1.250	0.500	1.750	3.000
Va1+Va2(Va1)	1.500	1.500	0.000	2.750	3.000
Va1+Va2(Va2)	1.500	1.500	0.000	0.500	0.500
Va1+Vt(Va1)	1.500	1.500	1.250	2.250	3.000
Va1+Vt(Vt)	1.250	1.250	0.000	0.000	0.000
Va2	1.000	1.000	0.500	0.250	1.000
Vd	1.250	2.750	1.500	0.000	1.000
Vd+Va2(Va2)	0.000	0.000	0.500	0.000	0.250
Vd+Va2(Vd)	1.250	1.250	0.500	0.250	1.000
Vd+Vt(Vd)	2.000	2.000	1.250	0.500	1.250
Vd+Vt(Vt)	0.000	0.000	0.250	0.000	0.000
Vt	0.750	0.250	0.250	0.250	0.750
water(Va)	0.250	0.250	0.250	0.000	0.000
water(Va2)	0.000	0.000	0.000	0.000	0.000
water(Vd)	0.250	0.000	0.250	0.000	0.000
water(Vt)	0.000	0.000	0.000	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3508	0.1961	0.7845

Variate: top

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	1.200	1.300	0.450	1.750	0.400

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	0.500	1.050	0.100	0.700	1.000

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	0.150	0.350	0.150	0.000	0.100

Species	water(Vt)
	0.000

Sampledate	1	2	3	4	5
	0.594	0.781	0.406	0.375	0.719

Species*Sampledate	1	2	3	4	5
Va1	0.750	0.750	0.750	1.250	2.500
Va1+Va2(Va1)	1.000	1.000	0.500	1.250	2.750
Va1+Va2(Va2)	1.250	0.500	0.000	0.250	0.250
Va1+Vt(Va1)	1.250	1.250	0.750	2.500	3.000
Va1+Vt(Vt)	1.000	1.000	0.000	0.000	0.000
Va2	1.000	0.500	0.500	0.000	0.500
Vd	0.750	2.000	1.250	0.250	1.000
Vd+Va2(Va2)	0.250	0.250	0.000	0.000	0.000
Vd+Va2(Vd)	0.500	2.000	0.250	0.250	0.500
Vd+Vt(Vd)	1.500	2.250	1.000	0.250	0.000
Vd+Vt(Vt)	0.000	0.500	0.250	0.000	0.000
Vt	0.000	0.250	0.750	0.000	0.750
water(Va)	0.000	0.250	0.250	0.000	0.250
water(Va2)	0.000	0.000	0.000	0.000	0.000
water(Vd)	0.250	0.000	0.250	0.000	0.000
water(Vt)	0.000	0.000	0.000	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3264	0.1825	0.7299

Variate: tuberstem

Rep stratum	**F.Prob**
Species	<.001
Sampledate	0.126
Species.Sampledate	0.914

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	0.000	0.568	1.228	1.158	0.161

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	0.350	0.000	0.935	0.442	0.179

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	-0.068	0.658	0.277	-0.052	-0.091

Species	water(Vt)
	-0.052

Sampledate	1	2	3	4	5
	0.356	0.356	0.356	0.283	0.428

Species*Sampledate	1	2	3	4	5
Va1	0.000	0.000	0.000	0.000	0.000
Va1+Va2(Va1)	0.568	0.568	0.568	0.496	0.642
Va1+Va2(Va2)	1.225	1.225	1.225	1.153	1.309
Va1+Vt(Va1)	1.157	1.157	1.157	1.070	1.250
Va1+Vt(Vt)	0.160	0.160	0.160	0.072	0.250
Va2	0.250	0.250	0.250	0.250	0.250
Vd	0.000	0.000	0.000	0.000	0.000
Vd+Va2(Va2)	0.932	0.932	0.932	0.860	1.016
Vd+Va2(Vd)	0.441	0.441	0.441	0.369	0.516
Vd+Vt(Vd)	0.180	0.180	0.180	0.107	0.250
Vd+Vt(Vt)	-0.067	-0.067	-0.067	-0.139	0.000
Vt	0.758	0.758	0.758	0.500	1.016
water(Va)	0.277	0.277	0.277	0.205	0.349
water(Va2)	-0.051	-0.051	-0.051	-0.124	0.016
water(Vd)	-0.089	-0.089	-0.089	-0.162	-0.025
water(Vt)	-0.051	-0.051	-0.051	-0.124	0.016

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3357	0.1877	0.7507

Variate: tubereye

Rep stratum	**F.Prob**
Species	<.001
Sampledate	0.004
Species.Sampledate	0.475

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)	Va1+Vt(Vt)
	0.000	0.862	0.862	0.396	0.271

Species	Va2	Vd	Vd+Va2(Va2)	Vd+Va2(Vd)	Vd+Vt(Vd)
	0.000	0.000	-0.084	-0.084	-0.102

Species	Vd+Vt(Vt)	Vt	water(Va)	water(Va2)	water(Vd)
	0.380	0.459	-0.101	-0.101	-0.101

Species	water(Vt)
	-0.101

Sampledate	1	2	3	4	5
	0.162	0.162	0.162	0.034	0.278

Species*Sampledate	1	2	3	4	5
Va1	0.001	0.001	0.001	0.000	0.000
Va1+Va2(Va1)	0.859	0.859	0.859	0.734	1.000
Va1+Va2(Va2)	0.859	0.859	0.859	0.734	1.000
Va1+Vt(Va1)	0.396	0.396	0.396	0.039	0.750
Va1+Vt(Vt)	0.271	0.271	0.271	0.039	0.500
Va2	0.001	0.001	0.001	0.000	0.000
Vd	0.001	0.001	0.001	0.000	0.000
Vd+Va2(Va2)	-0.079	-0.079	-0.079	-0.203	0.017
Vd+Va2(Vd)	-0.079	-0.079	-0.079	-0.203	0.017
Vd+Vt(Vd)	-0.096	-0.096	-0.096	-0.221	0.000
Vd+Vt(Vt)	0.381	0.381	0.381	0.257	0.500
Vt	0.459	0.459	0.459	0.250	0.666
water(Va)	-0.095	-0.095	-0.095	-0.220	0.000
water(Va2)	-0.095	-0.095	-0.095	-0.220	0.000
water(Vd)	-0.095	-0.095	-0.095	-0.220	0.000
water(Vt)	-0.095	-0.095	-0.095	-0.220	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.2905	0.1624	0.6496

Table A.1.3 Statistical program (Genstat) output data for analysis of variance in field experiment 2002.

Variate: soil

Rep stratum	**F.Prob**
Species	<.001
Sampledate	0.003
Species.Sampledate	0.001

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	2.350	3.000	0.500	3.000

Sampledate	1	2	3	4	5
	1.750	2.125	2.312	2.375	2.500

Species*Sampledate	1	2	3	4	5
Va1	0.750	2.000	3.000	3.000	3.000
Vd	3.000	3.000	3.000	3.000	3.000
water(Va)	0.250	0.500	0.250	0.500	1.000
water(Vd)	3.000	3.000	3.000	3.000	3.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3449	0.3856	0.7713

Variate: roots

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	1.650	2.950	0.000	2.700

Sampledate	1	2	3	4	5
	1.250	1.562	1.937	2.125	2.250

Species*Sampledate	1	2	3	4	5
Va1	0.000	0.750	2.000	2.500	3.000
Vd	2.750	3.000	3.000	3.000	3.000
water(Va)	0.000	0.000	0.000	0.000	0.000
water(Vd)	2.250	2.500	2.750	3.000	3.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.2080	0.2326	0.4651

Variate: lower

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	1.850	2.150	0.400	2.250

Sampledate	1	2	3	4	5
	0.813	1.188	1.875	2.188	2.250

Species*Sampledate	1	2	3	4	5
Va1	0.000	1.250	2.250	2.750	3.000
Vd	1.000	1.750	2.500	2.500	3.000
water(Va)	0.750	0.000	0.750	0.500	0.000
water(Vd)	1.500	1.750	2.000	3.000	3.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3195	0.3572	0.7145

Variate: mid

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	1.400	1.850	0.350	1.750

Sampledate	1	2	3	4	5
	0.500	0.750	1.562	1.625	2.250

Species*Sampledate	1	2	3	4	5
Va1	0.000	0.500	1.750	1.750	3.000
Vd	0.750	1.250	2.000	2.250	3.000
water(Va)	1.000	0.000	0.750	0.000	0.000
water(Vd)	0.250	1.250	1.750	2.500	3.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3173	0.3548	0.7095

Variate: top

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	1.350	1.250	0.300	1.300

Sampledate	1	2	3	4	5
	0.437	0.750	1.125	0.688	2.250

Species*Sampledate	1	2	3	4	5
Va1	0.000	0.250	1.750	1.750	3.000
Vd	0.500	1.250	1.000	0.500	3.000
water(Va)	0.750	0.500	0.250	0.000	0.000
water(Vd)	0.500	1.000	1.500	0.500	3.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3996	0.4468	0.8936

Variate: tuberstem

Rep stratum	**F.Prob**
Species	<.001
Sampledate	
Species.Sampledate	

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	2.497	2.248	0.004	1.500

Sampledate	1	2	3	4	5
	1.562	1.562	1.562	1.562	1.563

Species*Sampledate	1	2	3	4	5
Va1	2.497	2.497	2.497	2.497	2.500
Vd	2.248	2.248	2.248	2.248	2.250
water(Va)	0.005	0.005	0.005	0.005	0.000
water(Vd)	1.500	1.500	1.500	1.500	1.500

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.5871	0.6564	1.3128

Variate: tubereye

Rep stratum	**F.Prob**
Species	<.001
Sampledate	
Species.Sampledate	

Tables of means

Species	Va1	Vd	water(Va)	water(Vd)
	2.497	1.998	0.253	0.502

Sampledate	1	2	3	4	5
	1.312	1.312	1.312	1.312	1.313

Species*Sampledate	1	2	3	4	5
Va1	2.496	2.496	2.496	2.496	2.500
Vd	1.998	1.998	1.998	1.998	2.000
water(Va)	0.254	0.254	0.254	0.254	0.250
water(Vd)	0.503	0.503	0.503	0.503	0.500

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3817	0.4268	0.8535

Table A.1.4 Statistical program (Genstat) output data for analysis of variance in field experiment 2003.

Variate: soil

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	0.011

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	2.500	2.100	1.100	1.650

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	2.000	1.100	2.350	1.850

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	1.600	2.450	2.700	2.050

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	2.700	2.850	1.600	2.000

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	1.400	2.250	2.850	2.300

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.950	0.600	2.250	0.700

Sampledate	1	2	3	4	5
	1.990	1.333	1.719	2.208	2.312

Species*Sampledate	1	2	3	4	5
Va1	2.000	2.500	2.250	2.750	3.000
Va1+Va2(Va1)	2.500	0.500	1.500	3.000	3.000
Va1+Va2(Va2)	1.000	0.000	0.000	2.000	2.500
Va1+Vt(Va1)	1.750	0.000	2.250	2.000	2.250
Va1+Vt(Vt)	2.250	0.250	2.250	3.000	2.250
Va2	1.250	0.250	1.500	0.750	1.750
Va2+4dVa1(Va1)	1.750	2.000	2.250	3.000	2.750
Va2+4dVa1(Va2)	2.250	1.250	1.250	2.250	2.250
Va2+4dVd(Va2)	1.250	1.250	1.500	1.500	2.500
Va2+4dVd(Vd)	2.500	1.500	3.000	3.000	2.250
Vd	3.000	2.250	2.250	3.000	3.000
Vd+Va2(Va2)	2.500	2.500	1.750	1.750	1.750
Vd+Va2(Vd)	3.000	1.750	2.750	3.000	3.000
Vd+Vt(Vd)	3.000	2.250	3.000	3.000	3.000
Vd+Vt(Vt)	2.000	0.000	2.000	2.000	2.000
Vt	1.750	2.000	1.500	2.250	2.500

Vt+4dVa1(Va1)	1.750	1.250	1.250	0.750	2.000
Vt+4dVa1(Vt)	2.500	2.250	1.500	2.000	3.000
Vt+4dVd(Vd)	2.750	3.000	2.500	3.000	3.000
Vt+4dVd(Vt)	2.250	1.000	2.500	3.000	2.750
water(Va)	1.000	0.750	0.500	1.250	1.250
water(Va2)	0.750	0.750	0.000	1.000	0.500
water(Vd)	2.250	2.250	1.500	2.500	2.750
water(Vt)	0.750	0.500	0.500	1.250	0.500

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.5251	0.2397	1.1743

Variate: roots

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	0.750	0.550	0.900	1.700

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	0.850	0.800	0.650	1.150

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	0.750	1.400	1.800	0.150

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	2.100	2.250	1.300	1.200

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	0.450	1.100	1.650	1.100

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.100	0.100	0.700	0.550

Sampledate	1	2	3	4	5
	1.469	0.406	0.969	0.927	1.260

Species*Sampledate	1	2	3	4	5
Va1	1.500	0.000	0.750	0.000	1.500
Va1+Va2(Va1)	1.250	0.000	0.000	0.500	1.000
Va1+Va2(Va2)	2.250	0.000	0.000	2.000	0.250
Va1+Vt(Va1)	2.000	0.000	0.750	2.750	3.000
Va1+Vt(Vt)	1.250	0.000	0.000	1.500	1.500
Va2	2.250	0.000	1.000	0.750	0.000
Va2+4dVa1(Va1)	1.000	0.000	1.000	0.250	1.000
Va2+4dVa1(Va2)	1.500	0.000	1.500	1.000	1.750
Va2+4dVd(Va2)	1.000	0.000	0.750	0.500	1.500
Va2+4dVd(Vd)	2.000	0.750	1.500	1.000	1.750
Vd	3.000	0.250	2.750	1.500	1.500
Vd+Va2(Va2)	0.000	0.000	0.000	0.250	0.500
Vd+Va2(Vd)	1.750	0.500	2.500	2.750	3.000
Vd+Vt(Vd)	2.750	0.500	2.500	2.500	3.000
Vd+Vt(Vt)	0.250	0.500	1.250	2.000	2.500
Vt	3.000	1.750	1.750	0.000	0.000
Vt+4dVa1(Va1)	1.500	0.000	0.500	0.000	0.250
Vt+4dVa1(Vt)	0.500	1.500	1.250	0.500	1.750
Vt+4dVd(Vd)	2.250	0.250	2.000	1.500	2.250
Vt+4dVd(Vt)	2.250	2.000	0.500	0.000	0.750
water(Va)	0.000	0.000	0.000	0.000	0.500
water(Va2)	0.500	0.000	0.000	0.000	0.000
water(Vd)	0.500	0.750	0.500	0.750	1.000
water(Vt)	1.000	1.000	0.500	0.250	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.4122	0.1881	0.9217

Variate: lower

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	1.550	1.150	0.600	1.450

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	1.100	1.600	0.650	2.400

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	1.600	1.150	1.900	1.250

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	2.150	1.450	0.400	1.250

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	0.100	1.250	2.000	1.500

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.150	0.100	0.400	0.250

Sampledate	1	2	3	4	5
	1.219	0.083	1.115	1.448	1.844

Species*Sampledate	1	2	3	4	5
Va1	1.250	0.000	0.750	2.750	3.000
Va1+Va2(Va1)	0.750	0.000	0.500	2.000	2.500
Va1+Va2(Va2)	0.500	0.000	0.750	0.750	1.000
Va1+Vt(Va1)	0.500	0.000	1.500	2.250	3.000
Va1+Vt(Vt)	0.750	0.000	1.250	1.250	2.250
Va2	2.500	0.000	2.500	2.750	0.750
Va2+4dVa1(Va1)	0.500	0.000	0.000	1.500	1.250
Va2+4dVa1(Va2)	3.000	0.000	3.000	3.000	3.000
Va2+4dVd(Va2)	1.750	0.000	0.500	2.750	3.000
Va2+4dVd(Vd)	0.500	0.000	1.000	1.750	2.500
Vd	3.000	0.250	2.500	0.750	3.000
Vd+Va2(Va2)	1.500	0.000	1.250	1.250	2.250
Vd+Va2(Vd)	3.000	0.000	2.500	2.250	3.000
Vd+Vt(Vd)	2.000	0.000	1.000	1.500	2.750
Vd+Vt(Vt)	0.000	0.000	1.000	0.250	0.750
Vt	1.250	0.000	1.000	1.250	2.250
Vt+4dVa1(Va1)	0.000	0.000	0.000	0.250	0.250
Vt+4dVa1(Vt)	1.000	0.000	1.500	1.750	2.000
Vt+4dVd(Vd)	2.000	0.750	1.750	2.500	3.000
Vt+4dVd(Vt)	1.750	0.000	2.000	1.750	2.000
water(Va)	0.750	0.000	0.000	0.000	0.000
water(Va2)	0.000	0.500	0.000	0.000	0.000
water(Vd)	0.500	0.000	0.250	0.500	0.750
water(Vt)	0.500	0.500	0.250	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.4467	0.2039	0.9989

Variate: mid

Rep stratum	F.Prob
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	1.150	0.950	0.200	1.200

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	0.950	1.150	0.400	1.350

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	0.850	0.900	1.400	1.050

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	1.700	1.000	0.300	0.850

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	0.050	0.700	1.050	0.900

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.200	0.100	0.250	0.250

Sampledate	1	2	3	4	5
	0.635	0.063	0.552	1.073	1.594

Species*Sampledate	1	2	3	4	5
Va1	0.250	0.000	0.500	2.000	3.000
Va1+Va2(Va1)	0.750	0.000	0.000	1.750	2.250
Va1+Va2(Va2)	0.500	0.000	0.000	0.000	0.500
Va1+Vt(Va1)	0.250	0.000	1.250	1.750	2.750
Va1+Vt(Vt)	0.750	0.000	0.750	0.750	2.500
Va2	1.500	0.000	2.000	1.500	1.250
Va2+4dVa1(Va1)	0.000	0.000	0.000	1.250	0.750
Va2+4dVa1(Va2)	0.750	0.000	1.250	1.750	3.000
Va2+4dVd(Va2)	0.250	0.000	1.000	1.000	2.000
Va2+4dVd(Vd)	0.750	0.000	0.250	1.250	2.250
Vd	2.000	0.000	1.500	0.500	3.000
Vd+Va2(Va2)	1.250	0.000	0.500	2.000	1.500
Vd+Va2(Vd)	2.250	0.000	1.500	1.750	3.000
Vd+Vt(Vd)	0.750	0.000	0.000	1.750	2.500
Vd+Vt(Vt)	0.000	0.000	0.500	0.500	0.500
Vt	0.500	0.000	1.000	0.750	1.000
Vt+4dVa1(Va1)	0.000	0.000	0.000	0.000	0.250
Vt+4dVa1(Vt)	0.000	0.000	0.750	1.250	1.500
Vt+4dVd(Vd)	0.750	0.250	0.000	1.750	2.500

Vt+4dVd(Vt)	0.500	0.000	0.250	2.000	1.750
water(Va)	0.750	0.250	0.000	0.000	0.000
water(Va2)	0.250	0.250	0.000	0.000	0.000
water(Vd)	0.250	0.000	0.250	0.250	0.500
water(Vt)	0.250	0.750	0.000	0.250	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3478	0.1587	0.7777

Variate: top

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	<.001

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	0.600	0.500	0.200	1.000

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	1.100	0.650	0.400	1.450

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	0.600	0.600	1.100	0.950

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	1.500	0.750	0.200	0.500

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	0.000	0.650	0.450	0.750

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.000	0.050	0.200	0.300

Sampledate	1	2	3	4	5
	0.354	0.073	0.635	0.688	1.271

Species*Sampledate	1	2	3	4	5
Va1	0.000	0.000	0.250	0.750	2.000
Va1+Va2(Va1)	0.000	0.000	0.000	0.750	1.750
Va1+Va2(Va2)	0.500	0.000	0.000	0.250	0.250
Va1+Vt(Va1)	0.000	0.000	1.000	1.250	2.750
Va1+Vt(Vt)	1.000	0.000	1.000	0.750	2.750
Va2	0.000	0.000	2.000	0.750	0.500
Va2+4dVa1(Va1)	0.000	0.000	0.000	1.500	0.500
Va2+4dVa1(Va2)	1.000	0.000	1.750	1.750	2.750
Va2+4dVd(Va2)	0.000	0.000	1.000	0.500	1.500
Va2+4dVd(Vd)	0.000	0.000	0.250	0.750	2.000
Vd	1.250	0.000	1.000	0.250	3.000
Vd+Va2(Va2)	1.000	0.000	0.250	1.500	2.000
Vd+Va2(Vd)	1.750	0.000	1.500	1.250	3.000
Vd+Vt(Vd)	0.000	0.000	0.250	1.250	2.250
Vd+Vt(Vt)	0.000	0.000	0.750	0.000	0.250
Vt	0.750	0.000	1.000	0.500	0.250
Vt+4dVa1(Va1)	0.000	0.000	0.000	0.000	0.000
Vt+4dVa1(Vt)	0.000	0.000	1.250	1.000	1.000
Vt+4dVd(Vd)	0.500	0.000	0.250	0.250	1.250
Vt+4dVd(Vt)	0.750	0.000	1.000	1.500	0.500
water(Va)	0.000	0.000	0.000	0.000	0.000
water(Va2)	0.000	0.250	0.000	0.000	0.000
water(Vd)	0.000	0.500	0.250	0.000	0.250
water(Vt)	0.000	1.000	0.500	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.2959	0.1350	0.6615

Variate: tuberstem

Rep stratum	**F.Prob**
Species	<.001
Sampledate	<.001
Species.Sampledate	0.105

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	0.996	0.376	0.128	0.996

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	0.376	0.376	0.500	0.624

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	0.128	0.376	1.245	0.376

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	0.872	0.996	0.128	0.624

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	0.252	0.624	0.500	0.748

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.128	0.003	0.252	0.003

Sampledate	1	2	3	4	5
	0.484	0.484	0.484	0.302	0.667

Species*Sampledate	1	2	3	4	5
Va1	0.994	0.994	0.994	0.500	1.500
Va1+Va2(Va1)	0.376	0.376	0.376	0.250	0.500
Va1+Va2(Va2)	0.129	0.129	0.129	0.250	0.000
Va1+Vt(Va1)	0.994	0.994	0.994	0.500	1.500
Va1+Vt(Vt)	0.376	0.376	0.376	0.250	0.500
Va2	0.376	0.376	0.376	0.250	0.500
Va2+4dVa1(Va1)	0.500	0.500	0.500	0.250	0.750
Va2+4dVa1(Va2)	0.623	0.623	0.623	0.500	0.750
Va2+4dVd(Va2)	0.129	0.129	0.129	0.250	0.000
Va2+4dVd(Vd)	0.376	0.376	0.376	0.500	0.250
Vd	1.241	1.241	1.241	0.750	1.750
Vd+Va2(Va2)	0.376	0.376	0.376	0.250	0.500
Vd+Va2(Vd)	0.870	0.870	0.870	0.750	1.000
Vd+Vt(Vd)	0.994	0.994	0.994	0.500	1.500
Vd+Vt(Vt)	0.129	0.129	0.129	0.250	0.000
Vt	0.623	0.623	0.623	0.500	0.750
Vt+4dVa1(Va1)	0.253	0.253	0.253	0.000	0.500
Vt+4dVa1(Vt)	0.623	0.623	0.623	0.250	1.000
Vt+4dVd(Vd)	0.500	0.500	0.500	0.250	0.750
Vt+4dVd(Vt)	0.747	0.747	0.747	0.250	1.250
water(Va)	0.129	0.129	0.129	0.000	0.250
water(Va2)	0.006	0.006	0.006	0.000	0.000
water(Vd)	0.253	0.253	0.253	0.000	0.500
water(Vt)	0.006	0.006	0.006	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.3127	0.1427	0.6992

Variate: tubereye

Rep stratum	**F.Prob**
Species	<.001
Sampledate	0.002
Species.Sampledate	0.830

Tables of means

Species	Va1	Va1+Va2(Va1)	Va1+Va2(Va2)	Va1+Vt(Va1)
	0.125	0.374	0.001	0.499

Species	Va1+Vt(Vt)	Va2	Va2+4dVa1(Va1)	Va2+4dVa1(Va2)
	0.125	0.001	0.001	0.250

Species	Va2+4dVd(Va2)	Va2+4dVd(Vd)	Vd	Vd+Va2(Va2)
	0.001	0.001	0.748	0.001

Species	Vd+Va2(Vd)	Vd+Vt(Vd)	Vd+Vt(Vt)	Vt
	0.623	0.250	0.001	0.125

Species	Vt+4dVa1(Va1)	Vt+4dVa1(Vt)	Vt+4dVd(Vd)	Vt+4dVd(Vt)
	0.125	0.374	0.125	0.374

Species	water(Va)	water(Va2)	water(Vd)	water(Vt)
	0.001	0.001	0.125	0.001

Sampledate	1	2	3	4	5
	0.177	0.177	0.177	0.104	0.250

Species*Sampledate	1	2	3	4	5
Va1	0.125	0.125	0.125	0.000	0.250
Va1+Va2(Va1)	0.374	0.374	0.374	0.250	0.500
Va1+Va2(Va2)	0.001	0.001	0.001	0.000	0.000
Va1+Vt(Va1)	0.498	0.498	0.498	0.250	0.750
Va1+Vt(Vt)	0.125	0.125	0.125	0.000	0.250
Va2	0.001	0.001	0.001	0.000	0.000
Va2+4dVa1(Va1)	0.001	0.001	0.001	0.000	0.000
Va2+4dVa1(Va2)	0.250	0.250	0.250	0.250	0.250
Va2+4dVd(Va2)	0.001	0.001	0.001	0.000	0.000
Va2+4dVd(Vd)	0.001	0.001	0.001	0.000	0.000
Vd	0.747	0.747	0.747	0.500	1.000
Vd+Va2(Va2)	0.001	0.001	0.001	0.000	0.000
Vd+Va2(Vd)	0.622	0.622	0.622	0.500	0.750
Vd+Vt(Vd)	0.250	0.250	0.250	0.000	0.500
Vd+Vt(Vt)	0.001	0.001	0.001	0.000	0.000
Vt	0.125	0.125	0.125	0.250	0.000
Vt+4dVa1(Va1)	0.125	0.125	0.125	0.000	0.250
Vt+4dVa1(Vt)	0.374	0.374	0.374	0.250	0.500
Vt+4dVd(Vd)	0.125	0.125	0.125	0.000	0.250

Vt+4dVd(Vt)	0.374	0.374	0.374	0.250	0.500
water(Va)	0.001	0.001	0.001	0.000	0.000
water(Va2)	0.001	0.001	0.001	0.000	0.000
water(Vd)	0.125	0.125	0.125	0.000	0.250
water(Vt)	0.001	0.001	0.001	0.000	0.000

Least significant differences of means (5% level)

Rep stratum	Species	Sampledate	Species*Sampledate
l.s.d.	0.2035	0.0929	0.4550