IDENTIFICATION OF *VERTICILLIUM* SPECIES AND CONTROL METHODS FOR VERTICILLIUM WILT OF POTATO IN MANITOBA

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ABSTRACT

Molina Oscar. PhD., The University of Manitoba, 2016. **Identification of *Verticillium* species and control methods for Verticillium wilt of potato in Manitoba.**
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Manitoba is the second largest potato-producing province in Canada, after Prince Edward Island. Although the Manitoba average yield is slightly higher than the Canadian average, there are commercial fields with lower yield as a result of the pressure of diseases like Verticillium wilt, caused by *Verticillium dahliae*. In Manitoba and elsewhere, there is increased interest in the use of soil fumigation and application of composted manures to reduce Verticillium wilt. However, accurate quantification of *V. dahliae* inoculum in soil is needed for disease control decisions as well as to determine success of practices to reduce pathogen levels in soil. The traditional wet plating method for determination of *Verticillium* levels in soil is often prone to errors, laborious and costly. Therefore, the objectives of this thesis research were to: (i) evaluate control measures including compost addition and soil fumigation on Verticillium wilt and yield of potato, cv. Norland and cv. Russet Burbank; (ii) investigate the presence and quantity of microsclerotia-forming *Verticillium* species and its relation to Verticillium wilt in potato; and (iii) evaluate pathogenicity of *V. tricorpus* on potato, cv. Russet Burbank. In a replicated field study, composted manure did not prove to consistently reduce Verticillium wilt. Fumigation with metam sodium resulted in a reduction of *V. dahliae* levels in soil; however, only the lowest rate at 374 L ha^{-1} reduced *Verticillium* inoculum

at planting by up to 40%, and increased marketable yield in cv. Russet Burbank by up to 24%. This result could potentially help growers to reduce environmental impact on organisms not targeted and the costs associated to the use of soil fumigant for the control of Verticillium wilt. Examination of soils and plants from 17 commercial potato fields determined the presence of the microsclerotia-forming *Verticillium* species *V. dahliae* as well as *Verticillium tricorpus* and *Verticillium klebahnii* in Manitoba. Nevertheless, the study did not focus on the presence of other non-producing microsclerotia species. The study optimized a real-time PCR method to identify and quantify *V. dahliae, V. tricorpus* and *V. longisporum* in soil and plant. A subsequent pathogenicity study of selected isolates of the *Verticillium* species demonstrated that only those of *V. dahliae* were pathogenic to potato.

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TABLE OF CONTENTS

Contents **Page**

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

cfu	Colony-forming unit
g	Gram(s)
mg	Milligram
Mg	Megagrams
Kg	Kilogram
Ng	Nanogram
Pg	Pictogram
Fg	Femtogram
mM	Millimolar
µM	Micromolar
ppm	Parts per million
L	Liter
mL	milliliter
µL	Microliter
µm	Micrometer
gal	Gallon
ha	Hectare
cv	Cultivar
C°	Celsius
PDA	Potato dextrose agar

CCM	Composted cattle manure
CSS	Composted separated pig slurry solids
AUWPC	Area under the wilt progress curve
mpg	Microsclerotia per gram
rDNA	Ribosomal DNA
gDNA	Genomic DNA
PCR	Polymerase chain reaction
C_T	Cycle threshold
RM	Rural municipality
MB	Manitoba

FOREWORD

This thesis has been prepared in manuscript format following the guidelines established by the Faculty of Graduate Studies and Department of Soil Science at the University of Manitoba. A general introduction and comprehensive literature review precedes the three manuscripts. Each manuscript consists of an abstract, introduction, materials and methods, results and discussion. Thereafter, a general discussion, conclusions and recommendations are presented. Chapters 2 and 3 will be submitted to a peer-reviewed Journal, to be decided in the future.

CHAPTER 1

1. INTRODUCTION

1.1 CONTEXT FOR THIS STUDY

Potato (*Solanum tuberosum L.*) is the world's most important non-grain food crop (Fiers et al. 2011), with production close to 381 million tonnes in 2014 (FAOSTAT 2017). Potato is the most produced vegetable crop in Canada; Canadian production accounted for more than 4.7 million tonnes in 2015 (Mukezangango 2015b). In Canada, Manitoba is the second largest potato producing province after Prince Edward Island, followed by Alberta, New Brunswick, Québec, and Ontario (Mukezangango 2015b). Although the average Manitoba yield has increased in the last decade by 10 to 22% (Mukezangango 2015a), there is a gap between actual and prospective potato yields (Sawatzky 2015). In North America, low yields are triggered principally by insufficiency of inputs, low quality seeds, post-harvest losses, rural infrastructure, and diseases (Zandstra 2007).

In Manitoba, several diseases can limit potato yield and quality (Shinners-Carnelley et al. 2003). Verticillium wilt is caused by the soilborne fungus *Verticillium dahliae* Kleb. (Powelson and Rowe 1993b). This disease is a serious threat to growers and processors, as 80 % of the potato productive area is planted with the cultivar Russet Burbank, which

is a processing potato cultivar moderately susceptible to this pathogen (Jansky and Rouse 2000).

Potato production is continuously under increased pressure by industry and consumers demanding safer and environmentally friendly practices to be incorporated into the potato production chain, without affecting the demanding standards for tuber yield and quality (Pasche et al. 2014). In Manitoba, current research efforts have been directed at the evaluation of organic amendments, green manures and the soil fumigant metam sodium to control *V. dahliae* (Molina et al. 2014). Indeed, application of composted cattle manure reduced incidence of Verticillium wilt while maintaining low levels of *Verticillium* propagules and improving available phosphorus in soil, which ultimately were shown to benefit potato yield (Molina et al. 2014).

Unfortunately, cultural management practices are not consistently effective in controlling diseases such as Verticillium wilt (Gudmestad et al. 2007). Additionally, the efficacy of metam sodium has also been unreliable in some cases, due to different factors such as application rates (Tsror et al. 2005) and concentration of the active ingredient (Molina et al. 2014). Therefore, it is necessary to have a better understanding of the suppressive effect of composted manures and the optimal application rate of metam sodium that could be integrated to the production practices. This would consistently help control Verticillium wilt under field conditions, while reducing environmental impact.

Soil fumigation is gaining attention among potato producers in Manitoba, due to the successful reduction of Verticillium wilt and increased yields observed in other potato producing areas in North America (Taylor et al. 2005). Growers know that metam sodium can help to reduce propagule densities below the threshold levels for economic damage. The main source of inoculum of *V. dahliae* is the long-term survival structure called microsclerotia. Thus, the first step in successful management of Verticillium wilt is to identify the *V. dahliae* propagule density in the soil. Unfortunately, quantification of *V. dahliae* inoculum using the traditional wet and dry plating methods has a variety of limitations such as low recovery of *V. dahliae* microsclerotia from soil (Wheeler et al. 1992), which at times is only of 30-50% of the total *V. dahliae* inoculum in soil (Goud and Termorshuizen 2003; Termorshuizen et al. 1998).

In addition, the wet plating method relies on the formation of microsclerotia-originated colonies on semi-selective media (Goud and Termorshuizen 2003), but it fails to differentiate similar microsclerotia-forming colonies produced by other *Verticillium* species such as *V. tricorpus* and *V. longisporum*, that can coexist in the soil (Goud et al. 2003), leading to an overestimation of *V. dahliae* inoculum in the soil. Fortunately, molecular methods, for quantitative detection of *V. dahliae* using real-time polymerase chain reaction (PCR) have evolved in recent years (Atallah et al. 2007; Bilodeau et al. 2012). Real-time PCR methods are becoming the preferred diagnostic tool, as they rapidly and accurately differentiate and detect *Verticillium* species in soil (Debode et al. 2011).

Therefore, it is important to gain insights on the *Verticillium* species that are producing microsclerotia while potentially co-habiting with *V. dahliae* in Manitoba potato soils and contributing to Verticillium wilt. It is necessary to determine if the presence of microsclerotia-forming *Verticillium* species can lead to overestimation of *V. dahliae* propagule densities in soil, and which methods can be used to accurately quantify *V. dahliae* inoculum. Moreover, it is important to consider if the presence of other microsclerotia-forming *Verticillium* species represent a threat to potato crops in Manitoba. Although microsclerotia-forming *Verticillium* species such as *V. longisporum* are known for their detrimental effect in *Brassicaceae* crops (Karapapa et al. 1997), other species, such as *V. tricorpus* have been found infecting potato plants (Robinson et al. 2006). Consequently, it is important to determine if any other microsclerotia-forming *Verticillium* species in Manitoba are pathogenic to potato.

Effective determination of *V. dahliae* propagule density in the soil, acquired through the results of this project, would help increase producer's probability of obtaining a maximum market value for their crop, by making an informed decision of which management strategy is more appropriate for the propagule density present in their potato fields.

1.2 GENERAL LITERATURE REVIEW

1.2.1 Verticillium wilt

Potato production is challenged by a broad diversity of fungal or fungal-like pathogens that persist in soil or in association with seed tubers (Fiers et al. 2011). Among the most important fungal diseases, Verticillium wilt has been recognized in many important potato producing areas in United States and Canada (Davis et al. 2001; Rowe and Powelson 2002). Verticillium wilt is a disease that affects the vascular tissues impeding water movement in the plant (Powelson and Rowe 1993a, b). The pathogens causing this disease occur commonly throughout potato-producing areas of the United States and Canada, which makes the disease endemic in many potato production areas (Powelson and Rowe 1993a).

The most important causal pathogens are the two fungi, *Verticillium dahliae* Kleb. and *V. albo-atrum* Reinke & Berthold. However, *V. dahliae* has been recognized as the most important causal pathogen (Rowe et al. 1987). Unfortunately, other pathogens can be involved, and cause what is called Potato Early Dying (PED) syndrome (Dung et al. 2014; Powelson and Rowe 1993b; Rowe et al. 1987). Nematodes have been commonly implicated (Rowe et al. 1987). The root lesion nematode, *Pratylenchus penetrans* (Cobb) has been found to interact with *V. dahliae* (MacGuidwin and Rouse 1990; Rotenberg et al. 2004). The synergistic interaction of the two pathogens cause severe development of Verticillium wilt symptoms and yield losses, even if the population density of both pathogens is low enough that either pathogen independently would have little or no effect

(Rotenberg et al. 2004). Other pathogens can also have a synergistic or additive interaction with *V. dahliae*. For instance, pectolytic bacteria *Pectobacterium carotovorum* subsp. *carotovorum* can cause similar symptoms to those caused by *V. dahliae*. However, when both pathogens co-infect a susceptible host, disease symptom development is greater (Dung et al. 2014).

Fields affected by Verticillium wilt exhibit chlorosis, necrosis, uneven death of lower leaflets, wilting and premature vine death during the period of greatest tuber development, reducing yield substantially (Rowe et al. 1987; Rowe et al. 1985). Verticillium wilt incidence develops over many years in long-established potato production areas in North America. In some cases, potato growers have come to consider early maturity a normal situation, and to accept lower yields (Rowe et al. 1987).

The economic impact of Verticillium wilt is large but also variable. In North America, Verticillium wilt can result in up to 50 % reduction in potato yield (Powelson and Rowe 1993b). Verticillium wilt is a real yield constraint in fields with a long history of potato production and intensive management (Rowe and Powelson 2002). Furthermore, the economic impact is very upsetting in fields planted with susceptible cultivars and when the crop is grown in short or continuous potato rotations (Davis et al. 1994). In Manitoba, growers have integrated management practices to reduce disease pressure such as longer crop rotation (3 years) to help manage the disease. However, Verticillium wilt is still a major problem responsible for lower yields and tuber quality (Selvanathan 2006; Shinners-Carnelley et al. 2003; Uppal et al. 2007). In Manitoba, eighty per cent of the

potato productive area is planted with the processing potato cultivar Russet Burbank. Although this cultivar is very important for the potato french fry industry, it is moderately susceptible to the pathogen *Verticillium dahliae* (Johnson and Dung 2010; Lynch et al. 2003; Pasche et al. 2013a; Rowe and Powelson 2002; Rykbost et al. 1990), contributing to the disease's detrimental effect.

Other aspects that have contributed to increased yield losses by Verticillium wilt are directly related to the agronomy of the crop. For instance, the large percentage of potato acreage cropped with cultivars moderately to highly susceptible to Verticillium wilt in productive areas in North America might has contributed to an increase in yield losses (Rowe and Powelson 2002) and increased *V. dahliae* inoculum density. Moreover, if a potato field has a short rotation (two-years), *V. dahliae* propagule density could have 2- to 4-fold higher populations than fields with longer rotations (Taylor et al. 2005).

1.2.2 *Verticillium dahliae*

Since the genus *Verticillium* was recognized by Nees von Esenbeckin in 1817, approximately 190 species have been designated under this genus (Inderbitzin et al. 2011; Isaac 1967; Pegg and Brady 2002). *Verticillium* is a member of the family *Plectosphaerellaceae*, which is in the phylum *Ascomycota* (Agrios 2005). *Verticillium* is a small genus, recently redefined with *V. dahliae* as the type species (Inderbitzin and Subbarao 2014). *Verticillium dahliae* belongs to the group of Deuteromycetes, fungi which do not have known sexual stage (Agrios 2005). Currently, the taxonomy of the genus embraces ten different species based on unique molecular and morphological

characteristics such as resting structure type and capability to cause wilt in plants (Inderbitzin et al. 2011).

Within the *Verticillium* group, *V. dahliae*, *V. albo-atrum*, *V. longisporum* and *V. tricorpus* are well known plant pathogens with their morphologies and geographic occurrences (Inderbitzin et al. 2011; Karapapa et al. 1997; Pegg and Brady 2002). The other species that belong to the *Verticillium* genus are: *V. isaacii*, *V. klebahnii*, *V. zaregamsianum*, *V. alfalfae*, *V. Nonalfalfae*, and *V. nubilium* (Inderbitzin et al. 2011; Inderbitzin and Subbarao 2014).

Verticillium dahliae is the most widely distributed member within the *Verticillium* group, with a broader host range than other pathogenic *Verticillium* species (Rowe et al. 1987). The number of hosts is close to 200, mainly dicotyledonous species including high-value annual and perennial crops (Johnson and Dung 2010) such as potato (*Solanum tuberosum*), tomato (*Lycopersicon esculentum* Mill.), cotton (*Gossypium hirsutum* L.) and strawberry (*Fragaria x ananassa*) (Pegg and Brady 2002).

Verticillium dahliae is both soil- and seed-borne. It produces only one cycle of disease and inoculum production during a single growing season (monocyclic disease) (Fradin and Thomma 2006). The primary inoculum of *V. dahliae* is a thick-walled-melanized and multicelled structure called microsclerotia. It serves as a resting structure allowing the fungus to survive in soil for up to 14 years (Lopez-Escudero et al. 2007; Pegg and Brady 2002; Wilhelm 1955).

1.2.2.1 Life cycle

The life cycle of *Verticillium dahliae* can be divided into three stages: dormant, parasitic and sometimes saprophytic. The dormant stage of *V. dahliae* is basically dominated by microsclerotia, which remain viable and dormant in the absence of excess carbon and nitrogen in the soil. Germination is activated when processes of fungistasis or mycostasis are interrupted, as nutrient concentrations rise with the presence of root exudates from either host or non-host plants (Huisman and Ashworth 1976; Mol 1995; Mol and Scholte 1995).

The parasitic phase of *V. dahliae* proceeds with the infection of a susceptible plant. The pathogen penetrates the susceptible host through the root tip or at the elongation region, which are areas where the root endodermis is not fully formed (Pegg 1981; Pegg and Brady 2002). The pathogen moves through the endodermis until it reaches vascular tissues. Once the vascular cylinder has been penetrated, the fungus produces conidia, which travel via passive transport through the xylem system (Pegg and Brady 2002). Following conidia sporulation in the root, adjacent stem vessel elements are colonized and fungal proliferation occurs in a cylindrical manner (Schnathorst 1981). Within a week after infection, fungal recognition by the host can occur, unless the host is susceptible, in which case, the pathogen overcomes this recognition, and continues colonization (Heinz et al. 1998). Xylem vessels are invaded, and hyphae grow and sporulation occurs. Conidia are dispersed within xylem, which becomes plugged (Rowe and Powelson 2002). Rapid infection soon follows, and plant begins to wilt and

symptoms of necrosis become visible, and the pathogens enters a saprophytic stage (Fradin and Thomma 2006).

1.2.2.2 Pathogen morphology

The resting structures found in the *Verticillium* genus are melanized hyphae, short chains of melanized rounded cells called chlamydospores, and microsclerotia (Issac 1953; Pegg and Brady 2002). Although some species are morphologically indistinguishable, the presence of microsclerotia has been used to differentiate *V. dahliae, V. longisporum, V. tricorpus* and recently *V. isaacii, V. klebahnii* and *V. zaregamsianum* from other species in the genus. The microsclerotia-forming *Verticillium* species are morphologically differentiated based on short conidia (*V. dahliae*), long conidia (*V. longisporum*) and presence of chlamydospores and resting mycelium (*V. tricorpus*, *V. isaacii*, *V. klebahnii*) (Inderbitzin et al. 2011; Issac 1949; Karapapa et al. 1997). The species *V. zaregamsianun*, *V. isaacii*, and *V. klebahnii* are morphologically indistinguishable from *V. tricorpus*, but may differ in pathogenicity, affecting lettuce and artichoke crops (Inderbitzin et al. 2011).

Other characteristics have been suggested to differentiate *Verticillium* species. Inderbitzin et al. (2011) indicated that the production of yellow hyphal pigments, while in culture, is common for the species *V. albo-atrum*, *V. tricorpus*, *V. isaacii*, *V. klebahnii*, and *V. zaregamsianum*. In contrast, *V. dahliae*, *V. nubilum*, *V. longisporum*, as well as the two recently described species *V. alfalfa* and *V. nonalfalfae*, which are morphologically similar to *V. albo-atrum*, do not produce such pigments.

1.2.3 Verticillium wilt management

Potato growers as well as research groups have been working on developing effective strategies to manage Verticillium wilt. Disease management is usually challenged by the persistence and nature of the microsclerotia in soil, the main infective propagules of *V. dahliae* under field conditions, and its interaction with a wide range of hosts and other microorganisms.

Resistant cultivars

Although use of resistant materials is a promising control method of Verticillium wilt (Jansky and Rouse 2000), its use is limited because no potato cultivar is completely resistant to Verticillium wilt (Simko and Haynes 2017). Despite this limitation, some cultivars have shown a relatively high level of partial resistance and late display of symptoms. Therefore, potato growers need more information about partial resistance to identify materials suitable for production (Simko and Haynes 2017). In addition to the general lack of resistance in commonly grown potato cultivars (Pasche et al. 2013b), potato crops are grown on contract; therefore, potato growers have limited choice in the cultivars they plant. Consequently, the moderately susceptible cv. Russet Burbank became the major cultivar growth in potato areas such as Manitoba, due to its desirable characteristics for the french fry industry, such as long and large tubers, low sugar ends, middle specific gravity, long storability and less bruising compared to other cultivars. In potato, partial resistance to Verticillium wilt is associated with the StVe1 resistance gene (Simko and Haynes 2017) However, no commercial potato cultivars have resistance to *V.*

dahliae. Though there are no resistant cultivars, certain cultivars such as Ranger Russet are tolerant to *V. dahliae*. Therefore, as resistant materials are unavailable, potato growers have to rely on other strategies to manage Verticillium wilt, such as elimination of microsclerotia of *V. dahliae*, or management practices that modify the rate of stem colonization by *V. dahliae* (Rowe et al. 1987).

Soil fumigation

Soil fumigation with broad-spectrum fungicides has become an attractive practice to manage Verticillium wilt, but both cost and environmental considerations have limited its use (Rowe et al. 1987). Despite the cost, there has been a growing interest in the application of metam sodium (Sodium methylaminomethanedithioate) for the control of *V. dahliae* in Manitoba. Soil fumigation with metam sodium has been the primary means of managing Verticillium wilt in many potato producing regions in the United States (Gudmestad et al. 2007; Rowe and Powelson 2002; Tsror et al. 2005). In some potato areas of the United States, soil fumigation with metam sodium has been combined with crop rotations, which has resulted in more reduction of Verticillium wilt (Gudmestad et al. 2007).

The efficacy of metam sodium in the control of Verticillium wilt has been inconsistent due to different factors such as application rate (Tsror et al. 2005), warm soil (temperatures between 13-15 ºC) at the time of application (Pasche et al. 2014), propagule density of *V. dahliae* (Gudmestad et al. 2007; Taylor et al. 2005), and concentration of the active ingredient (Molina et al. 2014).

Soil amendments

The use of organic amendments has been seen as an environmental alternative to improve soil quality, crop yield, plant health, and natural suppressiveness of soil against several phytopathogens (Lazarovits 2001). There is a growing interest in alternative management practices that could help to reduce the amount of pesticides used in food production, and ultimately production costs. Previous studies have shown successful disease suppression after application of soil amendments, such as green manure crops (Davis et al. 1996; Ochiai et al. 2008), liquid swine manure (Conn et al. 2005; Tenuta et al. 2002), meat and bone meal (Tenuta and Lazarovits 2004) and compost (LaMondia et al. 1999; Molina et al. 2014).

Among the organic amendments, compost is the one of the most suppressive material (Bonanomi et al. 2007). In Manitoba, the suppressive effect of organic amendments, green manures and composted cattle manure were compared (Molina et al. 2014). In those studies, mustard seed meal reduced microsclerotia inoculum and incidence of Verticillium wilt, and application of composted cattle manure resulted in reduced incidence of Verticillium wilt and higher potato yield. Other studies have also proven the potential beneficial effect of composted animal manures in cv. Russet Burbank and cv. Umatilla Russet in both saline and non-saline soils (Tenuta et al. unpublished).

Compost is agronomically attractive to many potato growers because it provides organic matter and serves as a nutrient source. The addition of composts to soils promotes

favorable soil physical-chemical properties such as: improved soil structure, water infiltration and retention capacity, buffering capacity, nutrient status as well as suppressive effect on many diseases caused by soilborne pathogens (Hoitink and Fahy 1986; Miller et al. 2012; Tester 1990). The use of compost has been suggested to decrease the incidence of disease caused by soilborne pathogens (Litterick et al. 2004). Despite the reported benefits of compost, some potato growers have also challenged the use of compost due to the potential introduction of unwanted pathogens and weeds. These undesirable traits are common when using immature compost that has not undergone proper biological decomposition of organic materials responsible for chemical stabilization and the sanitation of plant pathogens and weed seeds (Noble and Roberts 2004).

Several mechanisms have been associated with effective disease suppression using composted materials. For instance, compost can delay the decline of photosynthesis of leaves due to infection or age (Gent et al. 1999), alter the mineral composition of roots and leaves to reinforce the response to *V. dahliae* (LaMondia et al. 1999), introduce plant growth-promoting rhizobacteria reducing development of Verticillium wilt symptoms (Markakis et al. 2008), or maintain low *Verticillium* propagule density in soil (Molina et al. 2014). It has been suggested that the ability of compost based on animal manures to control Verticillium wilt can also be attributed to the ability of beneficial microorganisms present in the compost such as *Pseudomonas fluorescens* and non-pathogenic *Fusarium oxysporum,* to be established in the rhizosphere and reduce the severity of the disease (Malandraki et al. 2008).

The suppressive effect of compost varies with feedstock-origin, compost maturity, application-rate of the composted material (Bonanomi et al. 2007; Litterick et al. 2004; Yadessa et al. 2010) and timing and number of applications (Bailey and Lazarovits 2003). It has been reported that not all composts have the capability to exert beneficial effects; instead, they have been either ineffective or disease conducive to some pathogens (Bonanomi et al. 2010; Noble and Coventry 2005). From this, it emerged that composted animal manures have great potential for the control of Verticillium wilt but, at the same time, more investigation on application rates and alternative composted materials is needed to make the use of compost more likely.

1.2.4 Quantification of *Verticillium* propagules in soil

Effective management of Verticillium wilt relies on either changing the susceptibility of the host or reducing the population of *V. dahliae* in soil. Most potato growers in Manitoba need to fulfill market demands growing the processing potato cv. Russet Burbank. This situation drives potato growers to look for management practices that could help them to reduce *V. dahliae* levels. Implementation of any disease management practice begins with a good understanding of the conditions favorable for microsclerotial production, the threshold levels at which the pathogen causes disease, and the relationship between disease severity and propagule density (Pegg and Brady 2002). In cv. Russet Burbank, soil inoculum density of *V. dahliae* higher than 2 cfu g^{-1} can increase the incidence of stem infection by *V. dahliae* to 70 - 100% depending on the differences in aggressiveness of *V. dahliae,* physical and biological micro environment of

the root systems, the physiology of individual plants and the variability of the method used to estimate inoculum density of *V. dahliae* (Nicot and Rouse 1987). However, understanding of these factors associated with microsclerotial inoculum production, is not possible without an accurate quantification method.

Detection and proper quantification of potato fungal pathogens such as *V. dahliae* can be extremely difficult due to the hidden condition of the microsclerotia in the soil, the low levels of pathogen inoculum that could be enough to cause infection (Schaad and Frederick 2002), the similarity of propagules among different species (Inderbitzin et al. 2011), heterogeneous pathogen distribution patterns (Brierley et al. 2009), and the inaccuracy of the classical diagnostic methods (Fiers et al. 2011). Overall, these factors have contributed to the development of a number of methods to quantify *Verticillium* propagules, from traditional plating methods to the most recently developed molecular methods.

1.2.4.1 Traditional plating methods

Several plating methods have been described for quantifying *V. dahliae* in soil. Quantification of *V. dahliae* has been traditionally performed using classical dry or wet plating and incubation on semi-selective media (Kabir et al. 2004; Pegg and Brady 2002). Traditional methods involved spreading a known amount of soil onto a Petri plates of semi-selective media for *V. dahliae*, followed by incubation, which enables the microsclerotia to germinate and form colonies in the media (Goud and Termorshuizen 2003). However, despite the efforts to optimize these methods, only 30-50% of the

microsclerotia can be detected from soil samples (Goud and Termorshuizen 2003; Termorshuizen et al. 1998).

- **Dry plating**

Dry plating methods use an Andersen Air Sampler to distribute soil on the media (Butterfield and DeVay 1977). Soil is drawn by an air current through sieve plates with 1.18 and 0.83 mm openings, to allow homogeneous distribution onto Petri plates of semi-selected media. As an alternative to the Andersen Air Sampler, soil can be sprinkled by hand, randomly over a Petri plate (Goud and Termorshuizen 2003).

- **Wet plating method**

Wet plating method usually uses five grams of air-dried soil. The wet plating method suspends the soil in water agar and spreads one mL aliquots of the suspension over ten agar plates for each sample. After soil sample is plated, the incubation period can go from three to four weeks incubation period. Fewer microsclerotia colonies are observed when using larger amounts of soil (Goud and Termorshuizen 2003). This is due to the fact that semi-selective media still sustain the growth of a large number of other organisms that could compete for space and nutrients, or simply act antagonistically against *V. dahliae* (Goud and Termorshuizen 2003).

Although the wet plating method does not have lower detection limits than the dry plating method (Termorshuizen et al. 1998), the wet plating method has been commonly used for the detection of *V. dahliae* propagule density in soil in many potato regions

including Manitoba, where plant pathology laboratories have traditionally used only the wet plating method.

Overall, the wet plating method is very time consuming, and involves large areas for processing. The analysis starts with a soil drying stage from one to up to six weeks time period to kill conidia and mycelial fragments of *V. dahliae*. Then, soil is spread onto a Petri plates containing semi-selective media. However, the wet plating method is also subject to low reproducibility as germination of viable microsclerotia could suffer a temporary inhibition by fungistatic dormancy forces imposed by antagonistic microorganisms (Butterfield and DeVay 1977) or the presence of low concentrations of toxic compounds such as 2-propenyl isothiocyanate (2-propenil ITC) (Molina 2009). Such fungistatic dormancy could require up to at least five weeks of incubation before microsclerotia rebound and form colonies (Molina et al., unpublished data).

Additionally, reproducibility of the wet plating method has been limited by other factors like the brand of the agar (Goud and Termorshuizen 2003) and polygalacturonic acid (Kabir et al. 2004), the carbon source used to favor growth of *Verticillium* spp in the semi-selective media (Goud and Termorshuizen 2003), and pH of the media (Molina et al. unpublished data). Poor reproducibility of the wet plating method was reported in a multiple lab experiment where underestimation of *V. dahliae* microsclerotia were common in those labs using the wet plating method (Termorshuizen et al. 1998).

- **Sedimentation and flotation techniques**

For this method, the specific density of microsclerotia is used to separate them from the bulk soil (Goud and Termorshuizen 2003). When soil samples are resuspended in saturated solution of sucrose and then centrifuged, microsclerotia separate from smaller soil particles and conidia of soil fungi by sedimentation and decanting processes (Evans et al. 1967). Then, the supernatant containing most of the microsclerotia is decanted, sieved, resuspended in tap water and plated (Goud and Termorshuizen 2003).

Although these methods have been used in risk analysis studies, they have several limitations associated with processing time and accuracy (Goud and Termorshuizen 2003). The results obtained through these methods can be influenced by several factors, including amount of soil plated, soil characteristics (e.g. texture), culturable soil microbiota, and the skill of operators to differentiate typical star-shape microsclerotia colonies formed from plated *V. dahliae* microsclerotia (Goud and Termorshuizen 2003; Termorshuizen et al. 1998).

Termorshuizen et al. (1998) reported the difficulty of distinguishing the microsclerotia-forming colonies produced by *V. dahliae* from those of *V. tricorpus*, among different specialized laboratories conducting traditional quantification of *V. dahliae* in soil. In fact, isolates producing microsclerotia have been erroneously and recurrently classified as *V. dahliae* (Barbara and Clewes 2003). This means that quantification of *V. dahliae* may be prone to overestimation, considering that soil can contain multiple microsclerotia-

forming *Verticillium* species, such as *V. dahliae*, *V. longisporum*, and *V. tricorpus* (Goud et al. 2003; Klosterman et al. 2009).

Overestimation of *V. dahliae* levels in soil presents another possible drawback for disease management, as practices such as soil fumigation could be decided based on incorrect levels. As a result, potato growers could experience increased production costs and environmental drawbacks, as fumigation eliminates a large fraction of beneficial microorganisms (Kinkel 1993). This is potentially unfavorable, as the presence of *Verticillium* species that don't harm potato has been suggested as a biological control agent against *V. dahliae* (Robinson et al. 2007). *Verticillium tricorpus* is usually found simultaneously with *V. dahliae* (Robinson et al. 2007), and has also been reported as a potential biological control (Davis et al. 2000).

1.2.4.2 Molecular methods

Quantification of soilborne pathogen inoculum affecting potato has been recently facilitated by the implementation of polymerase chain reaction (PCR) methods, either in conventional or real-time PCR (Brierley et al. 2009; Debode et al. 2011; Gudmestad et al. 2007). Conventional PCR was initially used to overcome the factors that limit the utility of the traditional methods including lack of duplication, lengthy process, and difficult and subjective quantification due to the growth of fungal species such as *V. tricorpus*, *Colletrotrichum coccoides*, *Helminthosporium* spp., and *Alternaria* spp. (Mahuku and Platt 2002).

During PCR, a large number of identical copies of a specific target DNA sequence are generated, so that it can readily be analyzed. Conventional PCR does not quantify the concentration of DNA target immediately. Instead, it quantifies the concentration of PCR product produced as a result of a successful amplification, and relates this to the concentration of target DNA initially present at the start of the reaction. The drawback of this procedure is slight variation in the amplification phase can generate different concentrations of final product from exactly the same concentration of DNA template (McCartney et al. 2003).

PCR-based quantification was improved and simplified with the introduction of real-time PCR technology. In real-time PCR, accumulation of PCR products can be measured automatically after each cycle while the phases of the reaction are monitored. Real-time PCR used specific fluorescent probes or non-specific fluorescent DNA binding dyes to monitor amplification of DNA in real-time (Rasmussen 2001). As a result, fluorescent signals of the labeled amplification products are used in real-time PCR as the detection system (McCartney et al. 2003). Real-time PCR uses either intercalating fluorescent dyes such as SYBR® Green or fluorescent probes (TaqMan®) to measure the accumulation of amplicons in real-time during each cycle of the PCR.

In real-time PCR, the initial concentration of target DNA (template) is estimated based on the number of cycles required for amplification to exceed background (Rasmussen 2001); this point during the amplification is the cycle threshold, described as that cycle number at which a statistical significant increase in fluorescence is detected (McCartney

et al. 2003). Then, the target DNA is quantified by building a calibration curve that relates the cycle threshold to known concentrations of template DNA. This approach is used to measure expression of taxonomic and functional gene markers, as well as to quantify relative abundances of the fungus in the soil using taxon-specific real-time primers (Fierer et al 2005; Rastogi and Sani 2011).

Real-time PCR has become the preferred diagnostic method for *V. dahliae* quantification as it provides an accurate quantification of the pathogen's inoculum with high sensitivity (Bilodeau et al. 2012). In fact, several real-time PCR assays have been developed for the specific detection and quantification of *V. dahliae* in different hosts and soil (Gramaje et al. 2013). Real-time PCR assays have effectively used to quantify *V. dahliae* in spinach seeds (Duressa et al. 2012), tomato plants (Lievens et al. 2006), breeding potato germplasm (Atallah et al. 2007; Pasche et al. 2013b), olives (Markakis et al. 2009), strawberry (Bilodeau et al. 2012), and cabbage (Banno et al. 2011) fields.

Optimal detection and quantification of fungal plant pathogens using real-time PCR assays will largely depend upon high yield and quality of target DNA (Bilodeau et al. 2012; Pérez-Artés et al. 2005), and developing pathogen-specific DNA primers (Duressa et al. 2012; Li et al. 1999). As these elements converge together in a real-time PCR assay, a reliable alternative to the traditional plating method could become available, with enhanced reliability, specificity and the potential to give potato producers analysis results within days compared to the six to eight weeks with the traditional methods.

Soil DNA extraction

Soil DNA extraction is the starting point for the development or optimization of real-time PCR assays to detect pathogens (Sanzani et al 2014). DNA may be either contained within living or dead cells or may exist as extracellular DNA released from organisms during life or death (Wackernagel 2006). Therefore, extraction protocols are mainly designed to obtain DNA from all kind of cells present in the soil plus extracellular DNA (Total DNA). Extraction methods use relatively harsh steps suitable to efficiently disrupt the resting structures and all sort of microbial cells and even spores. Some of the most common methods are ultrasonic treatments, mechanical grinding (bead-mill), or hot sodium dodecyl sulfate (SDS) (Wackernagel 2006).

Extraction protocols need to consider the type of matrix (e.g., soil, manure, compost) and its characteristics, such as physical properties and chemical composition of the soil sample. The extraction of DNA from soil is complicated due to the presence of clay particles, humic substances and organic matter, to which DNA molecules can bind right after lysis (Hirsch et al 2010). Depending on the soil pH and CEC (Cation Exchange Capacity), DNA can be strongly adsorbed to minerals due to its negative charge. For instance, soils with divalent cations such as Ca^{2+} and Mg^{2+} are more prone to DNA adsorption than soils with monovalent cations, due to promotion of the bridging effect. Similarly, clay soils are more likely to have problems during the DNA extraction than sandy soils due to the presence of higher organic matter content and their hygroscopic propensity to bind water (Hirsch et al 2010).

Co-extraction of humic acids or other inhibitory substances may occur while DNA of pathogens is extracted from soil samples. In this case, several modifications of DNA extraction protocols have been developed such as the use of resin columns (Cullens and Hirsch 1998) or acid washed sand during the grinding process and skim milk (Pérez-Artés et al. 2005). Furthermore, DNA extraction and purification have been improved in order to commercialize reliable extraction kits. Commercial DNA extraction kits have been used to recover pathogen specific DNA from soil samples and eliminate potential PCR inhibitors (Whitehouse and Hottel 2007). According to Whitehouse and Hottel (2007), the commercial UltraClean™ extraction Kit (now called DNeasy PowerSoil™, QIAGEN) resulted in the most consistent and sensitive kits evaluated with a range of soils with different levels of soil pathogen. Similarly, the Power soil® DNA extraction kit was successfully used to extract DNA from microsclerotia from soil samples naturally infested and artificially inoculated with *V. dahliae*, *V. tricorpus* and *V. longisporum* (Debode et al. 2011).

Pathogen-specific DNA primers

The second important component in a real-time PCR assay is the development of pathogen-specific DNA primers (Duressa et al. 2012; Li et al. 1999). The selection of appropriated target DNA regions is also critical for the successful development of the primers. Such DNA regions should not contain intraspecific variation that would reduce sensitivity of the detection, as well as, be sufficiently variable to enable the differentiation of closely related species. Overall, a suitable target gene should be readily be amplified, sequenced, and multi-copied for sensitive detection (Kumar et al. 2016)

Over the last few years, several primers and real-time PCR protocols have been developed for *V. dahliae* (Gramaje et al. 2013). Sequences of the internally transcribed spacer region (ITS) of the ribosomal DNA (rDNA) have been used to design real-time PCR primers for different fungal pathogens in potato (Atallah et al. 2007; Banno et al. 2011; Cullen et al. 2001, 2002; van de Graaf et al. 2003). Atallah et al. (2007) developed a real-time PCR assay to detect and quantify *V. dahliae* in potato tissue using the designed primer pair VertBt-F and VertBt-R derived from the ß-tubulin gene. The fact that the nuclear gene ß-tubulin in *V. dahliae* occurs in single copy (Duressa et al. 2012), allows consistent pathogen quantification regardless of age and growth stage of the pathogen (Atallah et al. 2007). However, one potential limitation of the VertBt-F/R primer set could be the cross-amplification with *V. longisporum* (Duressa et al. 2012; Gramaje et al. 2013).

Primers have also been developed focusing on the intergenic spacer (IGS), region of rDNA occurring in multicopies (Bilodeau et al. 2012; Gramaje et al. 2013). Real-time PCR assays using primers that target high-copy-number sequences have reduced potential nonspecific amplification, and increased specificity and sensitivity (Bilodeau et al. 2012). However, primers targeting high-copy-regions have also shown cross-amplification with *V. tricorpus*, *V. albo-atrum* and *V. longisporum* due to the fact that the number of rDNA copies in a microorganism varies with its age and stage of growth (Gramaje et al. 2013).

Bilodeau et al. (2012) published a Taq-Man real-time PCR assay using the primers Vd-F929-947 and Vd-R1076-1094 based on the IGS region, which was used for the detection of *V. dahliae* in strawberry soil samples. Although this study reported variation in the copy number, from 24 to 73 rDNA copies, the variability of copy number variation among isolates of *V. dahliae* did not affect the accuracy of the assay using the Vd-F929-947 and Vd-R1076-1094 primers (Bilodeau et al. 2012). Other genes have been used to design specific primers as well. The trypsin protease gene (VTP_1) was used to detect and quantify *V. dahliae* in potato stem (Pasche et al. 2013b). Although sequences of VTP1 cDNA have been identified in a sequence analysis of genes that promote microsclerotia formation, this gene is present in *V. dahliae* isolates as well as *V. albo-atrum* and *V. tricorpus* (Dobinson et al. 2004).

The protocols by Atallah et al. (2007) and Bilodeau et al. (2012), were the most accurate, specific and efficient among eight real-time PCR protocols used for the detection of *V. dahliae* in olive trees during a study that compared real-time assays developed in the last 10 years (Gramaje et al. 2013). According to Gramaje et al. (2013), the detection limit for the two assays was very sensitive at 18 fg and 15 fg of *V. dahliae* DNA, respectively.

Real-time PCR assays for other *Verticillium* species have been developed, due to the economic importance of closely related species to *V. dahliae*, including *V. albo-atrum*, *V. longisporum* and *V. tricorpus*. For instance, Debode et al. (2011) conducted diagnostic studies that aimed for the development of real-time PCR assays for *V. tricorpus* and *V. longisporum*. They used primers for real-time PCR assays designed to target the

ribosomal DNA internal transcribed spacer for *V. tricorpus* and the ß-tubulin gene for *V. longisporum*. The assays were tested with artificially and naturally infested soil and showed high reproducibility and sensitivity. Later, Bilodeau et al. (2012) developed a TaqMan® assay to help identify colonies of *V. tricorpus* commonly found growing on Petri plates plated with soil that had been planted with lettuce. The primers for that study were designed based on the IGS region; however, the primers failed to detect *V. tricorpus* isolated from regions geographically distanced, which are genetically distinct from the California lettuce isolates (Bilodeau et al. 2012).

1.2.5 Situation in Manitoba

Growers and processors have noticed that commercial potato fields in Manitoba with a long history of potato production and sometime under short rotations, have failed to increase yield despite increased inputs. While potato growers have observed that those fields die early, with symptoms of Verticillium wilt, there has been a growing interest for treatments and best management practices for swine manure to mitigate water quality issues related to phosphorus (eutrophication) from livestock operations in Manitoba.

Composting offers a potential method to reduce the application of manure phosphorus onto the surrounding hog operation land and reduce the risk of excess P accumulation in that land. For instance, composting separated solids from liquid pig slurry concentrates N and P, kills animal pathogens, and may allow moving the nutrient enriched product to other agricultural areas where the final product can be used to manage soilborne pathogens, increase crop performance or improve soil quality, which is the case in the

potato areas. Two consecutive yearly applications of composted cattle manure has shown great potential in Manitoba for increasing potato yield and reducing Verticillium wilt incidence (Molina et al. 2014). Similar results were found when using composted cattle manure in fields planted with cv. Russet Burbank and cv. Umatilla Russet (Tenuta et al. unpublished). However, it is still unclear if other composted materials such as separated pig slurry solids, or if single applications of composts can be enough to reduce disease pressure.

In Manitoba, Verticillium wilt is caused by *V. dahliae*. Although *V. albo-atrum* has been associated with Verticillium wilt in productive areas of Southern Canada (Rowe et al. 1987), a survey conducted in Manitoba reported that *V. dahliae* is the causal pathogen associated with Verticillium wilt (Desjardins et al. 2003). *Pratylenchus* spp has been reported in several fields in Manitoba (Tenuta et al. unpublished). However, the species identified in Manitoba potato fields is *P. neglectus*, which does not reproduce on potato cv. Russet Burbank (Mahran et al. 2010). *Verticillium dahliae* isolates collected in Manitoba have a high degree of pathogenic variability, according to their ability to colonize potato cv. Russet Burbank plants and cause wilt and browning of the vascular tissue (Uppal et al. 2007).

Potato production is continuously under increased pressure by industry and consumers who demand safer and more environmentally sustainable practices to be incorporated into the potato production chain, without affecting the demanding standards for tuber quality (Pasche et al. 2014). Potato is one of the most pesticide-demanding agricultural

crops (Kromann et al. 2014). In this context, Manitoba potato producers are currently looking for environmentally-sound and cost-effective strategies to control *V. dahliae*, from incorporation of organic amendments to application of soil fumigants such as metam sodium. However, in order to use chemical pesticides conscientiously and to adopt alternatives to the use of pesticides, potato growers need accurate assessments of *V. dahliae* propagule density in their fields. For instance, soil fumigation may be used to reduce high levels of inoculum (Rowe et al. 1987), but it is important to know if those high levels of inoculum correspond to *V. dahliae* specifically, or to other similar microsclerotia-forming *Verticillium* species, which do not cause Verticillium wilt.

Inoculum levels of *V. dahliae* are currently estimated in Manitoba by traditional plating methods, which do not always include morphological examination of the *Verticillium* colonies. Morphological identification is only possible when the resting structures microsclerotia, chlamydospores, and dark mycelia, and yellow hyphal pigmentations are present (Inderbitzin and Subbarao 2014). Unfortunately, these characteristics are unstable and may disappear in laboratory cultures (Inderbitzin et al. 2011). Therefore, the lack of an accurate identification of the microsclerotia-forming colonies could potentially lead to overestimation of propagule density, if other similar microsclerotia-forming *Verticillium* species coexist in the same soil. Additionally, improper quantification of *V. dahliae* levels in potato fields can delay advances in development and adoption of effective practices for the control of Verticillium wilt. And ultimately, it can lead to high production costs, yield reduction, and disease dissemination as well.

DNA-based identification is becoming a widely used diagnostic tool. It offers a more accurate and sensitive method for inoculum quantification. However, there has not been any study in Manitoba that shows the usefulness of molecular methods for the quantification of *V. dahliae* inoculum in soils.

1.3 HYPOTHESES

The first hypothesis for this research was that single applications of composted cattle manure or composted separated pig slurry solids, or the soil fumigant metam sodium can effectively suppress Verticillium wilt and increase marketable yield in Manitoba potato fields. The second hypothesis for this research was that traditional wet plating method does not quantify propagule density of *V. dahliae* in soil accurately, due to the occurrence of other microsclerotia-forming *Verticillium* species that may not be pathogenic to potato. An alternative method of quantifying *V. dahliae* propagule density in soil using real-time PCR should more accurately relate to Verticillium wilt severity.

1.4 OBJECTIVES

This hypothesis was examined based on three major research objectives:

i) Evaluate the effect of single-application rates of composted cattle manure and composted separated pig slurry solids, and two application rates of metam

sodium on Verticillium wilt, and potato yield of cv. Norland. and cv. Russet Burbank.

ii) Investigate the presence and quantity of microsclerotia-forming *Verticillium* species and its relation to Verticillium wilt in commercial potato fields in Manitoba, and to evaluate the use of published real-time PCR methods for detection and quantification of *V. dahliae* and other microsclerotia-forming *Verticillium* species possibly present.

iii) Determine the role of microsclerotia-forming *Verticillium* species *V. tricorpus* found in Manitoba in the development of Verticillium wilt symptoms in commonly planted processing potato cv. Russet Burbank.

1.5 STRUCTURE OF THIS THESIS

This thesis document starts with a general introduction and a literature review chapter (Chapter 1) describing important elements of Verticillium wilt in potato such as causal pathogen and methods for pathogen quantification. Additionally, this chapter describes control methods to manage the disease and the current situation of Verticillium wilt in Manitoba.

A field study is presented in Chapter 2: “Field evaluation of metam sodium and composted animal manures for the control of Verticillium wilt of potato in Manitoba”. It describes three experiments conducted between 2012 and 2013, including eight different sites on commercial potato fields in Manitoba planted with the cv. Russet Burbank or cv.

Norland. In that chapter, the effect of three different rates of composted cattle manure and composted separated pig slurry and two rates of soil fumigant metam sodium on Verticillium wilt of potato was evaluated under field conditions.

Chapter 3 is an examination of *Verticillium* species producing microsclerotia in potato fields from Manitoba with the study: "Quantification of microsclerotia-forming *Verticillium* species in potato fields in Manitoba". The chapter describes a set of real-time PCR assays selected for the detection of *V. dahliae*, *V. tricorpus*, and *V. longisporum* in potato soils. The selection of real-time PCR assays is supported on the development of collections with different *Verticillium* species received from other laboratories, and isolates found in Manitoba during the field study in Chapter 2. Chapter 3 also contains studies that support the applicability and advantages of the real-time PCR assays for the management of Verticillium wilt in potato.

Chapter 4: "Lack of pathogenicity and interaction of two isolates of *Verticillium tricorpus* with *Verticillium dahliae* to potato cv. Russet Burbank". In that chapter, *V. tricorpus* is examined for pathogenicity on potato cv. Russet Burbank and interaction with the plant pathogen *V. dahliae*. Chapter 4 describes a growth chamber study and outlines a set of parameters associated to the development of Verticillium wilt in potato used to evaluate pathogenicity of *V. tricorpus* found in Manitoba potato fields.

The final chapter (Chapter 5) presents as a summary and general discussion of the findings of the thesis as a whole. It includes application and implications for these studies and presents consideration of future directions.

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CHAPTER 2

2. FIELD EVALUATION OF COMPOSTED ANIMAL MANURES AND METAM SODIUM FOR THE CONTROL OF VERTICILLIUM WILT OF POTATO IN MANITOBA

2.1 ABSTRACT

Verticillium wilt can severely reduce yields in potato production areas in North America. In Manitoba, the major causal agent of Verticillium wilt is the fungus *Verticillium dahliae.* We previously reported that composted cattle manure reduced Verticillium wilt incidence and increased potato yield in Manitoba. The objective of the current study was to evaluate the effect of different rates of composted separated pig slurry solids (CSS) and composted cattle manure (CCM) and the soil fumigant metam sodium on the density of *Verticillium* propagules in soil, frequency of *Verticillium* in plant tissue, Verticillium wilt severity and potato yield. The study had three and two sites planted to cultivar Russet Burbank in 2012 and 2013, respectively, in addition to three sites planted to Norland in 2012. Treatments were soil-amended to three levels (20, 40 and 80 Mg ha^{-1}) of the two composted manures, two application rates of soil fumigant metam sodium at 374 and 561 L ha^{-1}, a high rate of synthetic fertilizer to simulate N-P-K addition with compost treatment, and a grower practice standard fertility that served as a control treatment.

Overall, metam sodium at 374 L ha^{-1}, effectively reduced *Verticillium* inoculum at planting by up to 40%, and increased marketable yield in cv. Russet Burbank by up to 24%, whereas marketable yield of cv. Norland was not improved. Surprisingly, the higher rate of metam sodium did not increase marketable yield. The disease suppressive effect of single applications of compost varied among compost types and rates. In cv. Russet Burbank, there was no effect on frequency of *Verticillium*. However, severity of Verticillium wilt was reduced with CCM at 20 Mg ha^{-1} or CSS 40 Mg ha^{-1}. In cv. Norland, frequency of *Verticillium* and Verticillium wilt severity was lower with CSS at 80 Mg ha^{-1}. Application of metam sodium reduced *Verticillium* propagule density before potato planting. Reduction of Verticillium wilt severity in composted and fumigated treatments was associated with increased available soil phosphorus and nitrate and concentration of calcium and magnesium in the potato plant.

2.2 INTRODUCTION

Verticillium wilt is a disease endemic in many potato production regions in North America (Rowe and Powelson 2002). The fungus *Verticillium dahliae* Kleb. is the major contributor agent to this disease in many potato regions (Pasche et al. 2014; Rowe et al. 1987), including Manitoba where the nematode *Pratylenchus penetrans* has not been detected (Mahran et al. 2010), and *V. dahliae* has been consistently found as the causal agent of Verticillium wilt of potato (Desjardins et al. 2003).

Fields affected by Verticillium wilt exhibit chlorosis, necrosis, uneven death of lower leaflets, wilting, and premature vine death (Rowe et al. 1987) during the period of greatest tuber development, reducing yield substantially (Rowe et al. 1985). Effects on potato yield are more detrimental with susceptible cultivars and when the crop is grown in short or continuous potato rotations (Davis et al. 1994b). In Manitoba, eighty percent of the potato productive area is planted with the processing potato variety Russet Burbank, which is moderately susceptible to *V. dahliae* (Jansky and Rouse 2000). Surveys conducted in 2002 to 2004 (Tenuta et al. unpublished) found that Verticillium wilt represents a serious threat for potato growers and processors in Manitoba.

Verticillium dahliae possesses particular attributes that make its control very challenging: broad host range (Pegg and Brady 2002), high pathogenic variability (Uppal et al. 2007), easy introduction into non-infested fields through infected tubers (Johnson and Dung 2010), and fungal resting structures called microsclerotia (Rowe et al. 1987).

Microsclerotia allow the pathogen to survive in the soil for long periods of time in the absence of a host (Huisman and Ashworth 1976), and serve as the main infective propagule under field conditions (Wilhelm 1955).

Microsclerotia are produced in colonized susceptible host tissue during senescence and continue to develop until stems dry (Slattery 1981). Microsclerotia embedded in crop residues hold viability longer than those existing free in soil (Johnson and Dung 2010). Consequently, release of microsclerotia from decaying crop residues and further increase of *Verticillium* inoculum density is observed within a two year period (Mol et al. 1996; Taylor et al. 2005). Decomposition of infected stem can release up to 9000 microsclerotia per gram of decaying material (Slattery 1981), challenging the cultural and chemical strategies used to reduce *V. dahliae* propagule density.

Reported disease thresholds for *V. dahliae* in North America range between 8 to 20 colony-forming units (cfu) per gram of soil (Davis and Sorensen 1986; Nicot and Rouse 1987). Potato growers have focused their efforts for controlling Verticillium wilt by reducing *V. dahliae* propagule density below soil threshold populations at which potato production is not affected by the disease (Johnson and Dung 2010; Powelson and Rowe 1993). Soil fumigation with products like chloropicrin, 1,3-dicloropropene, but mainly metam sodium has been a method of managing Verticillium wilt in many potato production regions (Gudmestad et al. 2007; Powelson and Rowe 1993; Rowe and Powelson 2002; Tsror et al. 2005). Metam sodium label-rates from 410 to 670 L ha^{-1} are recommended for soil application. However, the efficacy of metam sodium in the control

of Verticillium wilt has also been inconsistent in some cases due to different factors such as soil application rate (Pasche et al. 2014; Tsror et al. 2005) and concentration of active ingredient (Molina et al. 2014).

Potato production is continuously under increased pressure by processors and consumers, who to be incorporated into the potato production chain, without affecting the demanding standards for tuber quality (Pasche et al. 2014). While many soilborne diseases have been controlled, in part by the conscientious use of chemical pesticides, alternatives to the use of pesticides can be very valuable.

Cultural practices such as compost can be integrated with good agronomic practices, such as irrigation scheduling and soil fertility management, in order to reduce the impact of Verticillium wilt, by altering the conditions that benefit the development of *V. dahliae* (LaMondia et al. 1999). Although the suppressive effect of compost have been generally limited and variable under field conditions (Noble and Coventry 2005), application of composts can suppress soilborne diseases within the first season after application (Stone et al. 2004). In Manitoba, two consecutive years of application of 40 Mg ha^{-1} (Molina et al. 2014) and a single application of 80 Mg ha^{-1} (Tenuta et al. unpublished) of composted cattle manure resulted in reduced Verticillium wilt incidence and increased potato yield. For instance, compost delays the decline of photosynthesis of leaves due to infection or age (Gent et al. 1999), alters the mineral composition of roots and leaves to reinforce the response to *V. dahliae* (LaMondia et al. 1999), introduces plant growth-promoting rhizobacteria reducing development of wilt symptoms (Markakis et al. 2008), or

maintains low *Verticillium* propagule density in soil (Molina et al. 2014). It has been suggested that the ability of compost, based on animal manures, to control Verticillium wilt can be attributed to the ability of beneficial microorganisms present in the compost like *Pseudomonas fluorescens* and non-pathogenic *Fusarium oxysporum*, to establish in the rhizosphere and reduce the severity of the disease (Malandraki et al. 2008).

In addition to the potential disease suppressive effect of compost, composting offers a potential method to reduce the application of manure phosphorus onto the surrounding hog operation land and reduce the risk of excess P accumulation in that land. For instance, composting separated solids from liquid pig slurry concentrates N and P, and could allow moving the nutrient enriched product to other agricultural areas where the final product can be used to manage soilborne pathogens, increase crop performance or improve soil quality, which is the case in the potato areas.

In view of all the above, the objectives of this study were: (i) to evaluate the effect of single-application rates of composted cattle manure and composted separated pig slurry solids on Verticillium wilt, and potato yield of the fresh market potato cv. Norland and the commonly planted processing potato cv. Russet Burbank, and (ii) to examine the effect of two application rates of metam sodium that would reduce Verticillium wilt, and increase potato yield of the cv. Norland and the cv. Russet Burbank.

2.3 MATERIALS AND METHODS

2.3.1 Experimental design

Field trials were conducted in 2012 and 2013 in commercial potato fields across the major potato production areas of Southern Manitoba (Figure 2.1). Trials were planted to the fresh market potato cv. Norland, and the processing potato cv. Russet Burbank, with experimental plots (experimental unit) that were 6 m long x 4 m wide, separated by 3 m between plots and 20 m between blocks. All plots were GPS referenced in 2011 and were established in coordination with the potato grower at each site. GPS data at each site was available and used to establish experiments according to planting patterns at each location.

Experiment with cv. Norland

An experiment with three experimental trials established on non-irrigated commercial fields in the Rural Municipalities of Stanley (site G) and Rhineland (sites F and H) was planted to cv. Norland in 2012 (Figure 2.1). The experimental design at each trial (site) was a randomized completed block design. Each site had 10 treatments and four replications, for a total of 40 plots. Chemical and physical characteristics are presented in Table 2.1.

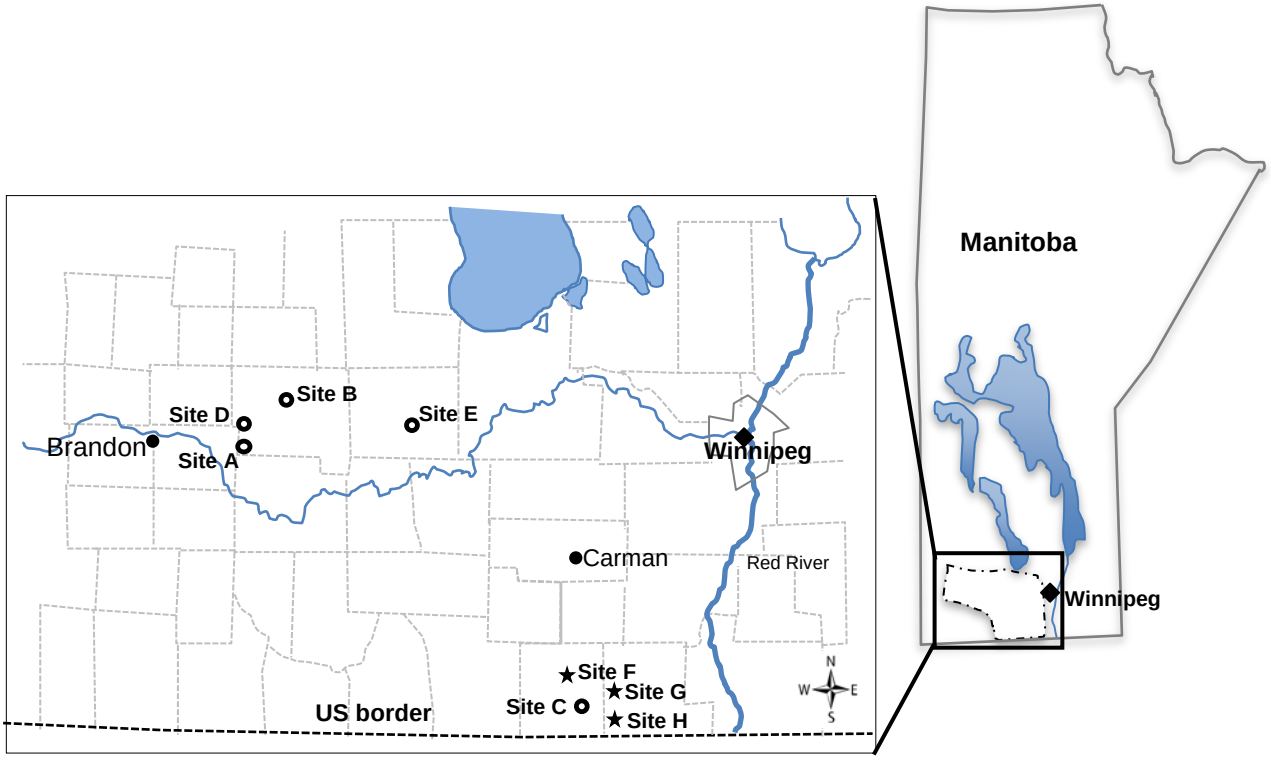

Figure 2.1 Location of the compost and soil fumigation trials. cv. Russet Burbank (O) sites A, B, C, D and E, and cv. Norland (★), sites F, G and H.

The following treatments were used: standard grower fertility practice (Appendix I) (Control); composted cattle manure 80 Mg ha^{-1} (CCM80); composted cattle manure 40 Mg ha^{-1} (CCM40); composted cattle manure 20 Mg ha^{-1} (CCM20); composted separated pig slurry solids 80 Mg ha^{-1} (CSS80); composted separated pig slurry solids 40 Mg ha^{-1} (CSS40); composted separated pig slurry solids 20 Mg ha^{-1} (CSS20); metam sodium (42% sodium methyldithiocarbamate, Vapam HL, Amvac Chemical Corporation) 374 L ha^{-1} (VAP40); metam sodium 561 L ha^{-1} (VAP60); and a high rate of synthetic fertilizer (FERT) (Table 2.2). All plots received the standard fertility management according to the grower's practices at each site (Appendix I). The treatment FERT received an additional

37.4 kg ha^{-1} N, 450 kg ha^{-1} P_2O_5, 62.7 kg ha^{-1} K_2O and 11.2 kg ha^{-1} sulfur (S), based on the potential nutritional contribution given by the highest rate of composted cattle manure. Cattle manure compost was kindly provided by Dr. Katherine Buckley at the Brandon Research station (AAFC), and separated pig slurry solid compost was provided by M.Sc. student, Jolene Rutter, at the Soil Science Department, University of Manitoba (Table 2.3).

Table 2.1 Chemical and physical properties from the commercial potato fields used in this study (depth 0-15 cm).

Year	Trials	N[a]	Olsen-P	K	S	Ca	Mg	Na	CEC	EC	pH	SOM	Texture			
		ppm							Meq $100g^{-1}$	dS m^{-1}		%	Clay	Sand	Silt	Class
													%			
cv. Standard Norland																
2012	**Site F**	6	19.6	195	32	3786	345	16	22.3	0.63	8.0	2.5	18	68	14	Sandy Loam
	Site G	14	19.9	240	100	3413	684	73	23.6	1.15	8.1	2.7	20	70	10	Sandy clay loam
	Site H	36	15.7	229	6	3228	312	17	19.3	0.67	7.9	2.6	15	74	11	Sandy Loam
cv. Russet Burbank																
2012	**Site A**	16	8	178	22	2669	616	29	19	0.62	7.8	2.8	11	80	8.6	Sandy Loam
	Site B	15	51	294	8	1238	205	8	13.8	0.30	5.5	2.7	17	70	13	Sandy Loam
	Site C	7	12	91	38	5987	585	71	35.2	2.52	7.8	4.4	25	50	25	Sandy clay loam
2013	**Site D**	7	21	200	2	980	120	13	8.1	0.21	6.2	1.5	9.7	82	8.3	Loamy sand
	Site E	29	24	230	57	4600	640	38	29.2	1.10	7.0	5.9	20	68	12	Sandy clay loam

[a] Extractable-N= Nitrate-N, Olsen extractable-P= sodium bicarbonate extractable-P; K= ammonium acetate extractable-K, water soluble S (SO_4^{-2}), CEC= cation exchange capacity; EC= electrical conductivity by 1:2 soil:water ratio method; pH (water extract); SOM: soil organic matter (acid digestion).

Table 2.2 Compost and soil fumigation treatments used during the study in potato commercial fields in Manitoba.

Description \ Potato material	cv. Norland	cv. Russet Burbank	
	2012	2012	2013
Standard fertility	Control	Control	Control
High fertility	FERT	FERT	FERT
Metam sodium 374 L ha^{-1} or 40 gal $acre^{-1}$	VAP40	VAP40	
Metam sodium 571 L ha^{-1} or 60 gal $acre^{-1}$	VAP60	VAP60	
Composted cattle manure 20 Mg ha^{-1}	CCM20	CCM20	
Composted cattle manure 40 Mg ha^{-1}	CCM40	CCM40	
Composted cattle manure 80 Mg ha^{-1}	CCM80	CCM80	
Composted separated pig slurry solids 20 Mg ha^{-1}	CSS 20		CSS 20
Composted separated pig slurry solids 40 Mg ha^{-1}	CSS 40		CSS 40
Composted separated pig slurry solids 80 Mg ha^{-1}	CSS 80		CSS 80

Table 2.3 Chemical analysis of composted cattle manure and composted separated pig slurry solids.

Parameter [a] \ Compost type	CCM [b]	CSS [c]	
		2012	2013
Bulk density (kg m^{-3})	701	473	521
Nitrogen (total) (g kg^{-1})	8.2	22.4	28.9
Phosphorus (total) (g kg^{-1})	2.3	13.1	2.4
Potassium (total) (g kg^{-1})	7.2	12.3	3.6
Sodium (g kg^{-1})	4.0	1.4	0.4
Organic matter (%)	20.3	43.8	48.1
Total ash (g kg^{-1})	796.9	561.6	393
Moisture (%)	34.2	55.1	60.6
C:N ratio	14:1	11:1	12:1
Total organic carbon (%)	11	24	34
pH (saturated)	7.2	6.4	5.5
Total solids (g kg^{-1})	658.5	448.7	394.3

[a] Results reported on a dry weight basis

[b] Composted cattle manure (CCM). Same product used in plots planted to cv. Russet Burbank-2012 and cv. Norland in 2012.

[c] Composted separated pig slurry solids (CSS). Two different CSS products were used in cv. Norland in 2012, and cv. Russet Burbank-2013, respectively.

Experiments with cv. Russet Burbank

Two experiments were established on irrigated commercial potato fields in 2012 and 2013, respectively. Each experiment had three and two different trials, respectively. Chemical and physical soil characteristics from the commercial potato fields are presented in Table 2.1. Experimental trials were a randomized completed block design. Unfortunately, composted separated pig slurry solids was not ready by the time trials were planted to cv. Russet Burbank in 2012. This was likely due to the fact that turning the compost in the winter with a windrow turner could expose the material to low air temperatures making it difficult to maintain thermophilic conditions required for the composting process (Rutter 2016). For this reason, a second trial was established, and a second batch of CSS was prepared during 2012 for the experiment established in 2013.

For the cv. Russet Burbank-2012 experiment, three trials were established on irrigated commercial potato fields. Two trials, sites A and B, were located in the Rural Municipality of North Cypress, and site C in the Rural Municipality of Stanley (Figure 2.1). Each trial in 2012 had seven treatments and four replications, for a total of 28 plots (Table 2.2). The treatments were: composted cattle manure 80 Mg ha^{-1} (CCM80); composted cattle manure 40 Mg ha^{-1} (CCM40); composted cattle manure 20 Mg ha^{-1} (CCM20), metam sodium 374 L ha^{-1} (40 gal $acre^{-1}$, VAP40); metam sodium 561 L ha^{-1} (60 gal $acre^{-1}$, VAP60); high fertility (FERT), and standard grower fertility practice (Appendix I) (Control). All plots received the standard fertility management according to

the grower's practices at each site (Appendix I). The FERT treatment received an additional 37.4 kg ha^{-1} N, 450 kg ha^{-1} P_2O_5, 62.7 kg ha^{-1} K_2O and 11.2 kg ha^{-1} sulfur (S), based on the potential nutritional contribution given by the highest rate of composted cattle manure.

In 2013, the cv. Russet Burbank-2013 experiment had two trials, sites D and E, located in the Rural Municipalities of North Cypress and North Folk, respectively (Figure 2.1). Each site had five treatments and four replications, for a total of 20 plots (Table 2.2). Chemical and physical characteristics are presented in Table 2.1. The following treatments were used in 2013: standard grower practice fertility (Appendix I) (Control); composted separated pig slurry solids 20 Mg ha^{-1} (CSS20); composted separated pig slurry solids 40 Mg ha^{-1} (CSS40); composted separated pig slurry solids 80 Mg ha^{-1} (CSS80); and high fertility (FERT), which received the same amount of nutrients supplemented for the treatment FERT in cv. Russet Burbank-2012.

2.3.2 Treatment application

In 2011, crop residues of wheat or canola were incorporated to 15-20 cm using a deep tiller or double disc cultivator in fall, before metam sodium treatments were applied in the fall of 2011. In the fall 2011, Metam sodium treatments were applied to site A on September 29, sites C and H on October 20, sites F and G on October 21 and Site B on October 22 (Figure 2.2). The product was injected using a commercial sweep injection system in sites A and B, and a unit built by Researchers at the University of Manitoba and AAFC in sites C, F, G and H, at a single depth of 20 cm with spray nozzles applying

the product directly into the soil. Metam sodium application was followed by compacters to smooth and pack the disturbed soil, and minimize methyl isothiocyanate (MITC) losses.

The soil temperature at the 15 cm depth for each site at time of application was 4, 4, 7, 7, 7, and 8 °C. Five soil samples from the upper soil (10-20 cm) were collected with a 2 cm diameter soil core probe at random from each plot before application to determine gravimetric moisture content 0.15, 0.15, 0.25, 0.17, 0.18, and 0.17 g water g^{-1} for sites A, B, C, F, G, and H respectively. Grower-cooperators at sites A and B were able to apply water within the first 30 min after application to prevent off-gassing and volatilization of MITC. Approximately 15-20 mm of water was applied via a pivot-irrigation system. This protocol could not be employed in the sites C, F, G, and H because growers did not have operational irrigation equipment at time of application (equipment stored for winter). For those sites, metam sodium was mixed with water (ratio 1:3) on-site and immediately applied at depth of 25 cm followed by packers to pack and seal the soil surface.

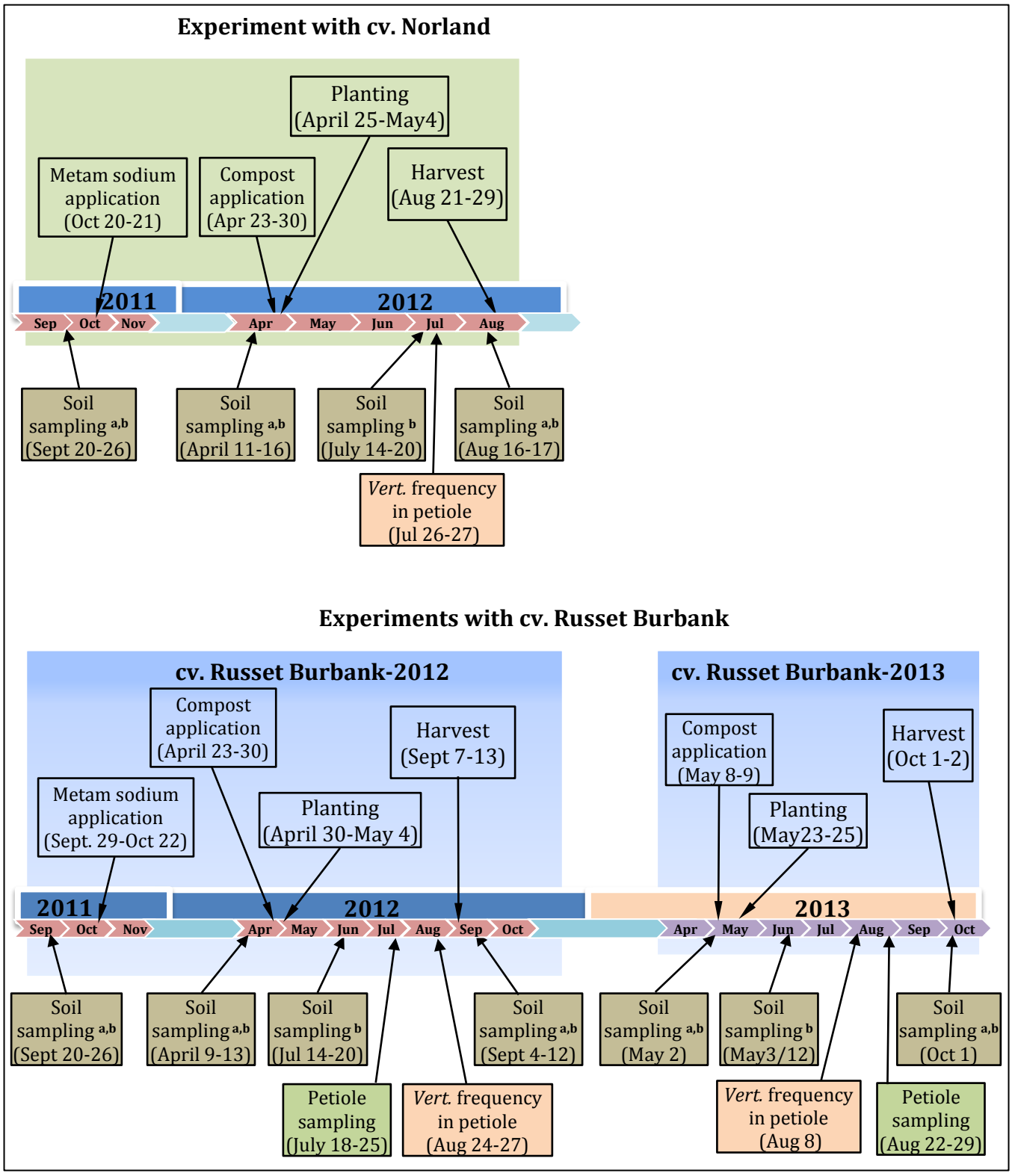

Figure 2.2 Experimental time-line in the cv. Norland and cv. Russet Burbank experiments. (Soil sampling purpose: ([a]) *Verticillium* propagule density, ([b]) soil chemical analysis).

The three rates of composted separated pig slurry solids and cattle manure were chosen based on the results of previous field trials showing the potential for composted cattle manures to reduce Verticillium wilt and increase potato yield (Molina et al. 2014). For compost chemical and physical analysis, three composite samples each comprised of ten cores were collected from each compost type used in 2012 and 2013. The core-samples were bulked, mixed by hand in a clean bucket, placed in polyethylene bags and transported on ice to the University of Manitoba, where samples were stored at 4 °C. Samples were stored for no more than 48h before sending to the commercial lab (A&L Canada Laboratories INC, London, ON) for analysis. Analysis of composted materials was for total nitrogen, total phosphorus, total potassium, sodium, organic matter, total ash, C:N ratio, total organic carbon, pH (saturated) and total solids (Table 2.3). Although two different batches of composted separated pig slurry solids were used in 2012 and 2013, most chemical properties were similar for both of them with the exception of phosphorus, potassium, and pH (Table 2.3).

On April 22 to May 4 2012, the separated pig slurry solids compost, cattle manure compost and fertilizer, for the CSS, CCM and FERT treatments (Figure 2.2), respectively, were spread by hand and incorporated into the first 20 cm with a rototiller before planting to potato. The compost and fertilizer for the CSS and FERT treatments, respectively, were applied the same way on May 8-9 2013. The FERT treatment included application of monoammonium phosphate (MAP) (254 kg ha^{-1}), potassium chloride (104 kg ha^{-1}), ammonium sulphate (47 kg ha^{-1}), and triple super phosphate (707 kg ha^{-1}). All plots at each site were rototilled to control experimental error.

2.3.3 Site preparation and management

In 2012, all sites were cultivated to 20 cm before application of compost treatments and planting. Sites A, B, C, F, G and H were planted on May 8, April 30, May 4, April 25, April 25 and May 4, respectively (Figure 2.2). Seed rows were 0.95 m apart, with four rows per plot, and within row spacing of 0.37 m. Center pivot irrigation systems were used to provide between 228 to 280 mm of water at each site planted to cv. Russet Burbank in 2012. Hilling was completed on 30 May 30, 22 May, 5 June, 4 May, 10 May and 21 May, for sites A, B, C, F, G, and H, respectively. Potato growers planted and hilled the sites.

In 2013, sites D and E were established in fields where residues of previous crops were incorporated in fall 2012 by growers. Fields were cultivated to 20 cm in late fall 2012 and spring 2013, before compost treatments were applied. Sites were planted on May 23 and 25, respectively (Figure 2.2). Potatoes were hilled on June 6 and 10. Other crop management practices followed appropriate standards for potato production in Manitoba. Synthetic fertilizers of NPK, that correspond to the standard fertility management, were applied to all plots by growers (Appendix I). Pesticides were applied by growers as required, to effectively control weeds, insects (Colorado potato beetle), and foliar diseases (late blight, early blight), using appropriate pesticides and rates (Manitoba Agriculture, Food and Rural Initiatives 2009).

2.3.4 *Verticillium* propagule density in soil

Verticillium propagule density in soil was determined three times at all sites planted to potato in 2012. Soil samples were collected on September 20-26, 2011 (before metam sodium application), April 9-13, 2012 (prior to planting potato on April 25-May 4, 2012) and on August 16-17, 2012 (cv. Norland) and September 6-12, 2012 (cv. Russet Burbank in 2012) (before potato harvest) (Figure 2.2). For the experiment with Russet Burbank in 2013, *Verticillium* propagule density was sampled twice, on May 2 and October 1, 2013, before potato planting and harvest, respectively.

Soil samples from each experimental unit were collected by taking 10 cores to a depth of 15 cm, with a 2.5 cm diameter soil probe and mixing them together. Composite samples were then placed in polyethylene bags, and transported on ice to the University of Manitoba. The samples were stored at 4 °C for one week before the *Verticillium* propagule density was determined by the wet plating method described by Molina et al. (2014).

A 200 g subsample of each soil sample was air-dried for seven days at room temperature and then 5 g suspended in 100 mL of sterilized agar-water (1%), and agitated for 2 min using an orbital shaker at 60 rpm. One milliliter of the suspension was placed onto a plate of Sorensen's NP-10 medium (Sorensen et al. 1991). Each sample was plated ten times. Plates were left open to eliminate the excess of water and then sealed with parafilm and incubated for 15 days in the dark at 22 °C. Plates were then rinsed with tap water to remove soil particles from the medium surface. Microsclerotia-forming colonies were

counted using a stereo microscope. Then, the individual counts from each plate were added together and multiply by 2, and expressed as colony forming unit (cfu) g^{-1} soil.

Additionally, the *Verticillium* propagule density change (%) was presented as the percent of change of *Verticillium* propagule density in soil before planting and harvest relative to the initial propagule density. The *Verticillium* propagule density change or inoculum change was calculated as ((cfu_f/cfu_i)x100), where $\mathbf{cfu_i}$= initial propagule density in fall-2011 (sites A, B and C) and Spring-2013 (Sites D, and E); and $\mathbf{cfu_f}$= final propagule density in Spring-2012 (only for VAP40, VAP60 and control treatments) and Fall-2012 (sites A, B and C) and Fall-2013 (Sites D, and E). Therefore, change in propagule density greater than 100% indicated an increase in *Verticillium* propagule density in soil compared to the initial inoculum density in Fall-2011 and Spring-2013 (cfu_i), for experiments cv Russet Burbank 2012 and 2013, respectively.

2.3.5 Frequency of *Verticillium* in petioles

The frequency of *Verticillium* in potato was determined according to Soltani et al. (2002). It was measured using petioles collected late in the growing seasons on August 24-27, 2012 (cv. Russet Burbank-2012), July 26-27, 2012 (cv. Norland) and Aug. 8 2013 (cv. Russet Burbank-2013) (Figure 2.2). Fifteen leaf petioles were collected, each from the lower third of each of 15 potato plants (single or group of several stems emerging from a single potato seed piece) in the middle two rows of each plot. A total of 720 and 600 petioles per site were collected from cv. Russet Burbank and cv. Norland trials, respectively. The petioles were rinsed with RO water and then sterilized by submergence

sequentially in 1.5% sodium hypochlorite, 95% alcohol and sterile water for one min each. Petioles were left to dry on sterile paper towels for five min in a laminar flow hood. Three sections from each petiole were plated onto Sorensen's NP-10 (Sorensen et al. 1991) containing chloramphenicol, streptomycin sulfate and chlortetracycline HCl. The plates were incubated at 22 °C in the dark for 15 days, and the presence or absence of *Verticillium* producing microsclerotia emanating from sections was determined using a stereo microscope. Frequency was calculated as the percentage of plants positive for *Verticillium*. A plant was rated as positive if *Verticillium* was present in at least one petiole section of the three plates.

2.3.6 Assessment of Verticillium wilt disease severity

The severity of Verticillium wilt was evaluated by estimating the percentage of the the foliage showing chlorosis, necrosis and/or wilt typical of *V. dahliae* infections. Fifteen plants in the middle two rows of each plot were rated individually. Disease severity was assessed only once for cv. Norland potato plants, on July 26-27, 2012 (12 weeks after planting), because this is a short-season cultivar, which requires vines to be killed to promote uniform development, tuber size and skin.

The disease severity for the late-season maturing cv. Russet Burbank, was assessed weekly (six times) for each site in 2012 and 2013. The rating began 10 weeks after planting until harvest. Based on weekly rating, the area under the wilt progress curve (AUWPC) was estimated for the plots planted to cv. Russet Burbank to quantify Verticillium wilt intensity over time. AUWPC was calculated using the equation Σ_i^{n-1}

$[(Y_i + Y_{i+1})/2](t_{i+1} - t_i)$, where Y_i = cumulative disease severity at the i^{th} observation, t_i = time (days after planting) at the i^{th} observation, and n = number of observations (Shaner and Finney 1977).

2.3.7 Soil chemical analysis

Soil samples for chemical analysis were taken three times from each treatment plot. Sites planted in 2012 were sampled on April 11-16, July 14-20, and September 4-12; while sites planted in 2013 were sampled on May 2, August 17 and October 1(Figure 2.2). Samples from each plot were collected by taking six cores with a 2.5 cm diameter soil probe from the hills and between plants, if present, at two depths: 0-15 cm for Olsen extractable-P, pH and electrical conductivity (by 1:2 soil:water ratio method), and 0-30 cm for concentration of NH_4^+ and NO_3^- in 2M KCl extracts (5:1 extractant:soil). The ten cores from each plot were mixed together for a composite sample, placed in polyethylene bags, and transported on ice to the University of Manitoba, where each bag was mixed again by hand and stored at 4 °C until analysis at the University of Manitoba Soil Ecology Laboratory. For each composite soil sample, a 5 g subsample was placed into a 50 ml centrifuge tube, then 25ml of 2M KCl extracting solution was added. The tube was capped and placed on a reciprocating shaker for 1 hour at 150 excursions per minute. A centrifuge was then used to spin the tubes for 3.5 minutes at 3,000 rpm. The resulting clear supernatant was then transferred to labeled scintillation vials for storage at 4 °C prior to analysis for the inorganic N ions.

Ammonium concentration of extracts was determined using a Technicon™ Autoanalizer II System (Pulse Instruments, Mequon, WI) using the automated phenate method. Nitrate analysis was also done with an Autoanalyzer II System but using the automated cadmium reduction azo-dye method. Nitrite analysis was done with a similar procedure as for NO3- except the reduction step was omitted.

2.3.8 Plant nutrient analysis

Petiole nutrient analysis was done twice in experiments cv. Russet Burbank-2012 and cv. Russet Burbank-2012, at the beginning of tuber initiation on July 18 and 25, 2012 and August 22 and 29, 2013, respectively (Figure 2.2). Analysis was not performed on the cv. Norland because this is a short-season potato cultivar, which has its vines killed early in the season to fulfill commercial characteristics. The petiole of the fourth or fifth leaf from the top of 25 potato plants was collected to determine plant nutrient status. The individual leaflets were removed from the petioles immediately after petioles were broken off cleanly by hand from the plant. Petioles were placed in labelled bags and transported on ice to the Agvise sample collection center, where samples were stored at 4 °C for no more than 24 hours until analysis at the Agvise Laboratories (Northwood, ND). Samples were analyzed for nitrate-N, phosphorus, potassium, sulphur, calcium, magnesium, sodium, zinc, iron, manganese, copper and boron.

2.3.9 Potato yield and quality

In cv. Russet Burbank, sites A, B and C were harvested on September 10, 7 and 13, 2012, and sites D and E on October 1 and 2, 2013, respectively (Figure 2.2). Potato

tubers were obtained by harvesting the two middle rows (five m long) of each plot, eliminating the first and last half meter of the rows. Plots were mechanically harvested and tubers were placed in mesh bags.

The potato tubers were transported to the Canada-Manitoba Crop Diversification Centre (CMCDC) near Carberry, Manitoba, where tubers were weighed and stored at 5 °C for up to 30 days before samples were processed. Samples were then washed and weighed to determine weight loss during storage. The size categories for marketable yield of cv. Russet Burbank were determined by weight analysis: non-marketable tubers <85 g, regular 86–170 g, bonus 170.1– 340 g and overweight >340 g. Tubers were also evaluated for specific gravity using the weight in air and water method, fry color analysis by using USDA Fry Color Chart, green color, rot and hollow heart by tuber observation, and sugar end analyses by frying and comparing color with a standard chart.

In cv. Norland, potato plants were top-killed with Reglone Dessicant (Diquat ion) in sites F and H or mechanically with a flail vine shredder in site G on August 3 and 6, 2012, respectively. Harvest was completed on August 29, 21 and 22 in sites F, G and H, respectively (Figure 2.2). After harvest, tubers were immediately transported to the Horticulture Storage Research Building at the University of Manitoba, weighed and stored at 5 °C for up to 30 days before samples were processed. Tubers were then washed and weighed to determine weight loss during storage. Yield was classified base on tuber size: non-marketable tubers <¾ in, small 1½ - 2¼ in, No.1 2¼ - 3¼ in and large >3¼ in. Yield data was converted to Mg ha^{-1}. Additionally, cv. Norland tubers were assessed for

skin defects: color (discoloration), elephant hide (coarse russetting of the skin), surface cracking (superficial cracking), growth cracks, Rhizoctonia, russetting (smooth or rough), greening (occurs when tuber are expose to light), skinning and misshapen.

2.3.10 Statistical analysis

All statistical analyses were performed with the Statistical Analysis Software (SAS Institute, Cary, NC; release 9.2). Prior to analysis, *Verticillium* propagule density in soil, frequency of *Verticillium* and severity, AUWPC, soil chemistry and marketable yield data sets were checked for normality (PROC Univariate). AUWPC and yield were $\log_{10}$ transformed and frequency of *Verticillium* in petioles with the arcsine transformation before analysis of variance was applied. Standard errors (SE) were calculated using SAS software (Proc summary). Analysis was done for cv. Russet Burbank and cv. Norland separately. Each experiment planted to cv. Russet Burbank (2012 and 2013) were analyzed separately as well. When no significant interaction between treatment × site main effects were determined, data were pooled from all sites within the experiment. The data were analyzed by analysis of variance using the PROC MIXED procedure in SAS. Block within site was considered a random variable. When a factor effect or an interaction was significant, as indicated by a significant *F* test (P <0.05), differences between the respective means were determined (P <0.05) using the Bonferroni's procedure. Trends were indicated where *P* values were between 0.05 and 0.10. Finally, the relationships between AUWPC and all soil and plant parameters were assessed using simple regression analysis (n=21 and n=10). Additionally, Pearson's correlation analyses of Verticillium wilt severity, frequency of *Verticillium*, Verticillium propagule density,

soil chemical (Olsen extractable-P, extractable-N) and plant nutrient concentration (nitrate-N, potassium, sulfur, magnesium) using the PROC CORR procedure in SAS; and simple regressions across treatments were performed to determine the effect of *Verticillium* propagule density on the frequency of *Verticillium* in potato, AUWPC on marketable yield, as well as nutrient levels in soil and plants on AUWPC and marketable yield.

2.4 RESULTS

2.4.1 *Verticillium* propagule density

In 2012, the change in propagule density at planting compared to the previous fall in 2011, before metam sodium application (Figure 2.2), revealed a significant reduction with VAP40 and VAP60 treatments compared to the non-treated Control, in cv. Norland (P =0.01) and cv. Russet Burbank-2012 (P =0.0001) and (Table 2.4, Table 2.5). In spring, propagule density in the Control plots increased 35 and 24 % over the density in Fall-2011 (before the metam sodium treatments were applied), in cv. Russet Burbank-2012 (Table 2.5) and cv. Norland (Table 2.4), respectively. The analysis did not indicate a significant interaction between treatment and site in either potato cultivar. Nevertheless, the mean propagule density in the experiment cv. Russet Burbank-2012 in spring, compared to the previous fall, was reduced in site A and B by 22 and 32 %, respectively, while it increased in site C by 36 % (Table 2.5).

Table 2.4 Potato cv. Norland total marketable yield response to application of composted cattle manure, composted separated pig slurry solids, and the soil fumigant, metam sodium in 2012.

Trials	Treatment	Marketable yield (Mg ha^{-1})[a]			*Verticillium* frequency [c] (%)	Severity [c] (%)	Propagule density [d] (cfu g^{-1})	Inoculum change [e] (%)	
		Small	No 1	Total				Planting	Harvest
	Control	14.3 ±1.3 [b]	17.6±2.1	31.9±1.8	41±8 a	21±4 a	74±23	124±21 a	142±15 a
	VAP40	11.9±1.3	22.5±2.1	34.5±2.3	16±6 b	17±3 ab	97±30	77±15 b	64±9 b
	VAP60	12.5±1.0	16.1±2.7	28.7±2.7	34±9 ab	18±3 ab	100±32	61±10 b	60±10 b
	CCM20	11.4±1.1	19.5±4.2	31.2±4.4	20±6 ab	19±6 ab	79±18		122±22 ab
	CCM40	14.3±1.4	19.3±2.3	33.6±2.5	26±8 ab	17±3 ab	49±15		123±18 ab
	CCM80	13.0±1.3	19.0±3.8	32.1±3.6	29±8 ab	17±4 ab	77±15		125±22 ab
	CSS20	12.8±0.7	14.2±2.6	27.0±2.4	38±9 a	14±3 ab	90±24		102±19 ab
	CSS40	14.6±1.2	17.7±2.2	32.5±2.0	25±5 ab	14±3 ab	110±43		104±18 ab
	CSS80	13.5±0.7	14.0±2.5	27.7±2.4	16±6 b	8±2 b	78±21		85±18 ab
	FERT	11.4±0.9	18.0±2.6	30.2±2.4	25±9 ab	11±3 ab	74±23		114±19 ab
Site F		10.4±0.3 B	21.8±1.0 A	32.3±0.8 A	50±4 A	11±1 B	168±11	98±22	98±8
Site G		14.9±0.5 A	8.0±1.1 B	23.0±1.4 B	16±6 B	25±2 A	38±3	70±10	123±11
Site H		13.8±0.6 A	21.3±1.2 A	35.6±1.0 A	14±3 B	13±2 B	31±3	88±17	91±11
Treatment [f]		0.06	0.04	0.1	0.0005	0.05	nd	0.01	0.001
Site		0.004	0.0001	0.0006	0.001	0.0005	nd	ns	ns
Treatment x Site		ns	ns	ns	ns	ns	nd	ns	ns

[a] Potato tubers graded into size categories: small 1½-2¼ in diameter, No.1 2¼-3¼ in and large >3¼ in.

[b] Values are means ±1 standard error. Within columns means followed by the same letter are not significantly different (P >0.05) according to the Bonferroni's multiple comparison test.

[c] Frequency of *Verticillium* and severity of Verticillium wilt symptoms at13 weeks after planting.

[d] Initial *Verticillium* propagule density (cfu g^{-1} of dry soil) on September 2011.

[e] Inoculum change represents the *Verticillium* propagule density change (%) in soil before planting and harvest relative to the initial propagule density.

[f] Treatment significant probability. ns, not significant (P >0.05), nd, not determined. Trends are indicated where P values were between 0.05 and 0.10.

Table 2.5 Potato cv. Russet Burbank yield response to application of composted cattle manure, composted separated pig slurry solids and the soil fumigant, metam sodium in 2012 and 2013.

Year	Trials	Treatment	Marketable yield (Mg ha^{-1}) [a]				*Verticillium* frequency (%)	AUWPC [b]	Propagule density [c] (cfu g^{-1})	Inoculum change [d] (%)	
			Regular	Bonus	Overweight	Total				Planting	Harvest
2012	**All**	Control	14.4±2.0 [e]	15.3±0.9	2.4±0.4	32.1±2.4 b	68±10 a	529±78 a	76±25	135±10 a	186±47
		VAP40	16.9±1.5	20.0±1.4	3.1±0.8	40.0±2.2 a	40±10 b	331±57 ab	51±13	76±12 b	149±33
		VAP60	15.0±1.3	18.0±0.8	3.9±1.0	36.9±2.2 ab	32±10 b	303±48 b	65±13	71±14 b	75±12
		CCM20	16.3±1.0	17.3±1.4	3.4±0.6	37.0±1.9 ab	56±11 ab	346±48 ab	60±16		174±41
		CCM40	14.5±1.4	18.4±1.3	4.3±1.0	37.2±2.8 ab	52±9 ab	394±68 ab	80±19		172±30
		CCM80	15.1±0.9	19.5±1.9	3.7±0.7	38.3±2.4 ab	54±9 ab	406±71 ab	73±18		133±20
		FERT	16.0±1.3	16.3±1.3	2.6±0.4	34.9±2.3 ab	53±11 ab	315±49 ab	88±19		137±31
	Site A		19.0±0.8 A	19.8±0.9 A	4.1±0.5 A	42.9±1.2 A	29±5 B	566±42 A	41±4	78 ±10 B	134±23
	Site B		15.8±0.6 B	19.0±0.8 A	2.0±0.3 B	36.7±1.1 A	35±4 B	230±11 B	33±2	68±16 B	146±24
	Site C		11.5±0.4 C	14.8±0.8 B	4.1±0.4 A	30.4±1.2 B	83±3 A	372±36 B	133±10	136±8 A	162±16
		Treatment [f]	ns	0.05	ns	0.02	0.009	0.03	nd	0.0001	0.1
		Site	0.0005	0.02	0.04	0.003	0.0001	0.05	nd	0.002	ns
		Treatment x Site	ns	ns	ns	ns	ns	ns	nd	ns	ns
2013	**All**	Control	10.1±2.08	18.7±2.3	15.0±3.1	43.8±1.6	85±5	484±97	75±6	nd	595±261
		CSS20	12.7±2.83	18.1±1.6	14.9±3.5	45.7±1.8	72±10	445±73	88±10		312±133
		CSS40	14.3±3.19	21.1±2.9	12.2±3.3	47.6±3.1	79±8	373±64	74±8		649±289
		CSS80	13.0±2.2	18.9±1.7	14.8±3.7	46.7±3.2	80±6	392±54	52±6		644±210
		FERT	13.8±2.75	18.8±1.2	14.7±3.5	47.3±1.2	65±9	428±49	60±9		399±139
	Site D		18.9±1.2 A	23.0±0.9 A	6.8±1.1 B	48.6±1.1 A	81±5	578±29 A	41±5		43±5 A
	Site E		7.3±0.5 B	15.6±0.8 B	21.2±1.3 A	44.1±1.5 B	71±5	285±19 B	95±12		1046±79 B
		Treatment	ns	ns	ns	ns	ns	ns	nd		ns
		Site	0.0002	0.0008	0.0004	0.04	ns	0.0001	nd		0.0001
		Treatment x Site	ns	ns	ns	ns	ns	ns	nd		ns

[a] Potato tubers graded into weight categories for marketable yield regular 86–170 g, bonus 170.1-340 g and overweight >340 g.
[b] AUWPC. Area under the wilt progress curve (%-days).
[c] Initial propagule density (cfu g^{-1} of dry soil) on September 2011 (sites A, B and C) and May 2013 (Sites D, and E).
[d] Inoculum change represents the *Verticillium* inoculum density change (%) in soil before planting and harvest relative to the initial inoculum density.
[e] Values are means ±1 standard error. Within columns means followed by the same letter are not significantly different (P >0.05) according to the Bonferroni's multiple comparison test.
[f] Treatment significant probability. ns, not significant (P >0.05), nd, not determined. Trends are indicated where P values were between 0.05 and 0.10.

At harvest, the change in *Verticillium* propagule density in cv. Norland trials, from fall-2011 to harvest, for the composted treatments were not significantly different from Control and FERT treatments (P =0.001). The VAP40 and VAP60 treatments reduced propagule density by 34 and 40%, respectively, compared to that found before sodium metam application in fall 2011 (P =0.001, Table 2.4). The reduction of *Verticillium* inoculum in cv. Russet Burbank-2012 plots treated with metam sodium at the lowest rate (374 L ha^{-1} -VAP40) was no longer significant. *Verticillium* propagule density tended to be reduced only in plots treated with 561 L ha^{-1} (VAP60) compared to the control, FERT and compost treatments (P =0.1). *Verticillium* propagule density was very similar among sites in cv. Russet Burbank-2012. In cv. Russet Burbank-2013, *Verticillium* propagule density in compost and fertility treatments was not different to that in the Control. However, propagule density was significantly different between the two sites in cv. Russet Burbank-2013 (P =0.0001). Surprisingly, propagule density in site E was 10 times higher at harvest than the density initially measured in spring 2013 (95 cfu), while the level in site D was very similar (41-43 cfu) (Table 2.5).

2.4.2 Frequency of *Verticillium* and disease severity

In the sites planted to cv. Norland, there were significant treatment (P =0.0005) and site (P =0.001) effects on frequency of *Verticillium*. Frequency of *Verticillium* in petioles was 61% lower in plots treated with VAP40 and CSS80 compared to the control (P =0.0005). Across all three sites planted to cv. Norland, site G and H had the lowest frequency of *Verticillium* compared to site F (P =0.001) (Table 2.4).

In cv. Norland trials, the disease severity rating was taken early, as soon as first wilt symptoms were observed. Disease severity was relatively low (less than 33% of foliage showing wilting, necrosis or chlorosis) among all three sites and treatments (Table 2.4). The effect of the compost amendments, metam sodium and high fertility treatments on disease severity in cv. Norland was not significant. Only the CSS80 treatment tended to reduce severity compared to the control (P =0.05) (Table 2.4). When comparing the treatment response within the different sites, no significant interaction between treatment and sites was detected. However, severity was greater in site G than sites F and H (Table 2.4).

In cv. Russet Burbank experiments, treatment and site effects on frequency of *Verticillium* was observed only in 2012. Frequency of *Verticillium* in cv. Russet Burbank was reduced with the VAP40 and VAP60 treatments by 41 and 53 %, respectively, compared to the Control (P =0.009, Table 2.5). The non-significant interaction between treatment x site, confirmed the consistent effect of the metam sodium treatments on frequency of *Verticillium* in petioles among treatments. Nevertheless, frequency of *Verticillium* was almost three and two times greater in site C, than B and A, respectively (P =0.0001). None of the compost treatments used in the cv. Russet Burbank experiments had a significant effect on frequency of *Verticillium* compared to the Control (Table 2.4).

The treatment effect significantly affected development of wilting symptoms (AUWPC) in cv. Russet Burbank-2012, but not in cv. Russet Burbank-2013. The site was affected the development of wilting symptoms in both cv. Russet Burbank experiments. The treatment x site interaction was not significant in any of the experiments, suggesting that the response to the treatments was consistent across sites. Compared to the control, AUWPC was significantly reduced by the treatments VAP60 in cv. Russet Burbank-2012 (P =0.005). In cv. Russet Burbank-2013, neither composts nor FERT treatments reduced AUWPC. In cv. Russet Burbank-2012, AUWPC in site A was 59 and 34 % higher than in sites B and C, respectively (P =0.05). In cv. Russet Burbank-2013, AUWPC in site D was 50 % lower than in site E (P =0.0001, Table 2.5).

2.4.3 Potato yield

In cv. Norland, a significant treatment effect was observed in the No.1 tubers (P =0.05). The No.1 yield ranged from 14 Mg ha^{-1} (CSS20) to 22.5 Mg ha^{-1} (VAP40). Although the small and total marketable yield tended to be affected by the treatments (P =0.06 and 0.1, respectively), the yield in any of the treated plots was not different from the non-treated control. Total marketable yield ranged from 27 Mg ha^{-1} (CSS20) to 34.5 Mg ha^{-1} (VAP40). Although there was not a significant interaction for treatment and site, all marketable categories and total yield were affected by the intrinsic conditions at each site. The No. 1 tubers and total marketable yield were much greater in site F and H than site G (Table 2.5). Analysis did not reveal any treatment or site effect on any skin defect (data not shown).

The potato yield from the cv. Russet Burbank-2012 experiment revealed a significant treatment effect on bonus (P =0.05) and total marketable yield (P =0.02), but not for regular and overweight marketable yield (Table 2.4) or tuber quality (fry color, specific gravity, sugar ends, greening, rot and hollow hearth of tubers) (data not shown). Total marketable yield in plots treated with VAP40 treatment were 25 % higher than the control. A significant site effect was observed for all marketable categories and total yield (P <0.05). Regular, bonus, and total marketable yield tubers were significantly higher in site A than C. There was no interaction for effects of treatment and site on marketable yield.

In cv. Russet Burbank-2013, the treatment effect on marketable yield was not significant. Similar to what was observed in cv. Russet Burbank-2012, the site effect was significant for all marketable categories and total yield. Total marketable yield, regular and bonus categories were all higher in site D compared to site E. Only the overweight marketable yield category was higher in site E than D (Table 2.5).

2.4.4 Plant and soil nutrient levels

In cv. Russet Burbank-2012 experiment, the analysis of the petiole nutrients concentration showed that the treatment effect was only significant for phosphorus (P< 0.0001). The highest concentration of phosphorus in petiole was for the FERT treatment (0.46%) (Appendix II). The concentrations of other nutrients in petioles were not affected by the treatments. The site effect was significant for all nutrients except for sodium. In cv. Russet Burbank-2013, petiole nutrient concentration was not affected by the

treatments effect, except for phosphorus ($P< 0.001$) and zinc ($P< 0.05$) (Appendix II). The site effect was only significant for phosphorus, sulfur, magnesium, zinc, iron, manganese and copper. There was no significant interaction between treatment and site for petiole nutrients in cv. Russet Burbank experiments.

In cv. Russet Burbank-2012, treatment effect was significant for Olsen extractable-P in soil measured at mid-season and harvest (Appendix III). At mid season, Olsen extractable-P was higher in treatments CCM80 (103 mg kg^{-1}) and FERT (114 mg kg^{-1}), compared to other treatments ($P< 0.001$). At harvest, Olsen extractable-P was only significantly higher in treatment FERT (107 mg kg^{-1}) compared to the non-treated Control. Olsen extractable-P measure at planting, mid-season and harvest was higher in site B than A. In cv. Russet Burbank-2013, Olsen extractable-P at mid-season and harvest was higher in treatments CSS80 and FERT compared to the non-treated control. Nitrate-N and Olsen extractable-P were higher in site E than D, at mid-season and harvest.

2.4.5 Relationship among frequency of *Verticillium*, disease development, yield, and plant and soil nutrient levels

Single linear regressions analysis showed a relationship between Verticillium wilt (AUWPC) and Olsen extractable-P and extractable nitrate in soil before harvest, in both experiments planted with cv. Russet Burbank (Figure 2.3). In cv. Russet Burbank-2012, the decreasing AUWPC was related with increasing water extractable-nitrate (R^2=0.35; P =0.004) and Olsen extractable-P (R^2=0.22; P =0.03) (Figures 2.3A, B). However, in cv.

Russet Burbank-2013, the same relationships between AUWPC and water extractable-nitrate (R^2=0.86; *P* =0.0001), and Olsen extractable-P (R^2=0.70; *P* =0.003) were stronger (Figure 2.3C, D). Additionally, in cv. Russet Burbank-2012, decreasing AUWPC was correlated with increasing concentration of nitrate-N (Pearson's correlation (r)= -0.85; *P* = 0.001) and potassium (r= -0.64; *P* = 0.002) in potato tissue. Moreover, the concentration of phosphorus in plant tissue was significantly and negatively correlated with frequency of *Verticillium* (r=-0.58, *P* =0.006) (Table 2.6).

Table 2.6 Pearson's correlation coefficients r for Verticillium wilt severity (AUWPC), frequency of *Verticillium*, Verticillium propagule density, selected soil chemical (Olsen extractable-P, extractable-N) and selected petiole nutrient concentration (nitrate-N, potassium, sulfur, magnesium) in cv Norland and cv. Russet Burbank experiments.

Year	Variables [a]	*Vert.* Freq.	cfu	Olsen-P	N	Petiole nutrient concentration				
						NO_3-N	P	K	S	Mg
Experiment cv. Norland										
2012	**Severity**	-0.24[b] *ns*	-0.47 *ns*	-0.1 *ns*	-0.60 *(0.0004)*	nd nd	nd nd	nd nd	nd nd	nd nd
	***Vert.* Freq.**		0.72 *(0.0001)*	-0.39 *(0.03)*	0.57 *(0.009)*	nd nd	nd nd	nd nd	nd nd	nd nd
Experiments cv. Russet Burbank										
2012	**AUWPC**	0.02 *ns*	0.02 *ns*	-0.476 *(0.03)*	-0.59 *(0.004)*	-0.85 *(0.001)*	0.43 *(0.04)*	-0.64 *(0.002)*	0.77 *(0.0001)*	0.01 *ns*
	***Vert.* Freq.**		0.84 *(0.0001)*	0.37 *ns*	-0.07 *ns*	-0.41 *ns*	-0.58 *(0.006)*	-0.26 *ns*	0.14 *ns*	0.77 *(0.0001)*
2013	**AUWPC**	-0.78 *(0.007)*	-0.80 *(0.005)*	-0.83 *(0.003)*	-0.92 *(0.0001)*	0.68 *0.02*	-0.52 *ns*	-0.22 *ns*	-0.82 *(0.003)*	-0.87 *(0.0009)*
	***Vert.* Freq.**		-0.54 *ns*	-0.43 *ns*	-0.41 *ns*	0.47 *ns*	0.39 *ns*	0.13 *ns*	-0.17 *ns*	-0.60 *ns*

[a] Variables: severity (%) and AUWPC (area under wilt progress curve), Vert. freq.: Verticillium frequency in potato stems; soil chemical: Olsen-P: Olsen extractable phosphorus (mg kg^{-1}) and N= nitrogen water extractable nitrate-N (mg kg^{-1}); petiole nutrient concentration: NO_3-N=nitrate-N (ppm), K= potassium (%), S= sulfur (%), Mg= magnesium (%).

[b] Pearson's correlation coefficients and value within parenthesis indicate significant probability. ns, not significant (*P* >0.05), nd, not determined.

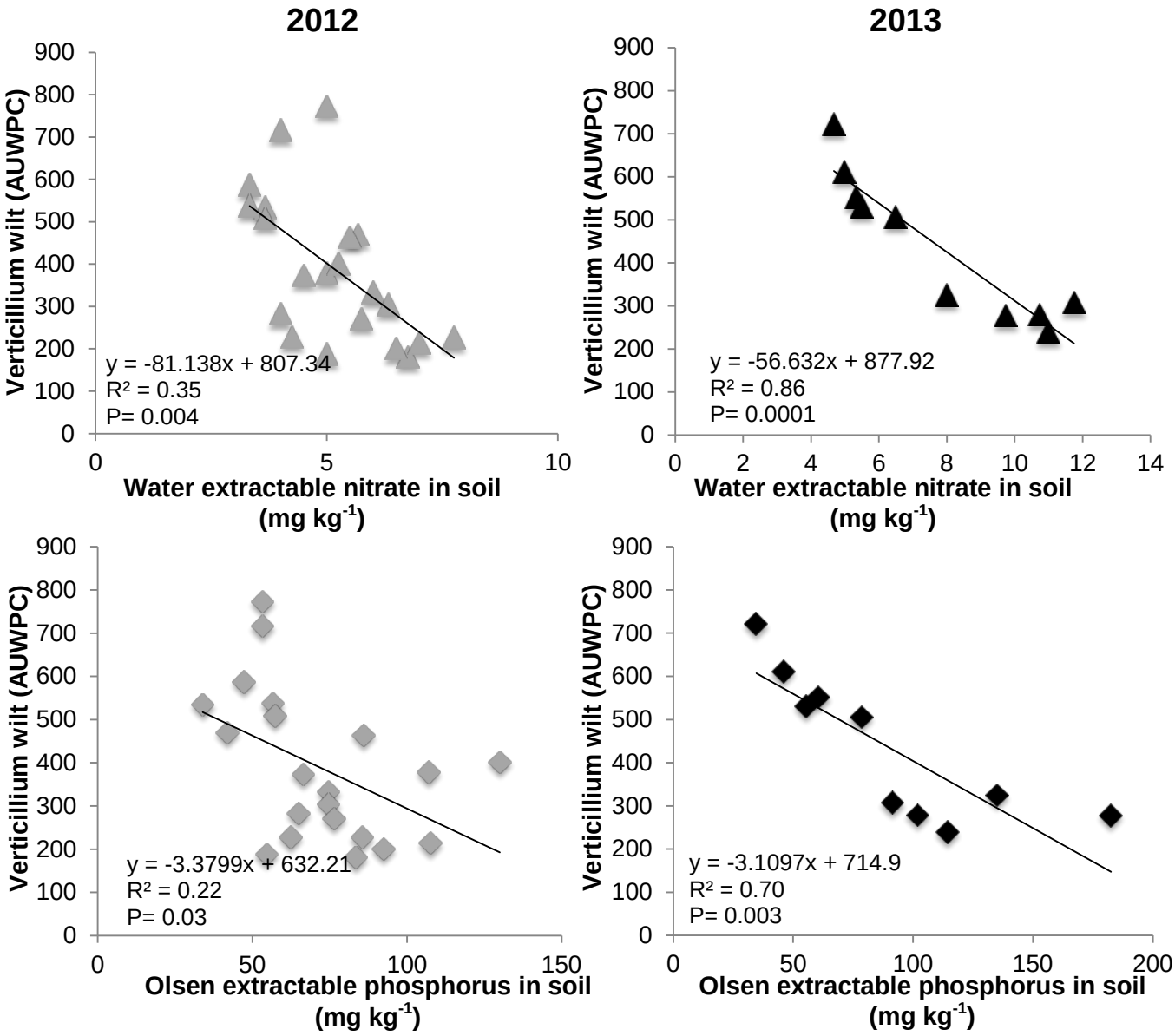

Figure 2.3 Relationships between Verticillium wilt severity (AUWPC) and water extractable nitrate-N and Olsen extractable phosphorus in soil before harvest in cv. Russet Burbank-2012 (**A, B**) and cv. Russet Burbank-2013 (**C, D**) experiments.

In cv. Russet Burbank-2013, decreasing AUWPC was significantly and negatively correlated with concentration of sulfur (r= -0.82; *P* = 0.003) and magnesium (r= -0.87; *P* = 0.0009). Correlation between *Verticillium* propagule density before planting and the development of Verticillium wilt symptoms was absent in cv. Russet Burbank-2012 and negative in cv. Russet Burbank-2013 (r =-0.80, *P* =0.005) (Table 2.6).

In the cv. Norland trials, decreasing severity was strongly correlated with water extractable nitrate-N in soil measured in summer (r=-0.60; *P* =0.0004). In this experiment, severity was not associated with any of other evaluated variables, including the *Verticillium* propagule density and frequency of *Verticillium*. This possibly as a result of the severity values being low at the time of evaluation, which was done early, before the crop was top-killed.

Based on linear regression analyses, *Verticillium* propagule density before planting partially explained the variation of frequency of *Verticillium* in petioles in the sites planted to cv. Russet Burbank. The relationship was significant in cv. Russet Burbank-2012 (R^2 =0.69; *P* =0.0001; Figure 2.4A), but not in cv. Russet Burbank-2013 (R^2 =0.29; *P* =0.11; Figure 2.4B), when the significant increment of cfu at the end of the season in site E did not result in more frequency of *Verticillium* in petioles or Verticillium wilt severity, expressed as AUWPC (Table 2.5).

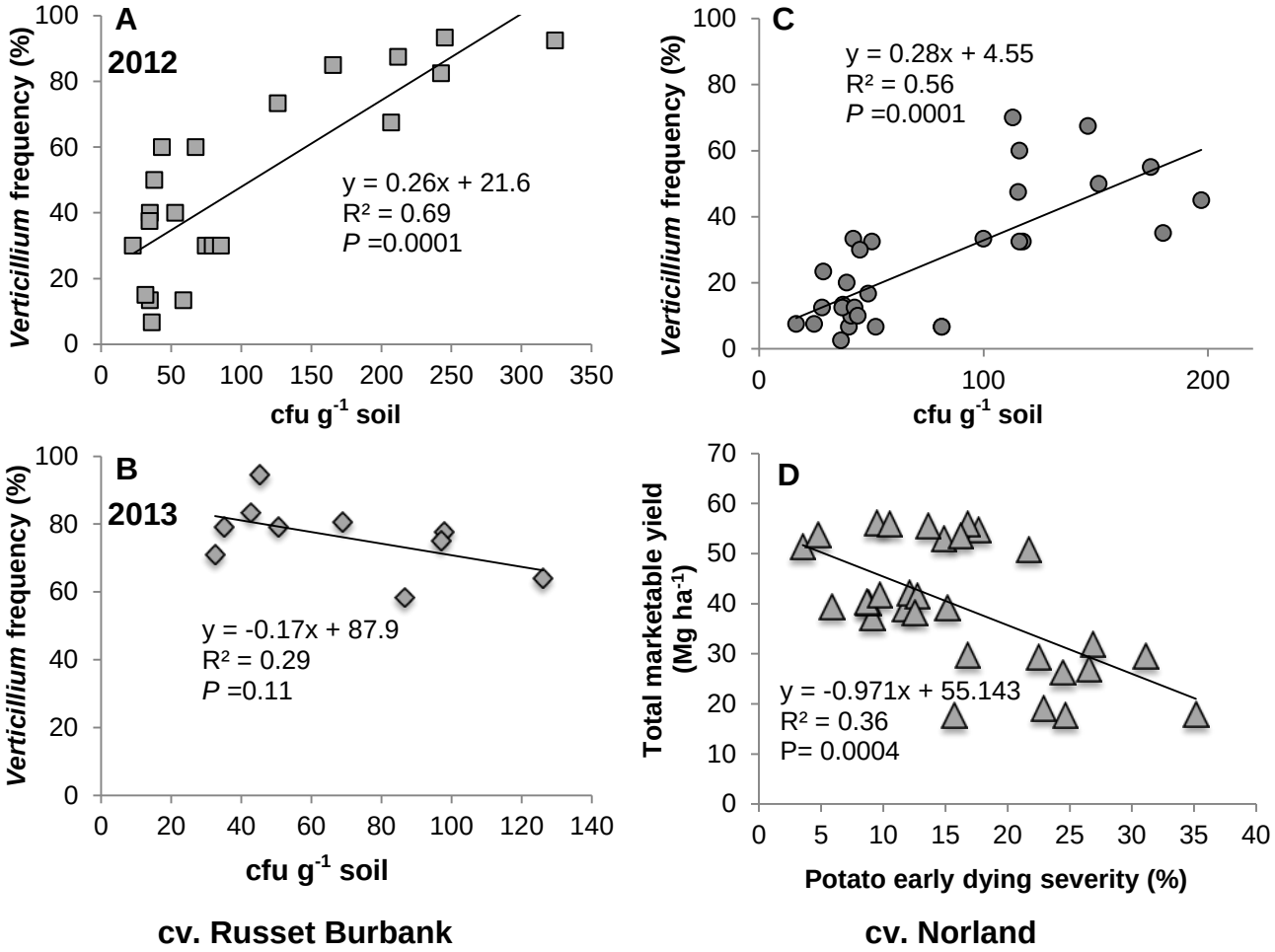

Figure 2.4 Relationships in cv. Russet Burbank trials between frequency of *Verticillium* in petioles (%) and *Verticillium* propagule density (cfu g^{-1}) before planting in cv. Russet Burbank-2012 **(A)** and cv. Russet Burbank-2013 **(B)**; and in cv. Norland trials between frequency of *Verticillium* (%) and *Verticillium* propagule density (cfu g^{-1}) before planting **(C)** and the cv. Norland total marketable yield (Mg ha^{-1}) and disease severity (%) **(D)**.

In the cv. Norland trials, 56% of the total variation of frequency of *Verticillium* was explained by the *Verticillium* propagule density observed before planting (R^2=0.56; *P* =0.0001; Figure 2.4C). Although Verticillium wilt severity was not high, 36% of the total marketable yield variation was predicted by the severity of the disease (*P* =0.0004; Figure 2.4D).

In experiment cv. Russet Burbank-2012, 61% of the marketable yield variation was explained by the frequency of *Verticillium* (R^2 =0.61; *P* =0.0001; Figure 2.5A), while the marketable yield was not predicted by frequency of *Verticillium* in cv. Russet Burbank-2013 (R^2 =0.05; *P* =0.5; data not shown).

In cv. Russet Burbank-2012, total marketable yield was significantly predicted by petiole phosphorus (R^2=0.57; *P* = 0.0001; Figure 2.5B) and to a smaller degree by extractable nitrate in soil (R^2=0.33; *P* = 0.007; Figure 2.5C). In cv. Russet Burbank-2013, there were significant relationships between the Olsen extractable-P in soil at mid-season and the marketable regular (R^2=0.43; *P* = 0.04), bonus (R^2=0.49; *P* = 0.02) and overweight (R^2=0.59; *P* = 0.009) categories (Figures 2.5D, E and F). Likewise, these relationships were observed between the Olsen extractable-P in soil at harvest and marketable regular (R^2=0.49; *P* = 0.02), bonus (R^2=0.52; *P* = 0.02) and overweight (R^2=0.66; *P* = 0.004) categories.

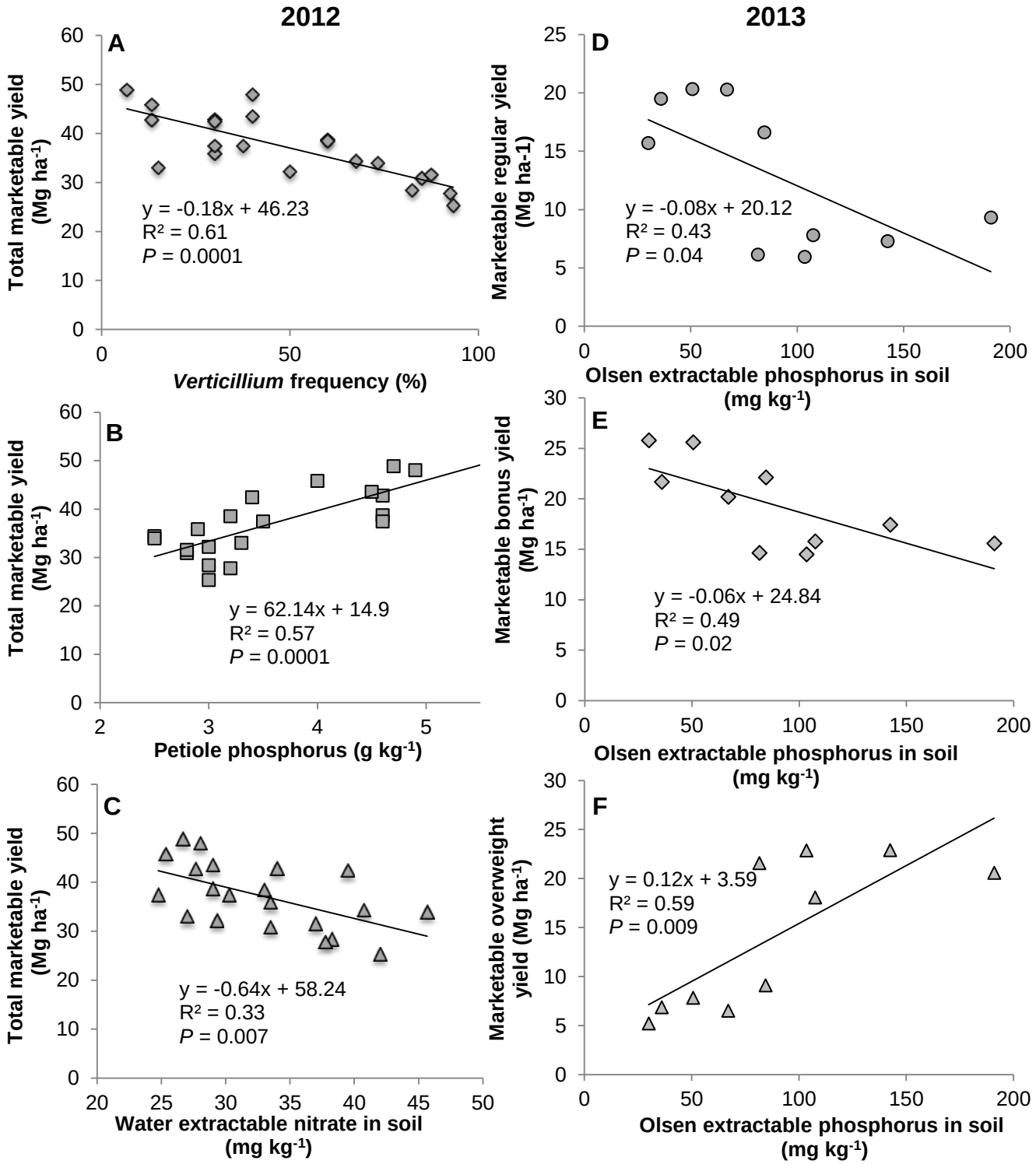

Figure 2.5 Relationships in the cv. Russet Burbank-2012 experiment between total marketable yield (Mg ha^{-1}) of cv. Russet Burbank and: **(A)** frequency of *Verticillium*, **(B)** petiole phosphorus (mg kg^{-1}) and **(C)** water extractable nitrate-N (NO_3^--N) at mid-season; and in cv. Russet Burbank-2013 between the potato tuber categories of marketable yield (Mg ha^{-1}) **(D)** regular (86-169 g), **(E)** bonus (170-340 g) and **(F)** overweight (>340g) and Olsen extractable phosphorus in soil at mid-season.

2.5 DISCUSSION

2.5.1 Compost effect on Verticillium wilt and *Verticillium* propagule density

In the current study, different rates of composted cattle manure, composted separated pig slurry solids and metam sodium were applied to soil naturally infested with *Verticillium* spp to evaluate the one-year effect of these soil treatments on *Verticillium* propagule density, frequency of *Verticillium,* Verticillium wilt severity and yield. The overall effect of the single application of composted cattle manure or composted separated pig slurry solids on *Verticillium* propagule density and frequency of *Verticillium* in cv. Russet Burbank was not significant. Severity of Verticillium wilt in cv. Russet Burbank was significantly reduced when soil was treated with 20 Mg ha^{-1} of composted cattle manure or 40 Mg ha^{-1} of composted separated pig slurry solids, in cv. Russet Burbank 2012 and 2013 respectively. In cv. Norland, frequency of *Verticillium* and Verticillium wilt symptoms were reduced with the highest rate of composted separated pig slurry solids only.

The implication of these data is that the level of disease suppression of single applications of composted materials varied with different composts, application rates and potato cultivars. These results are consistent with the varying suppressive effect observed in several pathosystems due to the type of compost, application-rate and target (Bonanomi et al. 2007; Litterick et al. 2004; Yadessa et al. 2010). Bonanomi et al. (2010) found that in a large number of cases, application of compost resulted in disease

suppression; however, in approximately 40% of the cases, the effect was disease conducive or not suppressive at all.

Another factor contributing to the inconsistent suppressive effect of the compost treatments is the fact that field studies with longer-term studies could result in a more predictable suppressive effect due to the increase of total or active carbon fractions or microbial biomass, which regulate soil physical properties, nutrient mineralization, and microbial communities shifts, composition and activities (Litterick et al. 2004; Stone et al. 2004; Stone et al. 2003). Although single applications can suppress soilborne diseases within the first season after application (Stone et al. 2004), there have been cases where single applications did not have any suppressive effect and had a detrimental effect on yield (Malandraki et al. 2008). For example, longer-term application could be required for compost to allow proper establishment of biocontrol agents in the rhizosphere where they can provide plants a more reliable suppression of vascular diseases (Nejad and Johnson 2000). For instance, introduction antagonistic bacteria such as *Pseudomonas fluorescens* DF37, and *Bacillus pumilus* M1, which have reduced Verticillium wilt incidence and severity, possibly after triggering potato resistance mechanisms (Uppal et al. 2008).

Direct killing of microsclerotia of *V. dahliae* had been associated with production and temporary accumulation of ammonia and nitrous acid in sandy soils with low organic matter content, after the application of high nitrogen content organic amendments with relatively low C:N ratio (below 15-10) such as meat and bone meal (Tenuta and

Lazarovits 2002, 2004). Although the compost products used in this study had a C:N ratio within the range from 11 to 14 (Table 2.3), decomposed products such as compost are very stable and mineralize nitrogen very slowly (Bonanomi et al. 2010), thus do not generate ammonia and nitrous acid toxicity.

In the particular case of Verticillium wilt, the ability of composted animal manures to control the disease can be attributed to the ability of beneficial microorganisms present in the compost such as *Pseudomonas fluorescens* and non-pathogenic *Fusarium oxysporum*, to establish in the rhizosphere and reduce the severity of the disease (Malandraki et al. 2008). This observation points out that control of soilborne pathogens using compost is rarely achieved with the reduction of pathogen inoculum levels in soil.

In fact, this same result has been observed in previous studies where compost failed to reduce pathogen inoculum (Bonanomi et al. 2010). In that study, 57 different cases of using compost for the control of soilborne pathogens, such as *Pythium* spp, *Phytophthora* spp. and *Fusarium* spp, were analyzed together, and in only 25% of the studies, the disease suppressive effect was associated with a decrease in pathogen population. The later suggests that long-term applications of compost have better chances of reducing soilborne populations, and eventually preventing large-scale diseases, than single applications. This might explain why the single applications of composted cattle manure or composted separated pig slurry solids in our study did not reduce *Verticillium* propagule density and frequency of *Verticillium* in potato plants.

2.5.2 Soil fumigation effect on Verticillium wilt and *Verticillium* propagule density

Soil fumigation with metam sodium is commonly implemented for the management of Verticillium wilt (Pasche et al. 2014; Rowe and Powelson 2002; Tsror et al. 2005). Since an estimated 80% of the Manitoba potato fields are planted to the susceptible cv. Russet Burbank, metam sodium has gained lot of interest due to its wide spectrum of activity towards soilborne pathogens, but particularly *V. dahliae*. In cv. Russet Burbank-2012, the application of metam sodium at rates 0.8x and 1.2x of recommended (467 L ha^{-1}), significantly reduced *Verticillium* propagule density (before potato planting), frequency of *Verticillium* and severity of Verticillium wilt in cv. Russet Burbank. The suppressive effect of the two rates was not different and the reduction in *Verticillium* inoculum, frequency of *Verticillium* and disease severity did not result in higher potato yields when soil was treated with the higher rate. This is consistent with previously published data showing that a rate of 514 L ha^{-1} is as effective as higher rates (701 and 935 L ha^{-1}) (Hamm et al. 2003). In fact, the a comparatively low rate of 373 L ha^{-1} is as effective as higher rates (Pasche et al. 2014)

The lack of positive yield response with the metam sodium at 561 L ha^{-1} is important not only for economic reasons, but because of the potential significant negative impacts that high rates might have on the environment (Gudmestad et al. 2007). Unnecessarily high rates could directly and significantly increase the methyl isothiocyanate (MITC) emissions and non target exposure since the rate of metam sodium is a factor directly involved in the breakdown to MITC and further loss due to volatile emissions (Sullivan

et al. 2004). Xie et al. (2015) stated that soil fumigants that effectively suppress diseases could significantly reduce non-target soil fungi, bacteria and actinobacteria. Therefore, high application rates could directly reduce beneficial species such as vesicular-arbuscular mycorrhizal (VAM) fungi, that otherwise could help potato plants to defend against *V. dahliae*, (Liu 1995). Schreiner et al. (2001) reported that soil fumigation with metam sodium directly reduced VAM fungi in soil. Although this could likely happen at recommended doses, in that study, metam sodium was applied at a high rate, almost 2x higher than recommended. This ultimately will contribute to make soil fumigation a requirement for further years, in order to keep pathogens under control (Daayf 2015).

Results of the trials planted to cv. Norland indicated that *Verticillium* propagule density was significantly reduced after metam sodium was applied. However, the effect on frequency of *Verticillium* and disease severity in the cv. Norland varied, as only the lowest rate (40 gal $acre^{-1}$) had a significant effect on frequency of *Verticillium*, while the severity did not respond to the fumigation treatments at all. The overall inconsistent disease suppressive effect of either compost or metam sodium treatments in cv. Norland is probably due to measuring frequency of *Verticillium* and Verticillium wilt severity soon after first wilt symptoms were spotted, before the crops were top-killed. The cv. Norland crops were top killed 88-100 days after planting, which is important for the table potato industry to control tuber size by stopping plant growth. In a previous study conducted in Idaho, 93 % Verticillium wilt severity was observed in plots planted to cv. Norland potato at 117 days after planting (Mohan et al. 1990). This suggests that wilt symptoms in our study may have not developed enough to identify a consistent disease

suppressive effect from the treatments. Davis et al. (1994a) also suggested that increasing applications of phosphorus and nitrogen will not reduce Verticillium wilt in cv. Norgold Russet as *V. dahliae* build-up will not be reduced until after natural or artificial vine killing.

These observations are consistent with the lack of relationship between frequency of *Verticillium* in petioles and disease severity and potato yield in cv. Norland. Although severity of Verticillium wilt symptoms could eventually reach levels that could reduce potato yield, as suggested by the significant, but weak, relationship observed between disease severity and yield loss, reduction of *Verticillium* propagule density using metam sodium does not necessarily translate into significantly more yield.

2.5.3 Relationships between propagule density, frequency of *Verticillium*, disease symptoms development and soil and plant conditions

In cv. Russet Burbank, the symptoms of Verticillium wilt developed under two scenarios, depending on the experiment (year). At first, in cv. Russet Burbank-2012, reduction of Verticillium wilt severity (AUWPC) was associated with increasing extractable soil NO_3-N and Olsen extractable soil P at the late stage of the crop (before harvest) and concentrations of nitrate and potassium in potato tissue. In cv. Russet Burbank-2013, reduction of Verticillium wilt severity (AUWPC) was associated with increasing concentrations of sulfur and magnesium in potato tissue, extractable soil NO_3-N and Olsen extractable soil P at the late stage of the crop (before harvest).

In both cv. Russet Burbank experiments, Olsen extractable soil P and extractable soil NO_3-N were associated with the development of Verticillium wilt symptoms. This result is consistent with previous studies showing the role of soil available phosphorus (Davis et al. 1990; Molina et al. 2014) and nitrogen (Davis et al. 1994a; Kelling et al. 2016; Lambert et al. 2005) on reduction of Verticillium wilt severity. Potato cultivars with limited degree of resistance to Verticillium wilt, such as cv. Russet Burbank, have shown improved resistance when nitrogen availability was increased (Davis 1985). Nitrogen has been identified as an important factor associated with reduction of incidence and severity of Verticillium wilt in cv. Russet Burbank and reduction of the rate of increase of *V. dahliae* inoculum (Davis et al. 1994a). However, high rates of nitrogen may potentially lead to slow tuber bulking during sprout development and vegetative growth, and ultimately reduce marketable yield, as observed in this study (Fig. 2.5C).

Phosphorus contributes to plant resistance against pathogen by increasing tissue phosphorus content, by accelerating tissue maturity or by increasing plant vigor (Prabhu et al. 2007). That was the case in cv. Russet Burbank-2012, where phosphorus concentration in tissue correlated negatively with frequency of *Verticillium*. Moreover, the Olsen extractable soil phosphorus data also revealed a positive relationship with total marketable yield. These results reveal the importance of available phosphorus for either Verticillium wilt control or potato yield in cv. Russet Burbank. It seems that phosphorus contributes to potato yield by increasing tissue phosphorus content or by accelerating canopy development and increasing leaf area tissue (Rosen et al. 2014), which is possibly contributing to plant disease suppression indirectly.

Potassium contributes to nitrogen metabolism and photosynthesis, and enhances nitrogen uptake and incorporation into proteins (Qu et al. 2011). Reduction of Verticillium wilt severity has been associated to increased concentrations of K (Pegg and Brady 2002), confirming the relationship observed in this study. DeVay et al. (1997) suggested that *V. dahliae* could disrupt the uptake and translocation of potassium in infected cotton plants, which ultimately resulted in reduced potassium concentration in petiole leaf.

Although magnesium has been associated to the reduction of Verticillium wilt severity in potato cv. Russet Burbank, the mechanisms implicated in such suppression are not clear (Ochiai et al. 2008). Magnesium is nearly as effective as calcium in preventing maceration of tissue by pectolytic enzymes produced by soft rot bacteria in potato (Jones and Huber 2007). Calcium helps to reduce pectin degradation in potato and contributing to the formation of enzyme resistant cell walls with Ca-pectate skeletons in susceptible hosts (Lambert et al. 2005; Pegg and Brady 2002).

Although sulfur accumulation has been detected in diverse members of the *Solanaceaum* family as a component of defense against *V. dahliae* (Cooper and Williams 2004; Novo et al. 2007), sulfur seems to accumulate in xylem parenchyma cells that are in direct contact with the xylem-invading *V. dahliae* (Williams et al. 2002). For instance, partially resistant pepper cultivars accumulated more sulfur in their vascular tissue than less resistant cultivars (Novo et al. 2007). However, in this study, sulfur concentrations were

within the range for recommended tissue concentration following the petiole analysis. Therefore, the sulfur concentration in this study may not have played a decisive role for Verticillium wilt symptom suppression in cv. Russet Burbank.

Relationships between Verticillium wilt severity and concentrations of nutrient in plant tissue and soil nutrients are not surprising given the role of nutrients in plants growth. For that same reason, nutrients are important factors in disease control, due to their role in the growth and development of plants and microorganisms (Agrios 2005). The nutritional status of the plant may either increase or decrease the resistance or tolerance of plants to pathogens by effecting changes in growth pattern, plant morphology and anatomy, and particularly chemical composition (Marschner 1986). In the present study, concentrations of nitrate-N, K, S and Mg were factors associated with the severity of Verticillium wilt in cv. Russet Burbank. The performance of the variables concentration of nitrate-N, K, S and Mg, were not affected by the treatments, and possibly affected by the site effect. On the other hand, the Olsen extractable soil phosphorus and nitrate-N in soil were affected by treatments, particularly extractable phosphorus which was 21 to 95% higher with CCM40, CCM80 and FERT treatments (cv. Russet Burbank-2012) and 36 to 95% higher with CSS40, CSS80 and FERT treatments (cv. Russet Burbank-2012) compared with the non-treated controls, respectively.

A similar result was previously observed after application of phosphorus fertilizer (Freeman et al. 1998; Hopkins et al. 2010) and two-years of application of 40 Mg ha^{-1} of composted cattle manure (Molina et al. 2014). The marketable yield data suggest a

general positive relationship between available P and total yield, and an additional effect on the tuber size distribution. Apparently, increases in bonus and overweight marketable yield were associated with increased available P. However, in this study, it was surprising to find that high levels of available phosphorus in soil increased the overweight marketable yield, at the expense of the bonus and regular marketable categories, which showed an inverse relationship with available P. This may be worth exploring as tighter row spacing could help to gain a more uniform tuber size and more tuber yield within the regular and bonus marketable weight categories.

In cv. Russet Burbank-2012, reduction of frequency of *Verticillium* in petioles was associated with low *Verticillium* propagule density before planting. In contrast, in cv. Russet Burbank-2013 there was no relationship between *Verticillium* propagule density and frequency of *Verticillium* (Figure 2.4B). This was not expected because the *Verticillium* propagules densities at the end of the season were approximately 10 times higher than the initial propagule density before potato planting (Table 2.5). This observation might be explained by the presence of propagules from other *Verticillium* species with the capability of producing microsclerotia colonies. This is very possible, because there are several *Verticillium* species capable of producing microsclerotia colonies (Inderbitzin et al. 2011). Unfortunately, the wet plating method, as any of the traditional methods designed to determine the level of *V. dahliae* in soil and the risk level of Verticillium wilt, does not easily distinguish between *V. dahliae* and closely related species, such as *V. tricorpus* (Duressa et al. 2012).

2.6 CONCLUSION

In summary, the suppressive effect of composted cattle manure and composted separated pig slurry solids varied among the rates. In general, composted materials had a positive or neutral effect on disease suppression, but did not stimulate development of Verticillium wilt symptoms or affect quality of potato tubers. However, the inconsistent suppressive effect could be associated to the single applications, which does not allow enough time for the suppressive mechanisms of compost to establish.

The results presented here support previous studies, which have suggested that nutrient management can decrease the severity of potato diseases, such as Verticillium wilt (Davis et al. 1990; Lambert et al. 2005). However, we suspect there may be other factors associated to the reduction of disease severity, such as build-up of organic matter and development of beneficial and antagonistic microbial activity in soil. This is an opportunity that needs to be explored in the future with multi-year applications of compost. Successive applications could promote mechanisms associated with suppression of soilborne pathogens and reduction of disease severity such as increase nutrient availability, build-up of total organic matter content, microbial activity and establishment of antagonistic organisms (Fiers et al. 2011; Hoitink and Fahy 1986; Hoitink et al. 1997; Kuter et al. 1983).

Reduction of frequency of *Verticillium* was an important factor associated with low Verticillium wilt severity. The application of metam sodium at 374 L ha^{-1}, plus adequate agronomic crop management, effectively reduced *Verticillium* inoculum by up to 40%,

and increased potato yield in cv. Russet Burbank by up to 24%. Surprisingly, higher rates failed to increase potato yield over that for the non-amended control. These results will offer more tools to help growers making a more effective and environmental decision on the amount of soil fumigant required to control Verticillium wilt and increase yield effectively.

Verticillium dahliae propagule density in soil should be a good indicator for whether to use metam sodium or not. However, the traditional wet plating method may not be an accurate and reliable method to quantify it, while other *Verticillium* species are present as well. In this study, the *Verticillium* propagule density data suggested that the traditional method does not estimate *V. dahliae* accurately, as fields with low propagule density were more severely affected by Verticillium wilt symptoms than fields with 10 times higher values. These results suggest that inoculum from other *Verticillium* species may contribute to overestimation of *V. dahliae*; therefore, a more reliable and accurate method that allows identification and proper quantifications of the *Verticillium* species is needed.

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CHAPTER 3

3. DETECTION AND QUANTIFICATION OF MICROSCLEROTIA-FORMING *VERTICILLIUM* SPECIES AND ITS RELATIONSHIP WITH VERTICILLIUM WILT IN POTATO FIELDS IN MANITOBA

3.1 ABSTRACT

Verticillium wilt is caused by the soilborne fungus *Verticillium dahliae.* This disease affects potato fields in Manitoba, where 80% of the fields are planted to the moderately susceptible cultivar, Russet Burbank. An accurate measurement of *V. dahliae* inoculum in soil is critical for disease prevention and management. In this study, we investigated the presence of microsclerotia-forming *Verticillium* species in potato fields in Manitoba, and compared published quantitative real-time PCR assays for *V. dahliae*, *V. tricorpus* and *V. longisporum* against a traditional plating method currently used to quantify *V. dahliae* in soil. The quantitative real-time PCR assays were capable of differentiation and quantification of the major microsclerotia-forming *Verticillium* species, *V. dahliae*, *V. tricorpus* and *V. longisporum*. Results from a total of 29 soil samples collected from 17 commercial potato fields showed that using the plating method, the presence of *V. tricorpus* in Manitoba potato soils could result in overestimation of *V. dahliae* propagule

density in 20% of soil samples, as observed in site E. Verticillium wilt severity in cv. Russet Burbank was negatively related to cfu from soil plating, while positive with the concentrations of *V. dahliae* genomic DNA in soils with both *V. dahliae* and *V. tricorpus* from two sites, selected from the field study in Chapter 2. The selected molecular diagnostic tool proved to have the accuracy to detect, identify and quantify inoculum of *V. dahliae* in soil and have a strong predictive value to Verticillium wilt severity, as *Verticillium* propagule density from soil plating proved to be a weak predictor of the development of the Verticillium wilt symptoms.

3.2 INTRODUCTION

Verticillium is a member of the family *Plectosphaerellaceae*, which is in the phylum *Ascomycota* (Agrios 2005). *Verticillium dahliae* belongs to the group of Deuteromycetes, fungi which do not have a known sexual stage (Agrios 2005). The genus *Verticillium* currently is comprised of 10 species, that differ by their morphological features, such as resting structures and capability to induce wilt disease in vascular plants (Inderbitzin et al. 2011). Resting structures are important for the survival of the fungus in soil. As the genus reproduces only asexually (Agrios 2005) and by anastomosis, but not sexual recombination, resting and dispersal structures are the primary characteristic to distinguish *Verticillium* species.

Resting structures in the genus *Verticillium* are represented by melanized hyphae, short chains of brown rounded cells called chlamydospores, and/or clusters of rounded and dark cells known as microsclerotia (Issac 1953; Pegg and Brady 2002). For instance, *V. albo-atrum* Reinke & Berthold was first differentiated by the production of brown-pigmented hyphae or resting mycelium (Issac 1949). The presence of microsclerotia has been used to differentiate *V. dahliae* Kleb. and *V. longisporum* (C. Stark) Karapapa from other species in the genus, while *V. tricorpus* I. Isaac is differentiated by the presence of resting mycelium, microsclerotia and chlamydospores simultaneously (Inderbitzin et al. 2011; Issac 1949; Karapapa et al. 1997).

Verticillium dahliae has a wide host range, including common agricultural crops such potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), cotton (*Gossypium hirsutum* L.) and strawberry (*Fragaria x ananassa*) (Pegg and Brady 2002). *Verticillium dahliae* can reside in soil as microsclerotia, which is produced inside the infected stem tissue, and eventually incorporated into the soil after harvest (Rowe and Powelson 2002). Microsclerotia allow the fungus to survive for many years, and serve as the main infective propagule under field conditions (Wilhelm 1955).

The reduction of microsclerotia has become the most suitable means to mitigate the effect of this monocyclic pathogen. In a previous study, reduction of Verticillium wilt incidence in cv. Russet Burbank was associated with low *Verticillium* propagule density of *V. dahliae* in soil treated with composted cattle manure. In contrast, non-treated plots planted to wheat, showed elevated *Verticillium* propagule densities, increased Verticillium wilt incidence in potato and ultimately lower potato yield (Chapter 2; Molina et al. 2014). Effective control strategies of *V. dahliae* rely on the precise detection and quantification of the propagule density of the pathogen in the soil. Reliable knowledge of the levels of *V. dahliae* in soil that could affect future yields and contribute to making more economical and environmentally-sound decisions is needed. For instance, this will be particularly useful in those potato regions with an increasing interest over the use of soil fumigants, like metam sodium and chloropicrin for the control of pathogens.

The wet plating method has been available to potato growers as a pre-planting inoculum level assessment. This test is based for the quantification of *Verticillium* microsclerotia-forming colonies growing on semi-selective medium (Goud and Termorshuizen 2003). As the test is reliant upon the formation of microsclerotia colonies, it could overestimate the level of *V. dahliae* inoculum due to the presence of other *Verticillium* species present in sampled soils which are able to produce very similar microsclerotia colonies on the medium (Goud et al. 2003).

Microsclerotia colonies formed on semi-selective medium can originate from microsclerotia as well as from other different fungal structures either free or contained in small pieces of decomposed plant debris (Butterfield and DeVay 1977). Resting structures such as microsclerotia, chlamydospores and dark mycelium are commonly produced by some *Verticillium* species and remain viable in the soil for several years (Isaac 1967). Although *V. dahliae*, *V. longisporum*, *V. zaregamsianum* Inderb. et al. sp. nov., *V. tricorpus*, *V. isaacii* Inderb. et al. sp. nov., and *V. klebahnii* Inderb. et al. sp. nov. all produce microsclerotia, the later four species produce resting mycelium and chlamydospores, as well (Inderbitzin et al. 2011).

Additionally, the wet plating method presents a variety of difficulties that make the quantification of *V. dahliae* very challenging due to low recovery of *V. dahliae* microsclerotia from soil (Wheeler et al. 1992), which at times is only of 30-50% of the total *V. dahliae* inoculum in soil (Goud and Termorshuizen 2003; Termorshuizen et al. 1998).

Previous studies conducted in the Soil ecology Lab at the University of Manitoba with wet and dry plating of soil on the semi-selective medium amended with pectin (polygalacturonic acid –PGA-) have showed a great deal of difficulties quantifying colony-forming units (cfu) of *V. dahliae*. Similar difficulties have been reported by others labs, such as variation among different lot or supply of PGA, variation in pH of medium, human error while scouting for cfu (Kabir et al. 2004; Termorshuizen et al. 1998), and the presence of other fungal species inhibiting the germination of microsclerotia (Goud and Termorshuizen 2003).

A Verticillium wilt survey conducted in 23 commercial cv. Russet Burbank fields in Manitoba (Tenuta et al. unpublished) indicated that the average propagule density obtained with the wet plating method was 73 cfu g^{-1} of dried-soil, with a very wide range from 0 to 389 cfu g^{-1} of soil (Coefficient of variation = 138). Counts are relatively high compared to some other potato growing regions, where the number of propagules estimated through the wet plating method ranged from 0 to 80 cfu g^{-1} air-dried soil (MacGuidwin et al. 2012; Nicot and Rouse 1987; Omer et al. 2008; Smith and Rowe 1984; Taylor et al. 2005). *Verticillium dahliae* is able to induce damage at relatively low inoculum densities. Soil propagule densities as low as 10 cfu g^{-1} air-dried soil have caused enough disease severity to significantly reduce yield (Davis and Sorensen 1986). Although potato fields in Manitoba have shown such high *Verticillium* propagule densities, severe Verticillium wilt and yield loss have not been consistently related (Chapter 2). There was no clear relation between low or high *Verticillium* propagule

densities from plating method and severity of Verticillium wilt in potato (Chapter 2). This situation has led us to suspect that *V. dahliae* propagule densities from plating method in Manitoba might be inaccurate.

Advances in molecular biology have created new diagnostic opportunities for the detection and management of *V. dahliae* in several pathosystems. For instance, real-time polymerase chain reaction (PCR) is becoming the preferred method of quantitative detection of plant pathogens in plant tissues and soil, due to its rapid, reliable and sensitive performance (Gachon et al. 2004). Real-time PCR offers an alternative for more specific and rapid detection of *Verticillium* species in soil than traditional culture based methods (Debode et al. 2011). Atallah et al. (2007) developed a real-time PCR assay to quantify *V. dahliae* in potato tissue using the designed primer pair VertBt-F and VertBt-R derived from the B-tubulin gene. More recently, Bilodeau et al. (2012) developed a TaqMan® real-time PCR assay based on the ribosomal DNA (rDNA) intergenic spacer (IGS) that was used for the quantitative detection of *V. dahliae* in strawberry soil samples.

The aims of this study were to: (i) investigate the presence of microsclerotia-forming *Verticillium* species in commercial potato soil in Manitoba, (ii) evaluate published real-time PCR methods for detection and quantification of *V. dahliae* and other microsclerotia-forming *Verticillium* species possibly present, and (iii) examine the relation between Verticillium wilt severity and microsclerotia-forming *Verticillium* species found in the soil.

The study started with sampling soil from 17 commercial fields in Manitoba. *Verticillium* propagule densities were quantified using the traditional wet plating method and soil genomic DNA (gDNA) extracted. *Verticillium* isolates were obtained from other research groups, and used as reference cultures. *Verticillium* isolates were collected from commercial potato soils, purified, confirmed, and used to create a pure culture collection. Microsclerotia of *V. dahliae* were produced to inoculate soils and further test the efficiency of the selected test to detect gDNA extracted from soil with *V. dahliae* microsclerotia. Both plating method and the selected real-time PCR assay were compared using soils from a previous field study in Chapter 2.

3.3 MATERIALS AND METHODS

3.3.1 Soil collection

Commercial potato fields were selected randomly over a broad geographic area from eight Rural Municipalities (RMs) that represent the major potato production regions of Manitoba. The fields had representation among the Reinland, Gnadenthal, Reinfeld, Neuenberg, Ramada, Hallboro, Long Plain and Wheatland soil series. Sampled fields included three fields planted to the table potato cv. Norland and 14 commercial potato fields planted to the processing potato cv. Russet Burbank, which included eight fields used in Chapter 2. Samples were collected during fall 2011 to spring 2013. Fifteen soil cores were taken from each field. A single core was obtained after walking 100 m into a field from the edge; then, a core was taken every 10 m in a zigzag pattern until 15 cores were collected. Cores were collected to a depth of 20 cm and bulked into a single soil

sample per site. Each sample was placed in a labeled plastic bag and transported in an insulated ice chest to the University of Manitoba.

Samples were passed through a 2 mm sieve, cleaned of roots and large plant debris, mixed thoroughly and air-dried at ambient temperature for one week. For each sample 200 g of air-dried soil was stored at 4 ˚C for up to four weeks before soil was plated on semi-selective medium to quantify *Verticillium* propagule density as cfu g^{-1}. Soil samples collected from commercial fields used in the Chapter 2 (sites B, F, D, and E), were stored at -20 ˚C for up to nine months, before soil gDNA extraction was performed.

3.3.2 Wet plating for cfu of *Verticillium* in soils

The *Verticillium* propagule density for all sampled fields was determined by using the wet plating on semi-selective Sorensen's NP-10 medium (Sorensen et al. 1991) supplemented with Pectin (polygalacturonic acid from orange, Sigma-Aldrich) to estimate *Verticillium* propagule density in soil. In short, 5 g of dried-soil were suspended in 100 mL of 0.01% agar water and 1 mL of the suspension spread onto plates of Sorensen's NP-10 medium supplemented with chloramphenicol, streptomycin sulfate and chlortetracycline HCl. Ten plates per sample were prepared and incubated in the dark at 22 ˚C for 21 days. After incubation, agar surfaces were gently rubbed by hand under running water to remove soil particles. Microsclerotia-forming colonies were counted under a stereo microscope. Then, the individual counts from each plate were added together and multiply by 2, and results expressed as cfu g^{-1} soil.

3.3.3 Soil genomic DNA extraction

Before soil gDNA extraction for the quantitative detection of *Verticillium* spp was conducted, each subsample of air-dried soil was pulverized individually using a mortar and pestle. The samples were then passed through a 20-mesh sieve to remove large stones. Total soil gDNA was extracted from 500 mg of pulverized soil using the MoBio PowerSoil® DNA Isolation extraction Kit (now called DNeasy PowerSoil™, QIAGEN). Each sample was processed with this kit, using a protocol modified from that in the kit's instructions to improve DNA yield. The cell disruption step was modified, using a mini-beadbeater-24 (BioSpec Products, Bartlesville, OK) at 3450 strokes min^{-1} for three min instead of using the recommended table-top vortex (MoBio Vortex adapter) for 10 minutes. Genomic DNA was eluted in 50 µL of the elution buffer (10mM Tris) (Solution-C6, QIAGEN). The quality and concentration of gDNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE). Extracted gDNA was then kept at -20 °C until use in PCR amplifications.

3.3.4 *Verticillium* isolates

In total, 20 isolates representative of three *Verticillium* species and *Gibellulopsis nigrescens* (Pethybr.) Zare, et al. (formerly *V. nigrescens*), were used as a reference to become familiar with the different *Verticillium* species and to verify specificity of real-time PCR assays for *V. dahliae, V. tricorpus* and *V. longisporum*. The reference isolates included six *V. dahliae*, one *V. tricorpus,* six *V. albo-atrum*, two *Gibellulopsis nigrescens*, six *V. longisporum* isolates (Table 3.1).

Table 3.1 Reference isolates of *Verticillium* species used in this study, and verification of specificity of real-time PCR assays for *V. dahliae*, *V. tricorpus* and *V. longisporum*.

Code	Host/ Source	Province/State	Year isolated	Real-time PCR assays [f]			Morphological and molecular identity verification [g]
				V. dahliae	*V. tricorpus*	*V. longisporum*	
V_104b[a]	Potato	PEI, CA	2006	-	-	-	*V. albo-atrum*
107724[b]	Potato	Ontario, CA	1965	-	-	-	*V. albo-atrum*
177115[b]	Potato	PEI, CA	1980	-	-	-	*V. albo-atrum*
216604[b]	Potato	PEI, CA	1992	-	-	-	*V. albo-atrum*
AT3[c]	Potato	Ontario, CA	1986	-	-	-	*V. albo-atrum*
Vd1396-9[a]	Potato	Manitoba, CA	2006	+	-	-	*V. dahliae*
241194[b]	Tomato	BC, CA	2010	+	-	-	*V. dahliae*
Dvd-E6[c]	Eggplant	Ontario, CA	1993	+	-	-	*V. dahliae*
Dvd-3[c]	Potato	Ontario, CA	1993	+	-	-	*V. dahliae*
Dvd-P2[c]	Potato	Ontario, CA	1995	+	-	-	*V. dahliae*
Dvd-S100[c]	Soil	Ontario, CA	1996	+	-	-	*V. dahliae*
226890[b]	Soil	Ontario, CA	1998	-	-	-	*G. nigrescens*
226891[b]	Soil	Ontario, CA	1998	-	-	-	*G. nigrescens*
Dvt-3[c]	Unknown	Ontario, CA	1999	-	+	-	*V. tricorpus*
VD624[d]	Cauliflower	California, USA	Unknown	-	-	+	*V. longisporum*
VD348[d]	Cauliflower	California, USA	Unknown	-	-	+	*V. longisporum*
243377D[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*
243377E[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*
243378D[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*
243378E[e]	Canola	Manitoba, CA	2014	-	-	+	*V. longisporum*

[a] Dr. Fouad Daayf. University of Manitoba, MB, Canada.
[b] Canadian Collection of Fungal Cultures, Ottawa, ON, Canada.
[c] Dr. Katherine Dobinson, Agriculture and Agri-Food Canada, London, ON, Canada.
[d] Dr. Krishna Subbarao University of California Davis Plant Pathology, Davis, CA, USA.
[e] Dr. André Lévesque, Agriculture and Agri-Food Canada, Ottawa, ON, Canada.
[f] Identity of the reference isolates was confirmed after morphological attributes (Presence of microsclerotia, resting mycelium and chlamydospores, and conidia size) were analyzed (Description is presented in Appendix II).
[g] Evaluation of species specificity of the real-time PCR assays for *V. dahliae* (Bilodeau et al. 2012), *V. tricorpus* (Debode et al. 2011) and *V. longisporum* (Banno et al. 2011). (+) indicates isolate amplified with protocol, and (-) indicates not amplification.

In this study, 82 *Verticillium* isolates were recovered from soil and diseased potato stems previously sampled from 17 commercial potato fields in Manitoba, screened for the presence of *Verticillium* and/or used for the field study in Chapter 2. To recover isolates from soil, soil samples that were plated to determine *Verticillium* propagule density were used to harvest a single microsclerotium from randomly selected colonies using a dissecting microscope. Microsclerotia were placed onto potato dextrose agar (PDA; Difco Laboratories, Spark, MD) plates supplemented with 0.02% streptomycin sulfate.

To recover isolates from potato plants, potato stems sections of 15-20 cm were washed with RO water and surface sterilized with 5% household bleach (0.053% Na hypochlorite), dipped 1 minute in 95% ethanol, rinsed in distilled water, and blotted dry on sterile absorbent paper. The stems were cut into small 3-4 mm width discs and placed onto the Sorensen's NP-10 (Sorensen et al. 1991) semi-selective medium supplemented with antibiotics. Plates with potato stems were incubated at 23±1 ˚C in darkness. After seven days, single microsclerotia were harvested and placed onto PDA plates supplemented with 0.02% streptomycin sulfate.

Ten days later, monosporic cultures were established from each isolate recovered from soil or diseased potato plants, by sub-culturing single conidia onto PDA plates without streptomycin hydrochloride (HCl). Isolates were kept in stock of fungus-colonized filter papers at -20 ˚C.

3.3.5 Morphological characterization of *Verticillium* species from cultures

The morphological features of the isolated cultures were examined on PDA plates and compared with the standard isolates *V. dahliae* (Vd1396-9), *V. tricorpus* (DVt3) and *V. longisporum* (VD624) (Table 3.1) in order to identify *Verticillium* species properly. Colony characteristics such as colour and occurrence of yellow discoloration of medium were recorded. The morphological features examined in the study were the presence of dark melanised mycelium growing throughout the plate, size (length and width) and shape (round or oval) of microsclerotia, presence and size (length and width) of chlamydospores and the colour, size and shape of the conidia for each isolate. Measurements were done using the Image-Pro program (Media Cybernetics, Rockville, MD) after taking pictures with a Megapixel Q-Color 3 imaging system (Olympus, Melville, NY) on either a microscope or stereo microscope.

3.3.6 *Verticillium* culture species identification by sequence analysis

The identification of the isolated fungi based on morphological characteristics was confirmed by means of the ribosomal RNA intergenic spacer (IGS) sequences. All isolates (isolates described in section 3.3.4) were sub-cultured on PDA without antibiotics at 23±1 ˚C in darkness for 14-18 days. Then, gDNA extraction from 300 mg of mycelium collected in a 1.5 mL sterilized tube was carried out following the protocol for fungal gDNA extraction as described by Mahuku and Platt (2002). Total gDNA was dissolved in 100 µL of 1xTE buffer (10 mM Tris, 1 mM EDTA, pH adjusted to 8.0).

The IGS region of each isolate was compared with those previously submitted to the National Center for Biotechnology Information (NCBI) (Qin et al. 2006). The IGS sequences (Approximately 1.8Kb size product) were amplified by PCR using the primers VdIGSF1 (5'GGGTCCTGTAAGCAGTAG 3') and VdIGSR1 (5' GAGCCATTCGCAGTTTCG 3') (Qin et al. 2006). Primers were synthesized by IDT (Integrated DNA Technologies, Coralville, IA). Reactions included 1x Thermo Scientific DreamTaq Green Buffer (Thermo Fisher Scientific, Waltham, MA), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 uM of each of the primers VdIGSF1 and VdIGSR1, 1 U of Thermo Scientific DreamTaq (Thermo Fisher Scientific, Waltham, MA) and 10 ng of gDNA template. PCR amplification was carried out in a C-1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA) programmed for 5 min at 95C, 35 cycles of 1 min at 95 °C, 50 sec at 60 °C and 2min at 72 °C, followed by 72 °C for 10 min. Aliquotes (5 µL) of each PCR product were visualized by electrophoresis using a 1.2% agarose gel containing 0.5 µL of 10,000X GelRed (Biotium, Hayward, CA, USA) dye and visualized on a UV transluminator GBox (Syngenem, Cambridge, UK). PCR products were sent to Macrogen Sequencing Service (Macrogen, Maryland, MD) for sequencing.

DNA sequences were edited and aligned using the DNAStar computer software package (DNAStar Lasergene, Inc., Madison, WI). A BLASTN search of the National Center for Biotechnology Information (NCBI) database was performed to compare the IGS sequences of the isolates with those available online. Sequences for each isolate were submitted to the NCBI. Sequence contigs were assembled using SeqMan Pro module of the DNAStar computer software package (DNAStar Lasergene, Inc., Madison, WI).

3.3.7 Real-time PCR primers and assay conditions

Two published real-time assays were evaluated for each of the *Verticillium* species *V. dahliae*, *V. tricorpus* and *V. longisporum*, in order to select the most sensitive and specific real-time PCR assays for the quantification of the *Verticillium* species (Table 3.2). Real-time PCR amplification was carried out in a CFX96 Real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Real-time PCR amplifications were performed using the 5 Prime Real Master Mix without rox (Fisher Scientific, Waltham, MA) for the TaqMan® assays, and SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA) for the SYBR® Green assays. TaqMan® probes were synthesized by BioSearch (Bioasearch Technologies, Inc., Novato, CA, USA), and primers by IDT (Integrated DNA Technologies, Coralville, IA).

Reaction mixture of the real-time assay with the TaqMan® probe (25 µL total volume) for *V. dahliae* and *V. tricorpus* was performed as described by Bilodeau et al. (2012). Cycling condition for the TaqMan® assay used with *V. dahliae* was optimized experimentally to increase specificity of the assay with 2 min at 98 °C followed by 40 cycles of 15 sec at 95 °C and 60 sec at 68 °C (Table 3.2). The reaction mixture of the real-time assays performed with the intercalating dye SYBR® Green (20 µL total volume) contained 2 µL of template gDNA dilution (1:10), 200 nM of each primer and 10 µL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Reactions were run in triplicate for each sample (Technical replication). Amplification was followed by melting curve analysis at temperatures ranging from 55 to 95 °C, with

temperature increasing by 0.5 °C every 10 s, to distinguish potential primer dimer and nonspecific amplification products.

Table 3.2 TaqMan® and SYBR® Green real-time PCR assays screened for detection of the microsclerotia-forming *Verticillium* species *V. dahliae*, *V. tricorpus* and *V. longisporum*.

Target	Chemistry	Primers	Protocol	Reference
V. dahliae	SYBR® Green	VertBt-F AACAACAGTCCGATGGATAATTC VertBt-R GTACCGGGCTCGAGATCG	2 min at 98°C, 40 cycles of 5 s at 98°C and 5 s at 60°C.	Atallah et al. (2007)
	TaqMan®	Vd-F929-947: CGTTTCCCGTTACTCTTCT Vd-R1076-1094: GGATTTCGGCCCAGAAACT Probe: [5′ 6-FAM] CACCGCAAGCAGACTCTTGAAAGCCA [3′ BHQ1]	2 min at 98°C, 40 cycles of 15 sec at 95°C and 1 min at 68°C.	Bilodeau et al. (2012)
V. tricorpus	SYBR® Green	VtF4: CCGGTGTTGGGGATCTACT VtR2: GTAGGGGGTTTAGAGGCTG	2 min at 95°C and 40 cycles of 5 s at 95°C and 5 s at 60°C.	Debode et al. (2011)
	TaqMan®	IGS_VtF1: TAGTAGAATACTAGATARCTAG IGS_VtR1: AGCCTAGGTCTTTATAGCTAG Probe: [CAL FluorRed 610] TCCTACTAATACCTTACTATAACTCTT AAGGTA [BHQ2]	90 s at 95°C and 40 cycles at 15 s at 95°C and 30 s at 57°C.	Bilodeau et al. (2012)
V. longisporum	SYBR® Green	VlTubF2: GCAAAACCCTACCGGGTTATG VlTubR1: AGATATCCATCGGACTGTTCGTA	10 min at 95°C and 40 cycles of 15 s at 95°C and 1 min at 60°C.	Debode et al. (2011)
	SYBR® Green	Vlsp-F1: AGCCTGAGTCACGAGAGATATGGG Vlsp-R4: CAAACCACGCCACTGCATTCTCGT	2 min at 98°C and 40 cycles of 5 s at 98°C and 5 s at 60°C.	(Banno et al. 2011)

3.3.8 Examination of specificity and sensitivity of real-time PCR assays using *Verticillium* isolates

The specificity of the real-time PCR assays was assessed using gDNA from 21 different fungal isolates (Table 3.1). All fungal isolates used for specificity testing were cultured and subject to DNA extraction using the methods describe above. The extracted gDNA was quantified and checked for purity using a NanoDrop 2000 Spectophotometer (Thermo Scientific, Wilmington, DE), and diluted to 1 ng μL^{-1}.

In order to determine the sensitivity of the primers (and probe) set used for each assay, we estimated the concentration of purified gDNA of Vd1396-9 (*V. dahliae*), DVt3 (*V. tricorpus*), PD624 (*V. longisporum*), respectively, using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE) and used them as a reference isolates. Ten-fold serial dilutions of the extract were prepared, ranging from 10-20 ng μL^{-1} to 10-20 fg μL^{-1} of gDNA to test the PCR assay's sensitivity. Each standard curve was generated by plotting the cycle threshold (C_T) values, which are inversely proportional to detected gDNA content, versus the log of concentrations of gDNA (10-fold dilution series) from cultured mycelia. The concentration of gDNA for unknown samples was extrapolated from the C_T value and the value obtained from the standard curve.

Real-time PCR assays were completed on the soil samples, fungal isolates (*V. dahliae, V. tricorpus*, *V. albo-atrum* and *V. longisporum*) and reference isolates. Relative values for target abundance in each soil samples were extrapolated from the standard curve generated from gDNA extracted from reference isolates. All real-time PCR assays were

monitored on a CFX96^{TM} real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA).

All amplification reactions were performed in 8-strip tubes (Bio-Rad Laboratories, Hercules, CA). All real-time PCR reactions were repeated three times for each sample, and always included a positive control accordingly (DNA from either *V. dahliae* or *V. tricorpus* or *V. longisporum*), and a negative control (NTC: no template control). In order to confirm the presence and interference of non-target fungal gDNA and PCR inhibitors in the real-time PCR assays with TaqMan® or SYBR® Green, any soil gDNA sample of C_T value higher than 37 (TaqMan®) or 35 (SYBR® Green) and any sample that had no amplification at all was spiked with gDNA of the target organism at concentration of 1 ng μL^{-1} and re-run.

3.3.9 DNA quantification of *Verticillium species* in artificially infested soils

To determine whether the presence of total soil gDNA affected the efficiency of the selected real-time PCR assay to detect gDNA extracted from soil with *V. dahliae* microsclerotia, an experiment was performed using soil with and without microsclerotia of *V. dahliae*. For this purpose, microsclerotia of *V. dahliae* were produced in the laboratory using the isolate Vd1396-9, which is highly pathogenic to potato (Uppal et al. 2007). The isolates were grown on semisolid Czapek- Dox medium supplemented with Pectin (polygalacturonic acid from orange, Sigma-Aldrich) in the dark at 24 °C (Hawke and Lazarovits 1994). After 3-4 weeks incubation, the culture turned black due to the presence of microsclerotia; then, microsclerotia was poured through mesh screens to

obtain microsclerotia 75 to 106 μm in diameter and stored at 4 °C until use. Then, microsclerotia were spread and separated with the help of a micro-spatula on a Petri plate to help individual counting under a stereo microscope.

Field soil with no detectable endogenous populations of *V. dahliae* was autoclaved and air-dried for 7 days (Soil E). Then, 5, 25, 50, 100, 150, and 250 individual microsclerotia per gram (mpg) were thoroughly mixed with the autoclaved field soil. Total gDNA from each spiked and non-spiked soils was extracted from triplicate 500-mg samples using the PowerSoil® DNA Isolation Kit. Real-time PCR reactions were conducted in triplicate using the real-time PCR assay that best performed in the specificity and sensitivity test for *V. dahliae*. A regression was generated to analyze the association between the *V. dahliae* microsclerotia per gram of soil (mpg) and the concentration of *V. dahliae* gDNA (pg g^{-1}) extracted from soil with and without microsclerotia.

3.3.10 Comparison of wet plating cfu counts for *V. dahliae* in soil and real-time PCR quantification of microsclerotia-forming *Verticillium* species in soils

The traditional soil wet plating method was compared against the more specific and sensitive real-time PCR assays examined previously, for species specificity and capability to detect low gDNA concentrations of *V. dahliae*, *V. tricorpus* and *V. longisporum*. For this study, we used ten soil samples collected in two commercial potato field-sites planted to potato in 2012 (site B and site F) from the field study in Chapter 2. Additionally, a 300g sample of air-dried Hallboro fine sandy loam soil, which carried no detectable endogenous population of either *V. dahliae*, *V. tricorpus* or *V. longisporum*,

was also included in the experiment, for a total of 11 soils. The propagule density (cfu g^{-1}) of dried-soil was estimated four times for each soil sample, by plating each sample on the Sorensen's semi-selective medium as describe above. Total gDNA from each soil sample was extracted from quadruple 500 mg samples using the PowerSoil® DNA Isolation Kit, as previously described. Real-time PCR reactions were conducted in triplicate for each extracted sample.

3.3.11 Relationship between gDNA of *V. dahliae* and development of Verticillium wilt symptoms in cv. Russet Burbank

Additionally, 40 soil samples from two commercial potato field-sites (Site D and E, experiment cv. Russet Burbank-2013) from Chapter 2 were used to determine if the level of *V. dahliae* quantified by the selected real-time PCR assay can be used to predict the severity of Verticillium wilt in the processing cultivar cv. Russet Burbank. The experiment (cv. Russet Burbank-2013) is described in Chapter 2 as a multi-site experiment, with a randomized completed block design. The experiment (cv. Russet Burbank-2013) had two sites (Site D and E). The soils belong to Wheatland and Long Plain series, with a sandy loam and a sandy clay loam texture, and 1.5 and 5.9% soil organic matter, respectively. Each location had 5 treatments and 4 replicated plots, for a total of 20 plots per location. The following treatments were established at each site: control (CON); composted pig slurry solids 20 Mg ha^{-1} (CSS20); composted pig slurry solids 40 Mg ha^{-1} (CSS40); composted pig slurry solids 80 Mg ha^{-1} (CSS80); and High Fertility (FERT).

The soil samples were collected two weeks before potato seed planting. In the current study, *V. dahliae* and *V. tricorpus* propagule density was estimated as pg of gDNA g^{-1} of soil, using the real-time PCR assays described above. Soil gDNA was extracted from each soil sample using the PowerSoil® DNA Isolation Kit. All real-time PCR reactions were conducted in triplicate for each sample.

Disease severity was assessed 10 weeks after planting and recorded on a weekly basis thereafter until crop was harvest as described previously in Chapter 2. The severity was evaluated using a modification of the procedure described by MacGuidwin and Rouse (1990). Symptoms of Verticillium wilt were assessed using a scale of 0= no symptoms, 1= 1-33% of the foliage showing wilting, necrosis or chlorosis, 2= 34-66%, 3= 66-99%, and 4= plant dead. Fifteen plants in the middle 2 rows of each plot were rated individually. Severity values were converted to area under the wilt progress curve (AUWPC) using the equation $\Sigma_i^{n-1}\ [(Y_i + Y_{i+1})/2](t_{i+1} - t_i)$, where Y_i = cumulative disease severity at the i^{th} observation, t_i = time (days after planting) at the i^{th} observation, and n = number of observations (Shaner and Finney 1977).

3.3.12 Statistical analysis

Linear regression analyses were performed to determine whether significant relationships existed between *V. dahliae* gDNA (pg g^{-1} soil) and propagule density (cfu g^{-1} soil) from soil plating and microsclerotia g^{-1} spiked-soil (mpg). The efficiency of each real-time PCR assay, slope and the coefficient of determination (R^2) were automatically calculated by the Bio-Rad CFX manager software v3.0 (Bio-Rad Laboratories, Hercules, CA). The

slope and R^2 were determined with the quantification of the standards described earlier. R^2 was considered as suitable when no lower than 0.96. The efficiency was considered satisfactory when not lower than 90% and above 105% (González-Salgado et al. 2009; Pfaffl et al. 2009). The efficiency was used as indicator of reproducibility of the real-time PCR assay and it was determined from the slope of the standard curve using the formula: $E= 10^{(-1/slope)}-1$ (González-Salgado et al. 2009). Verticillium wilt severity variability and deviation of plate counting and real-time PCR quantification of *V. dahliae* between samples obtained from Site D and E from Chapter 2 were analyzed using analysis of variance (ANOVA) with the PROC Mixed procedure (SAS Institute, release 9.2, Cary, NC). The data was analyzed as a one-way ANOVA, with block as a random effect. Site means were separated using the Bonferroni's procedure if the F-test was significant ($P<0.05$). Where data were not normally distributed, appropriate transformations were performed prior analysis. The relationship between Verticillium wilt severity, *Verticillium* propagule density from plating, and gDNA quantity of *V. dahliae* and *V. tricorpus* was analyzed using simple regression. All analyses were performed using the Statistical Software SAS (SAS Institute, release 9.2, Cary, NC).

3.4 RESULTS

3.4.1 Microsclerotia-forming *Verticillium* species

Three species of *Verticillium*-producing microsclerotia were found among 82 monosporic cultures produced from soil and potato stems from a total of 17 fields in Manitoba (Table 3.3). Direct identification of the *Verticillium* isolates on Sorensen's NP-10 medium plated with soil was difficult due to the similarity in the microsclerotia-

formed colonies, or for the presence of other fungal and bacterial colonies interfering with the visualization, or the physical perturbation that some colonies suffered before the examination under the microscope due to washing the excess of soil from the surface of the agar plates. According to the morphology on PDA media, the dominant species were *V. dahliae* and *V. tricorpus*, which comprised 68 and 28% of the total isolates (n=82), respectively. *Verticillium dahliae* was frequently recovered from soils and potato stems and represented between 24-100% (mean= 78%) of the total isolates obtained from each RM (Table 3.2).

Table 3.3 Number of isolates obtained from 23 and 59 *Verticillium* colonies obtained from potato plants and soil, respectively, from the eight major potato producing Rural Municipalities in Manitoba.

Rural	***V. dahliae***		***V. tricorpus***		***V. klebahnii***		***G. nigrescens*** [a]	
Municipality	Plant	Soil	Plant	Soil	Plant	Soil	Plant	Soil
Elton	4	3	2	-	-	-	-	-
North Cypress	3	17	2	5	-	-	-	-
North Norfolk	2	3	2	12	-	1	-	1
Portage la Prairie	4	2	-	-	-	-	-	-
Dufferin	1	1	-	-	-	-	-	-
Thompson	1	-	-	-	-	-	-	1
Stanley	1	10	-	-	-	-	-	-
Rhineland	1	3	-	-	-	-	-	-
Total[b]	17	39	6	17	0	1	0	2

[a] Not a *Verticillium* species, but formerly called *V. nigrescens*.
b Total number of isolates recovered from potato plants or soil.

Verticillium dahliae isolates were found in all sampled fields in all RMs. *V. tricorpus* was found in the western RMs of Elthon, North Cypress and North Norfolk, while *V. klebbanii* and *G. nigrescens* were recovered from two different fields in the RM of North Norfolk and Thompson, respectively (Table 3.3). Interestingly, in the North Norfolk RM

14 isolates (68%) of *V. tricorpus* were recovered, along with five isolates of *V. dahliae* (24%), one isolate of *V. klebanni* (5%) and one isolate of *G. nigrescens* (5%).

Colonies of *V. dahliae* isolates produced on PDA, were initially white and then black, due to the development of microsclerotia (Figure 3.1A). Microsclerotia were usually globose, oval to elongate (Figure 3.1D, E). Hyphae were hyaline and there was no presence of dark resting mycelium (Figure 3.1A, D). The size of the microsclerotia (length by width) ranged from 49.6 to 101 by 35.6 to 78 µm, with an average size of 50.4 by 84 µm (Appendix IV). Conidia were rounded to oval and hyaline. The size of the conidia ranged from 1.6 to 4 by 2.5 to 11 µm, with a mean of 2.73 by 5.4 µm (Appendix IV). Chlamydospores were absent.

Verticillium tricorpus isolates, collected from either plant or soil, produced microsclerotia on Sorensen's NP-10 and PDA media. The colonies on PDA were initially white, and turned to dark brown due to development of microsclerotia and melanized mycelia (Figure 3.2A). The isolates showed a yellow-orange discoloration around the edges of the colony when growing on PDA after two weeks (Figure 3.2A, D). Such discoloration was not always present in colonies growing on Sorensen's NP-10 media. The presence of dark mycelia was very noticeable (Figure 3.2A) connecting or not with the microsclerotia when growing on PDA (Figure 3.2C).

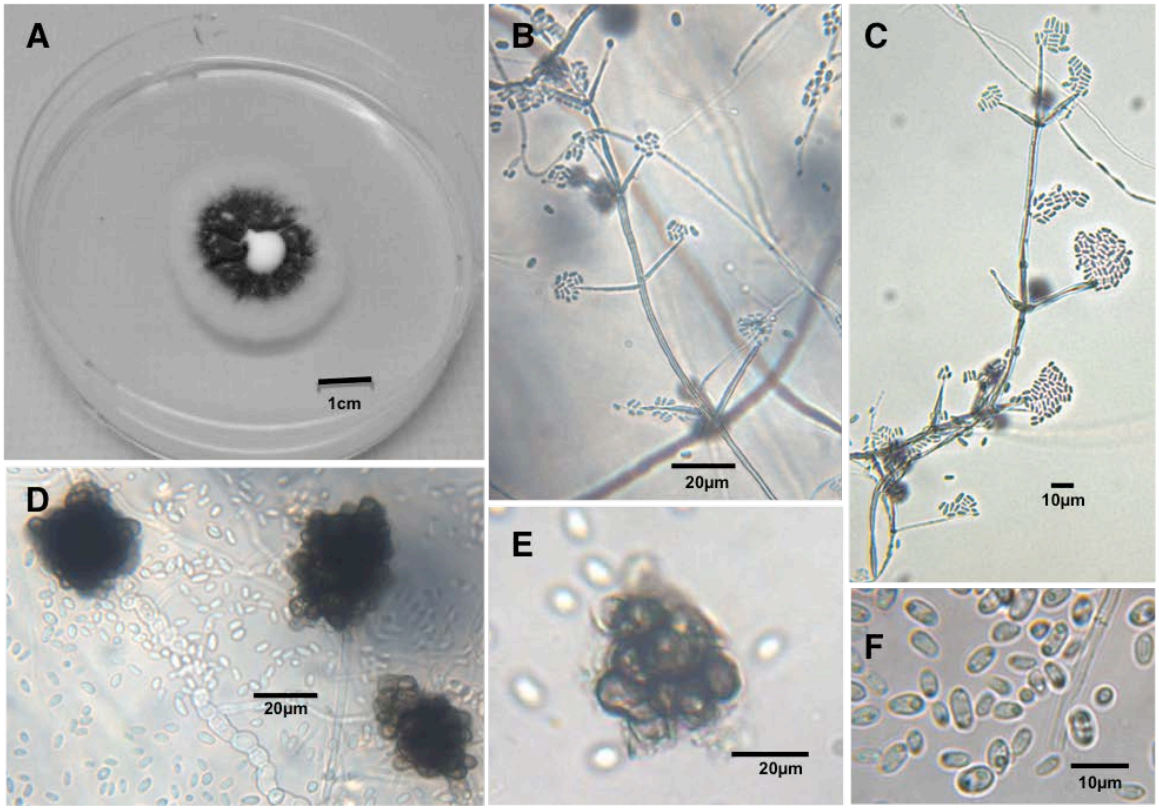

Figure 3.1 Morphology of *Verticillium dahliae* isolated from Manitoba fields. **A.** Colony after 18 days on PDA. **B, C.** Conidiophore and whorl phialide with conidia. **D, E.** Microsclerotia. **F.** Conidia.

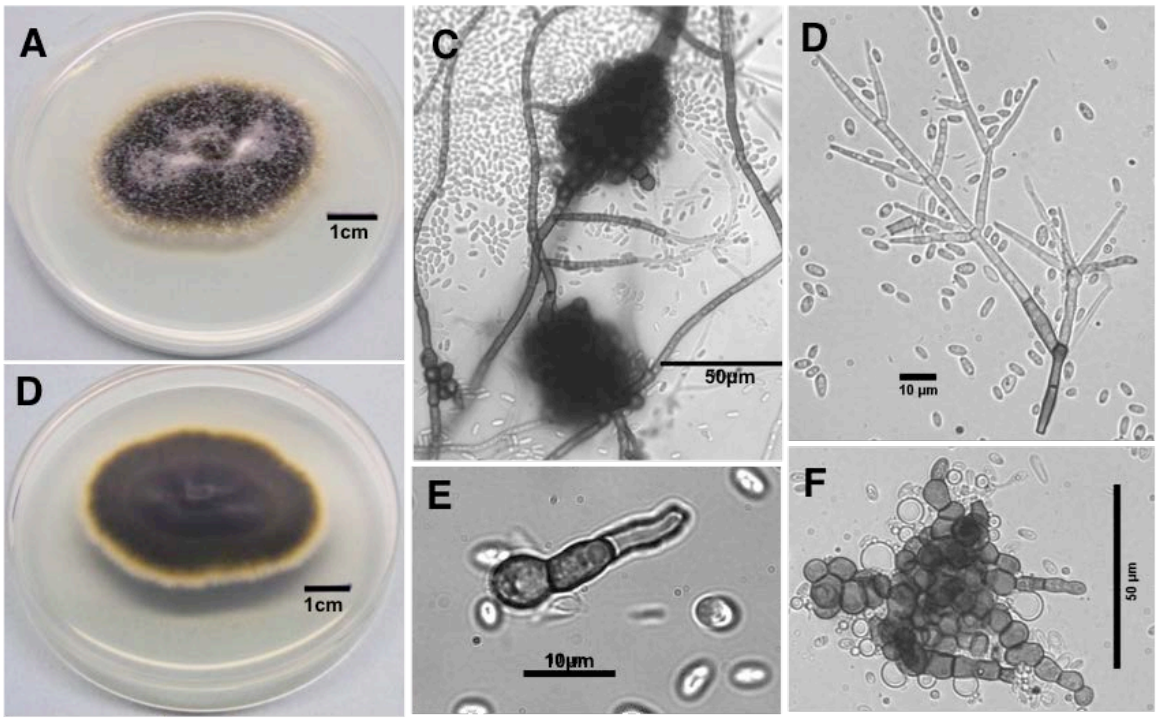

Figure 3.2 Morphology of *Verticillium tricorpus* isolated from Manitoba fields. **A.** Colony after 18 days on PDA, frontal view. **B.** Colony after 18 days on PDA, reverse view. **C.** Microsclerotia and dark mycelium. **D.** Conidiophore, whorl phialide and conidia. **E.** Chlamydospore. **F.** Microsclerotia.

Microsclerotia were usually rounded or elongate, ranging from 43.3 to 94.5 µm by 73 to 154 µm, with an average size of 64.7 by 100.8 µm. Conidia were hyaline and rounded to oval ranging from 2.3 to 4.6 µm by 3.4 by 11.2 µm, with a mean size of 3.3 by 5.7 µm (Appendix V). Chlamydospores were present with a mean size of 6.6 by 7.9 µm (Appendix V) and usually found in the same sample with microsclerotia and dark hyphae (Figure 3.2C, E).

Although *V. tricorpus* isolates were all morphologically similar, one of the isolates was identified as *V. klebahnii* after being sequenced (Table 3.2, Appendix V). A 1.7-1.9kb fragment of the IGS region of each monosporic isolate was sequenced. The sequences were compared with sequences deposited in the GeneBank ID database (Appendix IV). The comparison with the database confirmed the identification of the *V. dahliae* and *V. tricorpus* isolates, and allowed identification of those ambiguous isolates that were morphologically identical, such as *V. tricorpus* and *V. klebahnii*. *Verticillium klebahnii* colonies produced on PDA were initially white and then dark brown, due to the presence of microsclerotia, chlamydospores and melanized resting mycelium, exactly similar to the *V. tricorpus* isolates. However, the yellow-orange ring on the edges of the colony was not observed. Chlamydospores ranged from 4.5 to 7.6 µm by 8.8 to 10.3 µm, with a mean of 6.6 by 9.5 µm. The conidial size ranged from 2.4 to 3.1 by 3.6 to 8.2, with an average size of 2.8 by 5.6 µm (Appendix V). The size of the microsclerotia of the one isolate found ranged from 57.9 to 67 by 93.9 to 111 µm, with a mean size of 61.5 by 99.8 µm (Appendix III).

The *G. nigrescens* isolates were recovered while trying to pick single microsclerotia from colonies growing on Sorensen's medium plated with field soil. These isolates did not produce microsclerotia when growing on Sorensen's NP-10 or PDA media. *Gibellulopsis nigrescens* isolates were initially white and soon became brown when growing on PDA. Chlamydospores were also found as single or in short chains, with an average size of 5.6 by 7.8 µm. Conidia size ranged from 2.3 to 3.6 µm by 3.7 to 9.2 µm, with an average size of 3.1 by 6.3 µm (Appendix III).

3.4.2 Comparison of real-time PCR assays for the quantitative detection of *V. dahliae, V. tricorpus* and *V. longisporum*

The specificity of the primers (and probe for the taq-man assay) set of each real-time PCR assay (Table 3.1) was evaluated using a panel of gDNAs from related and unrelated fungi (Table 3.2). The real-time PCR assays with the primers VertBt-F/VertBt-R and Vd-F929-947/R1076-1094 for *V. dahliae*, VtF4/VtR2 and IGS-VtF1/IGS-VtR1 for *V. tricorpus*, and VlTubF2/VITubR1 and Vlsp-F1/Vlsp-R4 for *V. longisporum* were specific for the intended species respectively. None of the primer sets show cross-amplification with other *Verticillium* species.

Although the real-time PCR assays using the primers VertBt-F/VertBt-R, IGS-VtF1/IGS-VtR1, and VlTubF2/VITubR1 showed species specificity, low sensitivity of amplification occurred after four orders of magnitude of gDNA concentration (0.0002 ng ml^{-1} or lower) for Vd1396-9 (*V. dahliae*), DVt3 (*V. tricorpus*), PD624 (*V. longisporum*), respectively.

The real-time PCR assays with the primers Vd-F929-947/R1076-1094 for *V. dahliae*, VtF4/VtR2 for *V. tricorpus*, and Vlsp-F1/Vlsp-R4 for *V. longisporum* showed high sensitivity when screened with the respective reference isolates through serial dilutions of gDNA (Figure 3.3). The standard curves generated using a range of gDNA demonstrated that the indicated assays had good reproducibility of amplification with 99, 94 and 98% efficiency with six orders of magnitude of purified gDNA of *V. dahliae*, *V. tricorpus* and *V. longisporum*, respectively (Figure 3.3). Quantification showed a linear regression (R^2= 0.96, 0.97 and 0.99, for *V. dahliae, V. tricorpus* and *V. longispurum*, respectively) between the log of the gDNA concentration and the C_T value over the range of gDNA concentrations evaluated (Figure 3.3).

The minimum starting concentration of *V. dahliae* gDNA that could be accurately quantified with the real-time PCR assay using the primers Vd-F929-947/R1076-1094 was 0.1 pg, which corresponded with a C_T value of 36±0.02 (± standard deviation of C_T of technical replicates) (Figure 3.3A). Although the indicated real-time PCR assay detected gDNA dilutions as low as 0.01 pg, amplification was late, with C_T values of 39±0.04, or absent (Figure 3.3 A).

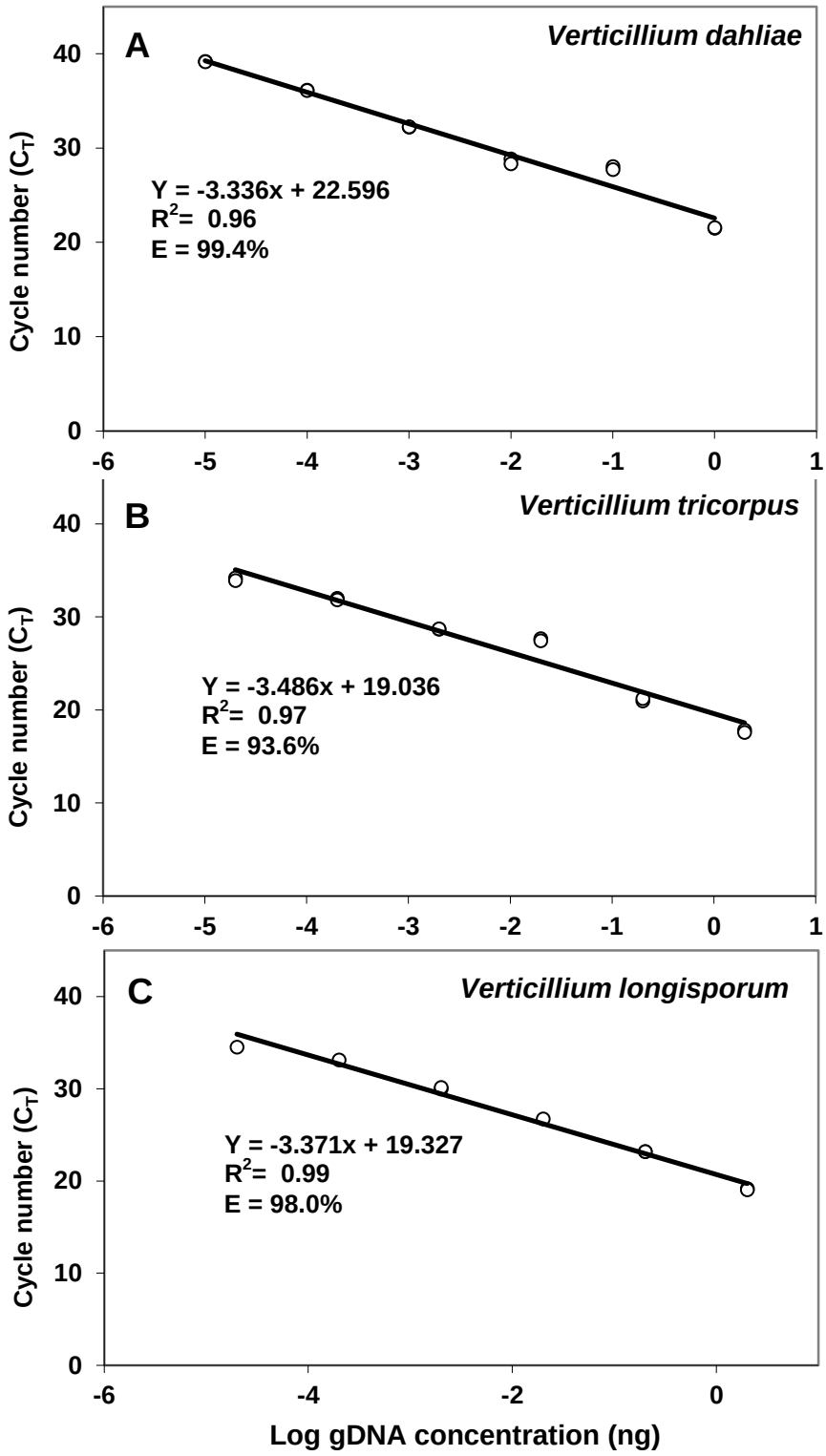

Figure 3.3 Standard curves showing amplification of successive 10-fold dilutions of **(A)** *V. dahliae* (Isolate *Vd*1396-9), **(B)** *V. tricorpus* (Isolate DVt3) and **(C)** *V. longisporum* (PD624) genomic DNA (gDNA).

Similarly, the real-time PCR assays with the primers VtF4/VtR2 for *V. tricorpus* and Vlsp-F1/Vlsp-R4 for *V. longisporum* quantified gDNA down to a concentration of 0.02 pg, which corresponded with C_T values of 34.2±0.1 and 34.5±0.07, respectively (Fig.3.3 B,C). At lower gDNA concentration below this point, detection was not possible. Therefore, the detection limits were fixed at the estimated C_T values corresponding to those concentrations at 37 for *V. dahliae,* and 35 for *V. tricorpus* and *V. longisporum*.

The confirmed two real-time PCR assays for *V. dahliae* (TaqMan® using primer set Vd-F929-947/R1076-1094 and probe 5′ 6-FAM) and *V. tricorpus (*SYBR® Green with primer set VtF4/VtR2), were then tested for their ability to identify the *Verticillium* isolates recovered from potato soil and plant material from Manitoba. The real-time assays effectively confirmed the identity of the isolates given by the morphological and molecular analysis (Table 3.3, S2).

3.4.3 Comparison of wet plating cfu counts for *V. dahliae* in soil and real-time PCR for the quantification of microsclerotia-forming *Verticillium* species in soil

Propagule density (cfu g^{-1}) from plating did not allow specific quantification of either *V. dahliae* or *V. tricorpus* due to the similarity in the colony morphology. Propagule density (cfu g^{-1}) in soils collected in site B and site F (Samples from field study in Chapter 2) ranged from 14 to 215 and 103 to 157 cfu g^{-1}, respectively. The concentration of *V. dahliae* gDNA ranged from 0 to 55 and 18 to 87 pg g^{-1} of soil, in site B and site F, respectively (Table 3.4). The real-time PCR assays for *V. dahliae* (primer set Vd-F929-

947/R1076-1094 and probe 5′ 6-FAM), *V. tricorpus* (primer set VtF4/VtR2) and *V. longisporum* (primer set Vlsp-F1/Vlsp-R4) confirmed the presence of *V. dahliae* or *V. tricorpus*, or both species producing microsclerotia in the subject soils. In fact, the real-time assays allowed differentiation of sites after *V. tricorpus* was only found in soil samples from site B with levels varying from 6.0 to 21.1 pg gDNA g^{-1} air-dried soil. *Verticillium longisporum* was not found in any of the samples from the two fields used in this particular experiment, or from any of the other sampled fields during the study.

Table 3.4 *Verticillium* propagule density estimated by wet plating (cfu g^{-1} soil) and genomic DNA concentration of microsclerotia-forming *Verticillium* species in some Manitoba soils by real-time PCR assays.

Soil ID[a]	**cfu**[b]	***V. dahliae***	***V. tricorpus***	***V. longisporum***
		Concentration (pg gDNA g^{-1} soil [b])		
Soil 522	54 (37)	0[c] (0)	15.1 (2.4)	0[d] (0)
Soil 524	90 (27)	9.5 (3)	21.1 (8.6)	0 (0)
Soil 531	14 (3)	0 (0)	10.7 (2.3)	0 (0)
Soil 534	179 (36)	75.4 (11.8)	6.8 (3.5)	0 (0)
Soil 536	215 (83)	55.1 (15.1)	6.0 (1.9)	0 (0)
Soil 566	157 (31)	26 (7.6)	0[d] (0)	0 (0)
Soil 570	171 (53)	87.4 (19.8)	0 (0)	0 (0)
Soil 571	103 (17)	17.9 (5.4)	0 (0)	0 (0)
Soil 594	177 (14)	53.8(14.7)	0 (0)	0 (0)
Soil 603	125 (25)	50.7 (11)	0 (0)	0 (0)
Soil E[e]	0 (0)	0 (0)	0 (0)	0 (0)

[a] Soil samples from two sites in field study Chapter 2. Soils 522, 524, 531, 534, and 536 from Site B, and Soils 566, 570, 571, 594, and 603 from site F.
[b] Soils were plated and extracted four times (n=4). Mean (standard deviation).
[c] C_T value for this soil was >37.
[d] C_T value for this soil was >35.
[e] Control soil, no *Verticillium* present.

In soils naturally infested, there was a relationship between *Verticillium* cfu estimated by the traditional plating method and the concentration of *V. dahliae* gDNA estimated through the real-time PCR assays (R^2= 0.68 *P*= 0.003, Figure 3.4A). The real-time assays allowed identification of soils with only V. tricorpus, particularly soils 522 and 531, where the traditional plating method did not allow differentiation of the species (Table 3.4, Figure 3.4A).

In soil (Soil E, Table 3.4) artificially spiked with *V. dahliae* microsclerotia of 75-30 µm diameter, gDNA of *V. dahliae* extracted from the spiked soil samples ranged from 0.3 to 118 pg g^{-1}, for densities of *V. dahliae* from 5 to 250 microsclerotia added per gram of soil (Figure 3.4B). There was a significant quadratic relationship (R^2= 0.99 *P*= 0.0001) between the concentration of *V. dahliae* gDNA extracted from the spiked soil samples and the number of microsclerotia of *V. dahliae* added (Figure 3.4B).

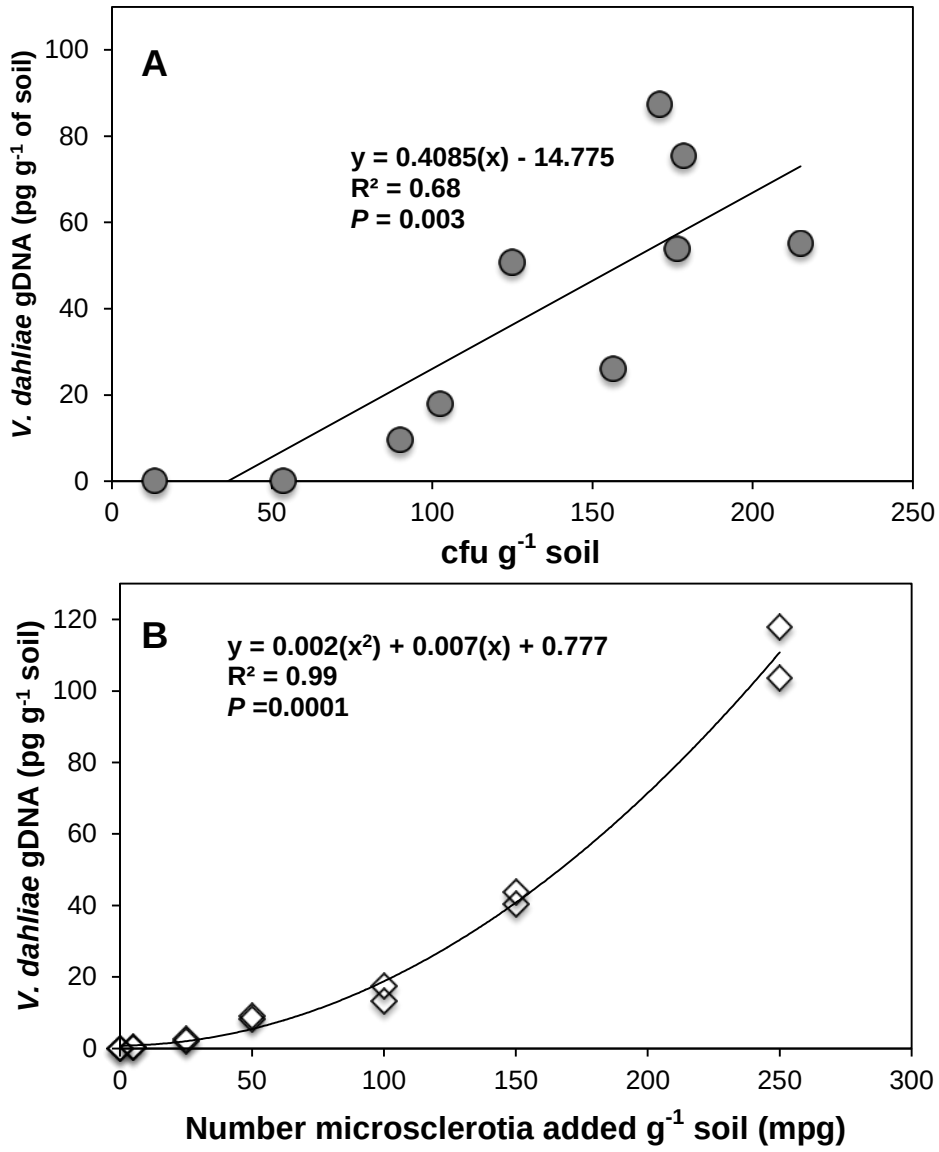

Figure 3.4 (A). Relationship between *Verticillium* propagule density (cfu) in soils naturally infested with *V. dahliae* and concentration of *V. dahliae* genomic DNA (gDNA) in soil. **(B).** Relationship between the number of *V. dahliae* (Vd1396-9) microsclerotia per gram (mpg) of spiked soil and the estimated concentration of *V. dahliae* genomic DNA (gDNA).

3.4.4 Effect of *V. tricorpus* on the quantification of *V. dahliae*

The average (n=20) *Verticillium* propagule density measured as cfu g^{-1} before all plots were planted to potato was two times higher in Site E than Site D (P =0.001, Figure 3.5A). Verticillium propagule density in site D ranged from 10 to 78 cfu g^{-1} of soil, and in site E from 14 to 206 cfu g^{-1} (Figure 3.5A). Real-time PCR assays detected *V. dahliae* and *V. tricorpus* in these two experimental sites. However, the levels of *V. dahliae* and *V. tricorpus* before potato planting were significantly different between sites (P=0.0001, Figure 3.5B). DNA detection for *V. dahliae* and *V. tricorpus* in Site D ranged from 0.2 to 62.3 pg g^{-1}, and 0.4 to 8.5 pg g^{-1} of soil, respectively. In Site E, the levels were 0.2 to 0.4 pg gDNA g^{-1} of soil for *V. dahliae*, and 2 to 8.5 pg g^{-1} of soil for *V. tricorpus* (Figure 3.5B). The real-time PCR analyses confirmed the presence of *V. dahliae* and *V. tricorpus* in all soil samples from sites D and E. Severity of Verticillium wilt, measured as AUWPC, was significantly different between sites (P= 0.001, Figure 3.5C).

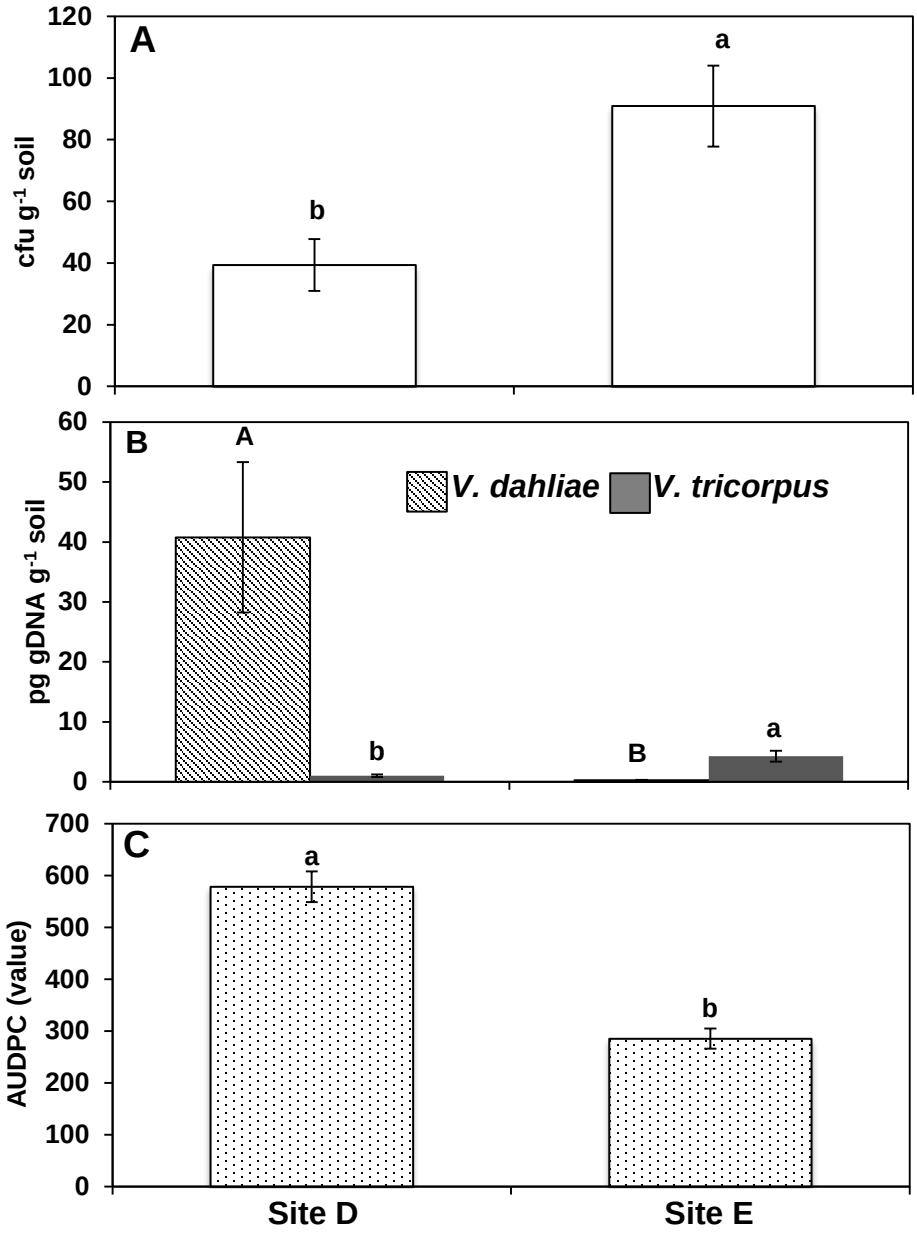

Figure 3.5 (A) Propagule density of *Verticillium* spp as cfu g^{-1} soil. **(B)** Propagule density of *V. dahliae* and *V. tricorpus* as pg genomic DNA(gDNA) g^{-1} soil. **(C)** Area under the wilt progress curve (AUWPC) in sites D and E from field study in Chapter 2. Means across sites within *Verticillium* specie followed by the same letter are not significantly different, according to Bonferroni's multiple comparison test (P>0.05). Error bars are ±1 standard error of the mean.

Verticillium propagule density in sites D and E, measured as cfu g^{-1} with the wet plating method, was a weak predictor of the development of Verticillium wilt symptoms (Figure 3.6A). AUWPC followed a significant negative linear relationship (R^2= 0.37, *P =0.04*, Figure 3.6A) in response to *Verticillium* propagule density. In contrast, there was a significant quadratic relationship (R^2=0.75, *P*= 0.003) between the concentration of *V. dahliae* gDNA g^{-1} of soil using the real-time PCR assay and the Verticillium wilt severity measured as AUWPC in the cv. Russet Burbank (Figure 3.6B). The AUWPC seemed to stay lower than 25 when the concentration of *V. dahliae* gDNA in soil was lower than 0.5 pg g^{-1}. However, when the concentration of *V. dahliae* in soil was higher than 10 pg g^{-1}, AUWPC increased to 505, and stayed within a range from 505 to 721 even after the inoculum increased up to 70 pg g^{-1}. In contrast, there was a significantly negative quadratic relationship between the level of *V. tricorpus* and AUWPC (R^2= 0.75, *P*= 0.003, Figure 3.6C). Potato plants in plots with more than 2 pg g^{-1} of *V. tricorpus* gDNA in the soil exhibited lower AUWPC, than those soil samples with lower levels of *V. tricorpus*.

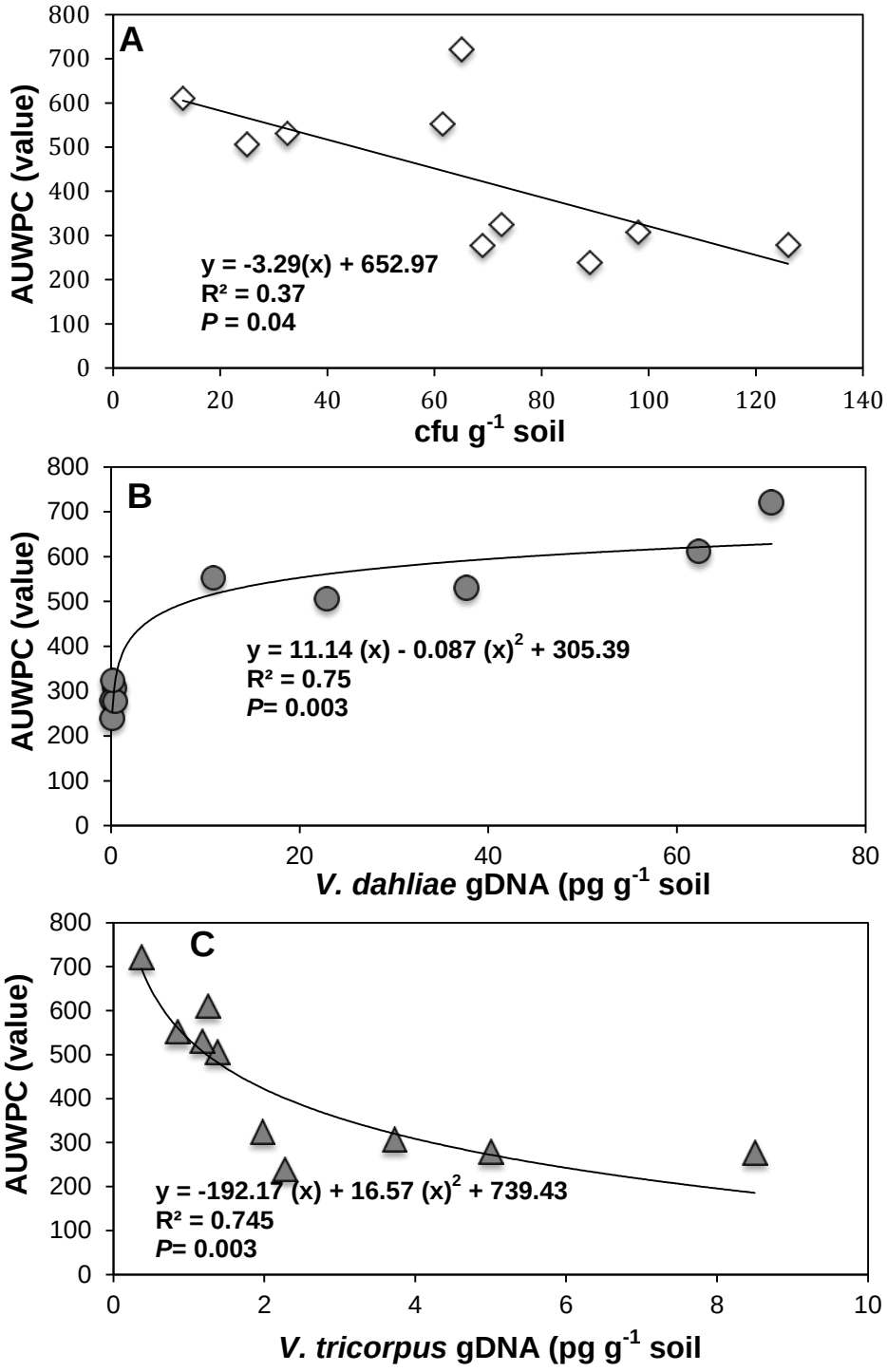

Figure 3.6 Relationships between AUWPC in sites D and E and: **(A)** *Verticillium* propagule density as cfu g^{-1} soil, **(B)** *V. dahliae* genomic DNA (gDNA) in soil (pg g^{-1} soil), and **(C)** *V. tricorpus* genomic DNA (gDNA) in soil (pg g^{-1} soil).

3.5 DISCUSSION

3.5.1 Prevalent microsclerotia-forming *Verticillium* species in potato fields

In the current study, three different fungal species were found producing microsclerotia in potato soil samples collected from seven rural municipalities in Manitoba, which represent the major potato production regions in Manitoba. In previous studies conducted in this province, 91% of the recovery isolates were identified as *V. dahliae* (Uppal et al. 2007). In the current study, morphological and molecular characterization confirmed the presence of two dominant species, *V. dahliae* and *V. tricorpus* representing 97% of the isolates recovery from soil or plant. Seventy six percent of the isolates produced microsclerotia as the only resting structure, and failed to produce dark mycelia and chlamydospores, which was consistent with the description given to *V. dahliae* isolates (Inderbitzin et al. 2011; Pegg and Brady 2002; Termorshuizen et al. 1998). Resting structures are important for the biology of *Verticillium* as well as its taxonomy. *Verticillium* species reproduce only asexually, as no sexual state is known (Inderbitzin et al. 2011; Usami et al. 2009). Microsclerotia are very important persistent dormant structures for *V. dahliae*, because they are the only means besides the mitotically-produced spores that allow the fungus to reproduce and survive long periods of time (Collado-Romero et al. 2008; Milgroom et al. 2014). Resting structures are traditionally used as the primary characteristic to distinguish *Verticillium* species.

The second most commonly found microsclerotia-forming *Verticillium* species in Manitoba was *V. tricorpus*, with a frequency of occurrence of 21% of the recovered isolates. It was detected and recovered from 3 of 8 major potato productive regions in

Manitoba, which indicates that *V. tricorpus* is fairly dispersed in Manitoba. Another *Verticillium* species found in Manitoba was *V. klebahnii*, for which identification was only confirmed after the IGS sequence was compared with sequences of *V. tricorpus*, *V. klebahnii* and *V. issacii* strains placed in the NCBI. Identification of these three species is very difficult using only the classical morphological and plated methods, because *V. klebahnii* and *V. isaacii* are morphologically indistinguishable from *V. tricorpus* (Inderbitzin et al. 2013).

3.5.2 The challenge of quantifying *Verticillium* species by culturing methods

Detection and further identification of *V. dahliae* species using the classical isolation and growth on semi-selective media methods can be laborious and challenging. For instance, microsclerotia-formed colonies of *V. tricorpus* can be very similar to those of *V. dahliae* (Bilodeau et al. 2012; Debode et al. 2011). Microsclerotia in *V. tricorpus* colonies form a scattered pattern with a few microsclerotia, compared with a more prolific and radial microsclerotia pattern for *V. dahliae* (Goud et al. 2003). However, in our experience colonies were usually found under mycelia produced by other fungi growing on the same medium. This pattern was probably the result of the slower growth rate of the *V. dahliae* and *Verticillium* species in general, on the growth media (Ausher et al. 1975).

In addition to those small formed colonies, colonies were often found disturbed, with only a few microsclerotia, after removing excess mycelia and soil to allow observation of colonies under the microscope. As a result, many colonies could have been misidentified and counted or not as *V. dahliae*, due to the fact that small colonies with fewer than 25

microsclerotia are very difficult to identify as either *V. dahliae* or *V. tricorpus* (Goud et al. 2003). Additionally, most characteristics of these *Verticillium* species are less pronounced when growing on soil plated media (Goud et al. 2003). Therefore, in the case of those species morphologically similar, such as *V. longisporum* and *V. dahliae*, individual quantification using the soil plating method, will only be possible after growing the isolates in pure culture, by looking at morphological traits such as the size of the conidia, which is larger in *V. longisporum* (Bilodeau et al. 2012; Karapapa et al. 1997). Nevertheless, the culturing of *Verticillium* isolates does not guarantee the identification of the specie, as some of the morphological characteristics could be compromised. For instance, *V. tricorpus* can produce smaller microsclerotia and fewer dark resting mycelia and chlamydospores, than is typical for this specie, and restrict the production of yellow-orange pigmentation when grown on PDA (Qin et al. 2008).

Interestingly, *V. longisporum* is another important *Verticillium* species that was recently found in Manitoba fields (Desjardins et al. 2015). *Verticillium longisporum* is an important pathogen, producing significant economic losses in canola (Karapapa et al. 1997), a crop commonly used in rotations with wheat and potato in Manitoba (Mohr et al. 2011). In this case, the presence of other *Verticillium* species that produce microsclerotia in the soil (e.g. *V. longisporum*), will contribute to overestimation of the levels of *V. dahliae*. This illustrates another difficulty associated with the traditional plating method; The colony morphology of the *V. longisporum* cannot be differentiated from that in *V. dahliae* when growing on NP-medium (Bilodeau et al. 2012), which is the same medium used in the plating method.

Verticillium tricorpus is mainly known as a saprophytic organism that thrives on decaying organic matter and as a causal agent of opportunistic infections on weakened plant hosts (Goud et al. 2003). Indeed, *V. tricorpus* is able to prompt wilt symptoms when a host plant has been challenged with other abiotic stresses, such as high soil nitrogen levels and waterlogging conditions (Isaac 1956). This could explain the presence of *V. tricorpus* in potato plants severely affected by Verticillium wilt, as plants were wilted and individual leaflets and stems were already dying, common symptoms of advanced stages of the disease (Rowe and Powelson 2002).

3.5.3 Importance of quantification of microsclerotia-forming *Verticillium* species in soil by real-time PCR

The lack of accurate identification and quantification of *V. dahliae* propagule density can contribute to higher costs for producing potatoes. The results presented in the current study suggest that the current wet plating method is not sufficiently specific to differentiate propagule density of *V. dahliae* from other microsclerotia-forming *Verticillium* species currently present in Manitoba soils, such as *V. tricorpus*, *V. klebahnii* and *V. longisporum*.

In the current study, we evaluated several real-time PCR assays and selected three assays for their specificity, sensitivity and reproducibility to quantify *V. dahliae*, *V. tricorpus* and *V. longisporum*, respectively. The real-time PCR assay selected for *V. dahliae* was the TaqMan® using primer set Vd-F929-947/R1076-1094 and probe 5′ 6-FAM

(Bilodeau et al. 2012). The specificity of the indicated real-time PCR assay was tested on a number of *V. dahliae* isolates, including reference isolates and those recovered from Manitoba soils, as well as on other *Verticillium* species. The real-time PCR assay selected for *V. dahliae* was confirmed as specific. The standard curve showed great linearity (R^2= 0.96) and amplification efficiency obtained from the curve slope was very close to 100% (99.4%), which is the best indicator of a reproducible assay (González-Salgado et al. 2009). The indicated real-time PCR assay achieved high sensitivity of amplification with high efficiency over five orders of magnitude of gDNA concentration, up to 0.1 pg gDNA of *V. dahliae*.

Although the real-time PCR assay for *V. dahliae* (using primer set Vd-F929-947/R1076-1094 and probe 5′ 6-FAM) was sensitive and able to detect 0.1 pg of gDNA of *V. dahliae*, Bilodeau et al. (2012) reported that the least gDNA concentration detected by the same assay was 3 fg. This difference could be explained by the fact that different DNA extraction methods were used in both studies. DNA extraction methods may cause markedly different yields of fungal DNA, as well as inhibitors, and thus can significantly affect the results of quantitative real-time PCR (Fredricks et al. 2005). In fact, the same real-time PCR assay was evaluated by Gramaje et al. (2013), who also used a DNA extraction method that was different from that used by Bilodeau. They reported a minimum detection limit similar to that achieved in the current study and encountered lost of reproducibility of amplification at gDNA concentrations of 0.01 pg and lower.

In the current study, the analysis of field soils spiked with microsclerotia of *V. dahliae* indicated that the real-time PCR with the primer pair Vd-F929-947/R1076-1094 and probe 5′ 6-FAM, in conjunction with the soil pulverizing step performed before soil DNA extraction using the MoBio PowerSoil DNA Isolation system, detected up to 5 microsclerotia per gram of soil, which corresponded to 0.3 pg of gDNA of *V. dahliae*. Although the indicated real-time PCR was not tested with a lower concentration of microsclerotia, the strong relationship between the microsclerotia added and the detected *V. dahliae* gDNA demonstrated accuracy in detecting microsclerotia in soil. This is particularly important for potato producers because the reported disease thresholds for *V. dahliae* in North America range between 8 and 20 microsclerotia per gram of soil (Davis and Sorensen 1986; Nicot and Rouse 1987). Therefore, the indicated real-time PCR can quantify *V. dahliae* microsclerotia at concentrations below those producing significant disease losses.

The selected real-time PCR assay has the potential to detect as little as one microsclerotium of *V. dahliae* (3 fg gDNA) (Bilodeau et al. 2012). However, this will likely depend on the ability to improve the soil DNA extraction process. If the DNA extraction method can increase yield DNA, without increasing PCR inhibitors in the DNA, detection limits will likely improve. For instance, the combination of the PowerSoil® DNA Isolation extraction system, used in the current study, with a density flotation-based extraction of microsclerotia using 100 g of soil allowed detection of *V. dahliae* in soil spiked with as little as 0.5 microsclerotia g^{-1} of soil (Debode et al. 2011).

The real-time PCR assays selected for *V. tricorpus* and *V. longisporum* were the SYBR® Green based-protocols using the primer pair VtF4/VtR2 (Debode et al. 2011) and Vlsp-F1/Vlsp-R4 (Banno et al. 2011), respectively. Real-time PCR assays were very sensitive, as both quantified gDNA of their corresponding pathogen up to a concentration of 0.02 pg g^{-1} of soil, for *V. tricorpus* and *V. longisporum*, respectively. The sensitivity of the indicated real-time PCR assays was similar to the sensitivity achieved in previous studies (Banno et al. 2011; Debode et al. 2011) and also similar to our observations for *V. dahliae*. Standard curves for both assays showed great linearity (R^2 >0.97) with high efficiency (94% and 98%, respectively) over five orders of magnitude of gDNA concentration. Efficiencies for an optimal reproducible real-time PCR should be between 90% and 105% (González-Salgado et al. 2009), which confirm the good reproducibility of the indicated real-time PCR assays.

Although the concentration of *V. dahliae* gDNA in soil was significantly related to the propagule density (cfu g^{-1}) estimated using the wet plating method (R^2=0.68 *P*=0.003) (Figure 3.4A), a stronger relationship was observed by Bilodeau et al. (2012) for cfu g^{-1} of *V. dahliae* and the C_T values obtained from the same real-time PCR assay (primer set Vd-F929-947/R1076-1094 and probe 5′ 6-FAM). In the current study, the weaker relationship was perhaps due to the presence of *V. tricorpus* propagules, as the real-time PCR assay using the primer set VtF4/VtR2 detected gDNA of *V. tricorpus* in some of the soil samples. Previous reports suggest that the presence of nonviable propagules in the soil do not compromise accurate quantification of *V. dahliae* in soil (Bilodeau et al. 2012). However, in our study, presence of other *Verticillium* species could account for

the cfu counts in samples where gDNA of *V. dahliae* was not detected. In our study, when soil without *Verticillium* was spiked with microsclerotia of *V. dahliae*, there was a very strong relationship between the concentration of *V. dahliae* gDNA detected and the number of *V. dahliae* microsclerotia added (R^2=0.99) (Figure 3.4B). Therefore, the weaker relationship between the *V. dahliae* gDNA and propagule density (cfu g^{-1}) in naturally infected soils is probably due to the presence of resting structures of *V. tricorpus*, as semi-selective media used to quantify *V. dahliae* also allows germination and growth of other *Verticillium* species, such as *V. tricorpus* (Atallah et al. 2007). Presence of microsclerotia formed colonies of *V. tricorpus*, could contribute to overestimate the propagule density of *V. dahliae* in soil, estimated as cfu g^{-1}.

A recent study by Tzelepis et al. (2017) suggested that the relationship between concentration of *V. dahliae* gDNA in soil and number of cfu needs to be examined cautiously since the number of cells and thereby the concentration of gDNA in each individual microsclerotium varies substantially. In addition, the relationship could also be affected by the type of method used to quantify cfu g^{-1}, as the dry plating method gives higher numbers of cfu g^{-1} than the wet plating method (Termorshuizen et al. 1998). This is likely associated with breakage of microsclerotia or clusters of microsclerotia in soil, as microsclerotia size can be reduced to less than 37µm by simple dry sieving (Ben-Yephet and Pinkas 1977).

Another potential limitation for the plating methods is that some microsclerotia present in the soil do not germinate when soil is plated on the semi-selective medium (Molina

2009). Microsclerotia plated onto semi-selective medium needs to germinate, grow hyphae and form new microsclerotia to create detectable microsclerotia-forming colonies. Despite the presence of carbon and nitrogen in the recovery semi-selective medium, which is used to break the microbiostasis that inhibits the germination of microsclerotia in soil (Goud and Termorshuizen 2003), microsclerotia still fail to germinate on the plated media. This has been observed after microsclerotia have been exposed to sub-lethal conditions such as low concentrations of NH_3 and HNO_2 (Tenuta and Lazarovits 2004) or by the inhibition enforced by other competing micro-organisms growing on the medium (Goud and Termorshuizen 2003). These results reflect the potential usability of DNA assays to quantify *V. dahliae* propagule density in soil, and draw attention to the importance of checking for the presence of other species that might compromise the accuracy of the molecular assay.

Our study compared the capability of the real-time PCR assay of *V. dahliae* gDNA in soil and the propagule density (cfu g^{-1}) to predict Verticillium wilt severity in two Manitoba fields naturally infested with *V. dahliae*, and history of Verticillium wilt. The two fields had very different *Verticillium* propagule densities. A quadratic relationship (R^2=0.82) was observed between the concentrations of *V. dahliae* gDNA in soil and Verticillium wilt severity in cv. Russet Burbank. In contrast, propagule density (cfu g^{-1}) counts had a weaker relationship with disease severity. Interestingly, the field with the highest propagule density (cfu g^{-1}) in soil was the same site with the lowest concentration of *V. dahliae* gDNA, but with the highest concentration of *V. tricorpus* gDNA.

Production of cfu from the different survival structures of *V. tricorpus*, resting mycelia, chlamydospores and microsclerotia, likely contributes to the higher propagule density (cfu g^{-1}) in soil, and ultimately to the negative relationship between propagule density (cfu g-1) in soil and disease development. Unfortunately, the role of the *V. tricorpus* isolates on the development of the disease in Manitoba soils is unknown, and needs further study. Elsewhere, negative relationships between *V. tricorpus* in soil and Verticillium wilt of potato have been reported (Davis et al. 2000; Davis and Sorensen 1985), suggesting that *V. tricorpus* could be a potential biological control against *V. dahliae*. However, *V. tricorpus* has also been found causing Verticillium wilt in potato (Nair et al. 2015), which indicates the possible presence of several pathotypes within this species (Ebihara et al. 2003).

3.6 CONCLUSIONS

A specific, sensitive and reproducible real-time PCR assay using the primer pair Vd-F929-947/R1076-1094 and probe 5′ 6-FAM (Bilodeau et al. 2012) was selected to quantify *V. dahliae* gDNA in soil. The real-time PCR assay was optimized with a soil-pulverizing step followed by soil DNA extraction using the MoBio PowerSoil DNA Isolation system to improve *V. dahliae* detection in soil. The real-time PCR assay detected as low as to 0.1 pg of gDNA from culture *V. dahliae* and 5 microsclerotia per gram of soil (or 0.3 pg of gDNA of *V. dahliae* g^{-1} soil), which is below the threshold level of inoculum needed to cause significant Verticillium wilt symptoms. Overall, quantification of *V. dahliae* using the real-time PCR with the primer pair Vd-F929-947/R1076-1094 and probe 5′ 6-FAM was a better predictor of Verticillium wilt

severity than propagule density (cfu g^{-1}) from the wet plating method. For accurate detection and quantification of *V. tricorpus* and *V. longisporum*, specific and reproducible real-time PCR assays using the primer pair VtF4/VtR2 (Debode et al. 2011) and Vlsp-F1/Vlsp-R4 (Banno et al. 2011) were selected for the two species respectively. Both real-time PCR assays were sensitive down to a concentration of 0.02 pg of gDNA g^{-1} soil.

Considering the presence of other *Verticillium* species producing microsclerotia, the relatively low propagule density, patchiness of the *V. dahliae* soil inoculum and the inaccuracy of the wet plating method, real-time PCR methods could be considered for the detection and quantification of *V. dahliae* propagule density in soil. The traditional wet plating method does not seem to properly estimate the level of *V. dahliae* in soil or to predict disease severity, due to the presence of other *Verticillium* species producing microsclerotia, particularly *V. tricorpus.* In this study, the *V. tricorpus* and *V. klebahnii* species were confirmed in Manitoba soils producing microsclerotia that contribute to overestimating the propagule density of *V. dahliae* measured as cfu g^{-1} with the wet plating method.

The evaluated real-time PCR assays were able to detect and quantify *V. dahliae* in artificially and naturally infested soils in Manitoba. Additionally, real-time PCR assays were successfully evaluated for the detection and quantification of *V. tricorpus* and *V. longisporum* in naturally infested soil from Manitoba.

The combination of the soil DNA extraction protocol and the real-time PCR assays used in this study proved to be a specific and sensitive method for the detection and quantification of the different *Verticillium* species found in Manitoba soils, particularly *V. dahliae*, *V. tricorpus* and *V. longisporum.* With these PCR assays, a larger number of samples can be processed in a much shorter period of time compared with the traditional wet plating method. Unlike the wet plating method, the real-time PCR assay allows reliable quantification of *V. dahliae* in field soils. Although the DNA extraction method and PCR assay was an excellent tool to correctly identify potato fields with high levels of *V. dahliae* in this study, a more inclusive study with a larger number of field samples is recommended to determine the effect of soil type and the patchiness distribution of the pathogen in the soil. This will help to make more economically and environmentally sound decisions on whether or not to use soil fumigants, such as metam sodium.

3.7 ACKNOWLEDGMENTS

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CHAPTER 4

4. LACK OF PATHOGENICITY AND INTERACTION OF TWO ISOLATES OF *VERTICILLIUM TRICORPUS* WITH *VERTICILLIUM DAHLIAE* TO POTATO cv. RUSSET BURBANK

4.1 ABSTRACT

Verticillium dahliae is a common soilborne pathogen found in Manitoba potato fields producing Verticillium wilt of potato. Previously in this thesis, *Verticillium tricorpus* was reported to occur with *V. dahliae* in potato fields affected by Verticillium wilt. The role of *V. tricorpus* as a pathogen or biocontrol agent of Verticillium wilt is not known. In this growth chamber study, potato cv. Russet Burbank plants were inoculated by root dip with two isolates of *V. tricorpus* from potato fields, alone and in combination with two isolates of *V. dahliae* from Manitoba, and grown for six weeks to determine the pathogenicity of *V. tricorpus* or its interaction with *V. dahliae*. The *V. tricorpus* isolates alone did not produce symptoms of Verticillium wilt. Although *V. tricorpus* gDNA was present in small concentrations in roots, and thus colonized roots, neither its DNA nor vascular discoloration of stem tissue was observed. In contrast, *V. dahliae* isolates produced highly colonized roots, vascular-stem discoloration, stem elongation, wilt and plant death. Only *V. dahliae* was re-isolated and its gDNA was detected in stems and

roots of inoculated and co-inoculated plants. The findings in this study suggest the *V. tricorpus* isolates examined were not pathogenic or protective against Verticillium wilt in potato cv. Russet Burbank under the assay conditions used.

4.2 INTRODUCTION

The genus *Verticillium* is considered a relatively small, but very important group of soilborne plant pathogenic fungi (Inderbitzin et al. 2011). After the recent taxonomic revisions of the genus, five plant-pathogenic species are recognized: *Verticillium dahliae*, *Verticillium albo-atrum*, *Verticillium longisporum*, *Verticillium tricorpus* and *Verticillium nubilum* (Klimes et al. 2015).

Verticillium tricorpus was first reported to affect tomato (*Solanum lycopersicum*) (Issac 1953). Since then, it has been isolated from tomato (Huisman 1988), lettuce (*Lactuca sativa*) (Powell et al. 2013), artichoke (*Cynara scolymus*) (Qin et al. 2008), and potato (*Solanum tuberosum*) (Ebihara et al. 2003; Nair et al. 2015; Platt and Bollen 1995). In Manitoba, *V. tricorpus* has been isolated from potato cv. Russet Burbank severely affected by Verticillium wilt, as well as from the soil of commercial potato fields (Chapter 3).

Although *V. dahliae*, *V. albo-atrum*, *V. tricorpus*, *Colletotrichum coccodes*, the nematode *Pratylenchus penetrans*, as well as bacteria in the genus *Pectobacterium* can be involved in wilt and early dying of potato (Dung et al. 2014; Korolev and Katan 1999; Pegg and Brady 2002; Platt and Bollen 1995), the role of *V. tricorpus* is not understood, particularly its ability to infect and colonize plants (Robinson et al. 2006). *Verticillium tricorpus* was considered to possibly be a weak pathogen of potato in comparison to *V. dahliae* and *V. albo-atrum* (Mahuku et al. 1999). Although it has been detected in soil

and infected potato plants expressing wilt symptoms and/or premature senescence, the intensity of the disease is lower than that reported for plant infected with *V. dahliae* or *V. albo-atrum* (Robb et al. 1993). *Verticillium tricorpus* has been suggested as the causal agent of opportunistic infections on weakened plant hosts (Goud et al. 2003). Indeed, *V. tricorpus* was able to induce wilt symptoms when a host plant, Snapdragon (*Antirrhinum majus*), was weakened by biotic or abiotic stresses, such as waterlogging conditions (Isaac 1956).

Verticillium tricorpus does not exhibit the expanding parasitic phase in the living host tissue as *V. dahliae* and *V. albo-atrum* do. It has been found to grow saprotrophically in soil, unlike *V. dahliae* and *V. albo-atrum*, which are restricted to the internal plant tissue and immediate vicinity of the roots of theirs hosts (Isaac 1967). *Verticillium tricorpus* is an asexually reproducing fungi, similar to other *Verticillium* species (Korolev and Katan 1999). It survives in soil by means of resting structures that also act as propagules for infecting the subsequent plant host. *Verticillium tricorpus* is characterized by the production of three forms of resting structures: melanized mycelium, chlamydospores, and microsclerotia (Issac 1953).

Verticillium tricorpus can occur simultaneously with other *Verticillium* species in soil (Korolev and Katan 1999; Mahuku et al. 1999; Tyvaert et al. 2014). *Verticillium tricorpus* was recovered from 28% of the soil samples collected from Manitoba potato fields surveyed in 2011-2013 (Chapter 3). Other studies have also reported the presence

of *V. tricorpus* concomitantly with *V. dahliae*, *V. albo-atrum* and *V. longisporum* in soil (Davis et al. 2000; Debode et al. 2011; Mahuku et al. 1999; Tyvaert et al. 2014).

In contrast, *V. tricorpus* has also been implicated as a potential biocontrol agent, reducing Verticillium wilt severity caused by *V. dahliae* or *V. albo-atrum* (Klosterman et al. 2009). Reduced Verticillium wilt severity and improved growth in lettuce and artichoke was observed when *V. tricorpus* was applied a week prior to the addition of *V. dahliae* to soil (Qin et al. 2008).

In potato cv. Russet Burbank, less symptoms of wilt occurred in field plots naturally infested with *V. tricorpus* and *V. dahliae* (Davis et al. 2000). Similarly, when the Verticillium wilt susceptible potato cv. Superior was co-inoculated with either *V. tricorpus* and *V. dahliae*, or *V. tricorpus* and *V. albo-atrum*, Verticillium wilt symptoms were reduced (Robinson et al. 2007). Robinson et al. (2007) suggested that due to competition for space and nutrients within the host plant, hyphal interference was the mechanism by which *V. tricorpus* might have reduced the pathogenicity of *V. dahliae* in potato.

The microsclerotia-forming *Verticillium* species *V. dahliae* and *V. tricorpus* have been detected in soil and plant samples collected in Manitoba potato fields (Chapter 3). Although the same fields showed symptoms of Verticillium wilt, the role of *V. tricorpus* in the development of Verticillium wilt symptoms in Manitoba fields remains unclear. To address this question, the pathogenicity of two *V. tricorpus* isolates from Manitoba was

investigated with potato cv. Russet Burbank inoculated alone and in combination with *V. dahliae* isolates. Pathogenicity was assessed by the effect of inoculation treatments on the moderately susceptible cultivar Russet Burbank to Verticillium wilt, and presence of the fungi in root and stem tissue.

4.3 MATERIALS AND METHODS

4.3.1 *Verticillium* Isolates

Two *V. tricorpus* isolates, MBVt22 and MBVt36, were selected randomly from the 22 isolates collected from commercial potato fields surveyed from 2011 to 2013 in Manitoba. The *V. tricorpus* isolates produced an irregular shaped microsclerotia, dark mycelium and chlamydospores, indicative of the species (Chapter 3). Two *V. dahliae* isolates were used in this study: the isolate Vd1396-9, previously described as highly pathogenic to cv. Russet Burbank (Uppal et al. 2007) and the isolate MBVd41 collected from Verticillium wilt-symptomatic potato cv. Russet Burbank, grown in a commercial field in Manitoba (Chapter 3). Isolate identification was confirmed after a sequenced fragment of the IGS region was compared with sequences of *V. tricorpus* and *V. dahliae*, respectively, deposited in the GenBank ID database (Chapter 3).

For each isolate, single spore (conidium) cultures were grown on potato dextrose agar (PDA) at 22 °C under dark conditions for two weeks. Conidia were harvested by flooding the Petri plate culture with sterile deionized water and scraping the PDA surface with a glass rod. After filtering through a triple layer of sterile cheesecloth, the suspension of conidia was adjusted to a density of 1×10^6 conidia mL^{-1} water.

4.3.2 Pathogenicity assay

The pathogenicity assay was arranged following a randomized complete block design with inoculated treatments of two isolates of *V. tricorpus* and two isolates of *V. dahliae*, three combinations of *V. tricorpus*/*V. dahliae* isolates, and two treatments of non-inoculated control treatments. Each treatment was comprised of four pots with one potato plant in each to serve as replicates. Plantlets were inoculated with either one of two isolates of *V. tricorpus* (MBVt36, MBVt22) or *V. dahliae* (MBVd41, Vd1396-9). In addition, three treatments of isolate combinations of *V. tricorpus* and *V. dahliae* together (MBVt36+MBVd41, MBVt22+Vd1396-9, MBVt36+Vd1396-9) were also examined. The two control treatments consisted of non-inoculated treatments with and without wounding.

The assay was conducted using potato cv. Russet Burbank. Plants were produced from certified seed pieces, freshly cut and planted into plastic pots (5 cm diameter) filled with Sunshine Growing Mix LA4 (60-70% sphagnum peat moss, horticultural perlite and dolomite lime). Pots were placed in a growth chamber at 22/18 °C day/night temperature with a 16 hour photoperiod. At two weeks, roots of plantlets were washed with reverse osmotic (RO) water to separate soil from roots. Plants were inoculated with *Verticillium* isolates by cutting root tips by 10-15 mm, and dipping the remaining root system in a suspension of conidia (150 mL) for one minute (Alkher et al. 2009). Plantlets cut and non-cut dipped in sterile water served as non-inoculated wounded (W/Control) and non-wounded (NW/Control) control treatments, respectively.

All plantlets were immediately transplanted to individual 15 cm diameter plastic pots containing a pasteurized soil-sand-peat-perlite mixture (4:4:4:1, v:v:v:v) and supplemented with a general purpose soluble fertilizer (NPK 20-20-20). Plants were grown in a growth chamber (Conviron CMP3244, Conviron Products Company, Winnipeg, MB) at 22/18 °C day/night temperature with 16 hour photoperiod (light intensity at the top of plants was between 1300 to 1400 microeinsteins m^{-2} sec^{-2}), and watered on alternate days.

4.3.3 Assessment of Verticillium wilt severity

Plants were assessed 2, 3, 4, 5 and 6 weeks after inoculation for Verticillium wilt development and progress (wilting, chlorosis and necrosis) using a 0-5 visual scale based on the percentage of plant tissue displaying the symptoms of *Verticillium* infection, where 0= no necrosis or chlorosis, 1=visible chlorosis with <1% necrosis, 2= up to 40% chlorosis and 1-20% necrosis, 3= up to 65% chlorosis and 20-35% necrosis, 4= 100% chlorosis and 35-70% necrosis, 5=100% chlorosis and 70-100% necrosis (Alkher et al. 2009). Verticillium wilt severity was calculated as follows: $[\sum_{i=0}^{n}(n\ x\ b)]\frac{x100}{T}x\ (N-1)$, where b is the chlorosis/necrosis grade (0-5 referring to the pre-developed scale), n is the number of leaves with necrosis grade b, N is the total number of chlorosis/necrosis grades used in the scale and T is the total number of leaves (Alkher et al. 2009). Based on the over-time Verticillium wilt severity the area under the wilt progress curve (AUWPC) was calculated using the equation $\Sigma_i^{n-1}\ [(Y_i + Y_{i+1})/2](t_{i+1} - t_i)$, where n= total number

of observations in weeks, Y_i= cumulative Verticillium wilt severity at the i^{th} observation week, and t_i= time (weeks after planting) at the i^{th} observation (Shaner and Finney 1977).

Additionally, stem vascular discoloration was used as an indicator of vascular tissue damage. This indicator was added to the study as potato plants could recover quickly from disease, by forming new vascular tissue (Alkher et al. 2009). In contrast, vascular discoloration is permanent and remains visible in the old xylem tissue. Vascular discoloration was determined on portions of lower (first 20 cm above ground), middle and upper (last 20 cm) stems of each plant. A visual scale 0-5 was used, where 0= no vascular discoloration, 1= 1-9% of the cross-section showing discoloration, 2= 10-24%, 3= 25-49%, 4=50-74%, and 5= 75-100% discoloration (Alkher et al. 2009).

4.3.4 Plant response

Plants were harvested six weeks after transplanting. Each plant was harvested individually for total tuber fresh weight, total tuber number, shoot fresh biomass, shoot height (cm) and internode spacing.

4.3.5 Quantitative detection of *V. tricorpus* and *V. dahliae* in-planta

The quantity of genomic DNA (gDNA) of *V. tricorpus* and *V. dahliae* in the potato roots and stems was determined using a real-time PCR assay developed previously (Chapter 3). Roots and stems were collected six weeks after inoculation. Stems were divided in three sections: lower, middle and upper, as described previously. From each section, a 10

cm long piece was obtained using sterile methods. Then, roots and stem pieces were washed individually with autoclaved distilled water, to remove residues.

Total DNA was extracted from approximately 200-300 mg of plant tissue using the DNA extracting protocol described by Henriquez (2010). Total gDNA was extracted from each stem section and roots. Extracted gDNA was then dissolved in 100 µL of 1xTE buffer (10mM Tris, 1 mM EDTA, pH 8.0). Genomic DNA concentration was measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 100 ng μL^{-1}. The concentration of *V. tricorpus* and *V. dahliae* gDNA in extracts was determined using the protocol described in Chapter 3 using a CFX96 real-time PCR detection system (Bio-Rad Laboratories, Hercules, CA). Values are reported as ng of gDNA per gram of plant tissue.

4.3.6 Statistical analysis

All analyses were performed with the Statistical Analysis Software SAS (SAS Institute, Cary, NC; release 9.2 for Windows). Prior to analysis, data sets were checked for normality (PROC Univariate). AUWPC data was normalized by square root transformation for analysis. Since data for vascular discoloration in non-inoculated and *V. tricorpus* treatments were nil, they were removed to facilitate the analysis. Means and standard error of the concentration of *V. dahliae* and *V. tricorpus* gDNA g^{-1} of plant tissue, were calculated. The data were analyzed by analysis of variance (ANOVA-PROC MIXED). Effect mean values on measures were compared using the Bonferroni's procedure if the F-test was significant ($P<0.05$). Contrasts were used to test the

significance of differences between *V. tricorpus* and *V. dahliae* isolates, as well as treatments with the two isolates combined.

4.4 RESULTS

4.4.1 Verticillium wilt

Potato plants non-inoculated or inoculated with *V. tricorpus* isolates showed no Verticillium wilt symptoms (Figure 4.1). Furthermore, AUWPC values in potato plants inoculated with *V tricorpus* were not different from those observed for non-inoculated plants (Table 4.1). In contrast, AUWPC in plants inoculated with *V. dahliae* alone and co-inoculated was greater than in plants non-inoculated or inoculated with *V. tricorpus* alone. Contrast analysis confirmed a significant decrease in AUWPC for plants inoculated with *V. tricorpus* alone compared to plants inoculated with *V. dahliae* or co-inoculated with both pathogens.

Potato plants inoculated with *V. dahliae* showed significant Verticillium wilt severity, particularly for isolate Vd1396-9. Although plants inoculated with MBVd31 showed significantly less foliar chlorosis and necrosis than those inoculated with Vd1396-9, inoculated plants with MBVd31 showed additional symptoms such as uneven death of lower leaflets and conspicuously longer stems.

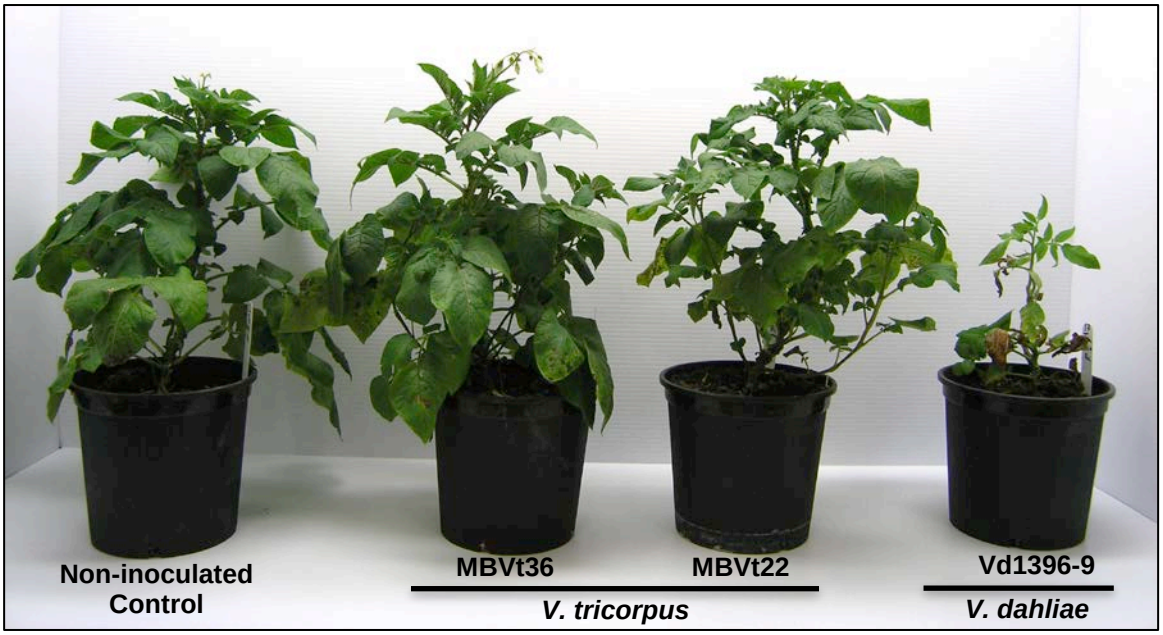

Figure 4.1 Russet Burbank response to *V. tricorpus* and *V. dahliae* inoculation.

Table 4.1 Area under the wilt progress curve, plant weight, tuber weight and number, height and internode space in potato cv. Russet Burbank after inoculation with *V. tricorpus* isolates alone and in combination with *V. dahliae* isolates.

Treatment [a]		AUWPC [b]	Biomass (g)	Tubers [c] (g)	Tubers [c] (#)	Height (cm)	Internode (cm)
Control	Non-wounded	6±(0.7) c [d]	89±3 a	81±6 a	9±1	30.3±0.8 b	1.7±0.05 b
	Wounded	10±(0.9) c	84±3 ab	85±4 a	9±1	27.6±0.5 b	1.4±0.05 bc
V. tricorpus	MBVt22	9±(1.5) c	77±1 abc	92±4 a	10±2	28.2±1.3 b	1.3±0.03 c
	MBVt36	9±(0.8) c	86±2 ab	91±5 a	11±1	29.9±0.7 b	1.4±0.03 bc
V. dahliae	MBVd31	69±(4) b	72±2 bc	41±10 b	11±2	40.3±1.8 a	2.1±0.12 a
	Vd1396-9	159±(6.7) a	11±2 d	26±3 b	6±1	14±1.8 c	0.8±0.06 d
Co-inoculation	MBVd31+ MBVt22	57±(6.6) b	69±6 c	45±9 b	7±1	43.5±1.6 a	1.8±0.11 ab
	Vd1396-9+ MBVt22	158±(10.6) a	5±2 d	20±4 b	7±1	17.5±2.9 c	0.8±0.07 d
	Vd1396-9+ MBVt36	150±(11.2) a	7±3 d	16±2 b	7±1	11.6±1.8 c	0.9±0.07 d
	***P* value [e]**	0.0001	0.0001	0.0001	ns	0.0001	0.0001
Selected contrasts (significant probability) [f]							
V. tricorpus vs *V. dahliae*		0.0001	0.0001	0.0001	ns	ns	ns
V. tricorpus vs Co-inoculation		0.0001	0.0001	0.0001	0.02	ns	ns
V. dahliae vs Co-inoculation		ns	ns	ns	ns	0.05	ns

[a] Non-inoculated control plants: non wounded (NW) and wounded (W). n=4 for each treatment

[b] Area under the wilt progress curve (AUWPC) and vascular discoloration in the lower, middle and upper parts of the potato stem six weeks after inoculation.

[c] Tuber weight (g total tuber per plant) and number (# =Number of tubers per plant).

[d] Values are means ±1 standard error of the mean.

[e] Within columns, means followed by the same letter are not significantly different (P >0.05) according to the Bonferroni's multiple comparison test.

[f] *V. tricorpus*= MBVt22 and MBVt36; *V. dahliae*=MBVd31 and Vd1396-9; Co-inoculation= MBVd31+ MBVt22;Vd1396-9+ MBVt22 andVd1396-9+ MBVt36

Potato plants inoculated with *V. dahliae* showed significant Verticillium wilt severity, particularly for isolate Vd1396-9. Although plants inoculated with MBVd31 showed significantly less foliar chlorosis and necrosis than those inoculated with Vd1396-9, inoculated plants with MBVd31 showed additional symptoms such as uneven death of lower leaflets and conspicuously longer stems.

The vascular discoloration was observed only in plants inoculated with *V. dahliae* (Figure 4.2). Similar to the responses in AUWPC, vascular discoloration in plants co-inoculated with *V. tricorpus* and *V. dahliae* was similar to that observed when plants were inoculated with only *V. dahliae* isolates (Figure 4.2). Potato plants non-inoculated or inoculated with *V. tricorpus* isolates alone did not show vascular discoloration. Interestingly, only plants inoculated or co-inoculated with Vd1396-9 showed vascular discoloration along the lower, middle and upper sections of stems. In contrast, potato plants inoculated with MBVd31 only showed vascular discoloration in the lower and middle section of stems.

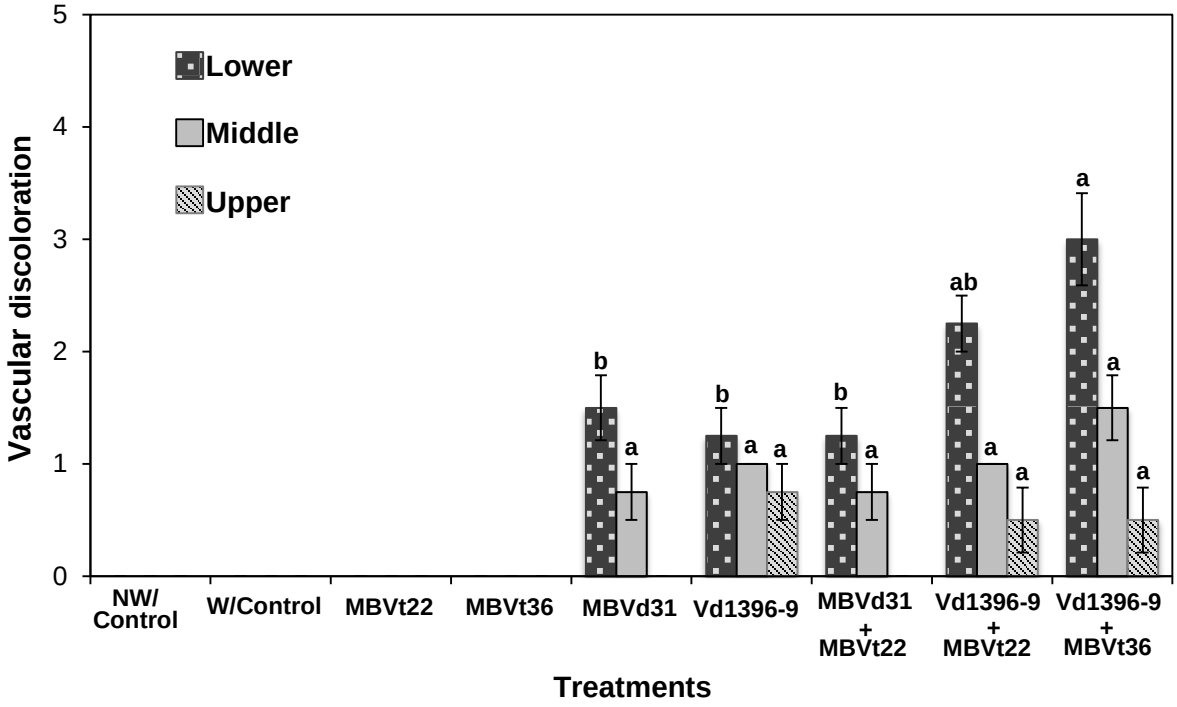

Figure 4.2 Vascular discoloration in the lower, middle and upper parts of potato stems six weeks after inoculation. Within the same part of the stem, means followed by the same letter are not significantly different, according to Bonferroni's multiple comparison test (P>0.05). Error bars are ±1 standard error. Non-inoculated control plants: non wounded (NW) and wounded (W). *V. tricorpus*= MBVt22 and MBVt36; *V. dahliae*=MBVd31 and Vd1396-9; Co-inoculation= MBVd31+ MBVt22;Vd1396-9+ MBVt22 andVd1396-9+ MBVt36

4.4.2 Plant response to individual and co-inoculated *Verticillium* species

The effect of the *Verticillium* inoculations on fresh foliar and tuber weight, height and internode length was significant, but not for tuber number per plant (Table 4.1). Potato plants inoculated with *V. dahliae* isolate Vd1396-9 or co-inoculated with isolate Vd1396-9 and the *V. tricorpus* isolates, exhibited significant stunting, reflected in lower biomass and tuber weight and plant height, relative to non-inoculated controls. The plants inoculated with *V. tricorpus* isolates and non-inoculated produced similar amount of

biomass, which was up to eight times the amount produced when plants were inoculated with *V. dahliae* Vd1396-9.

Verticillium tricorpus isolates did not reduce fresh foliar, and tuber weight and plant height, relative to the control (Table 4.1). Although the number of tubers per plant was similar within treatments, the weight of the tubers collected from plants inoculated with *V. dahliae* isolates or co-inoculated with *V. tricorpus* was lower than that of plants inoculated with *V. tricorpus* isolates and non-inoculated.

There was no difference in plant height between non-inoculated and inoculated plants with *V. tricorpus* (Table 4.1). The height of the plants inoculated with *V. dahliae* varied among isolates. Plants inoculated with *V. dahliae* isolate Vd1396-9 showed very short internodes. In contrast, plant inoculated with *V. dahliae* MBVd31 showed significant internode elongation, reflected in greater height of the plant (P =0.001) relative to the non-inoculated controls and inoculated plants with *V. tricorpus* isolates. Although the internode length in plants inoculated with *V. dahliae* MBVd31 and plants co-inoculated with *V. tricorpus* MBVt22 and *V. dahliae* MBVd31 was not significantly different, the internode length in the co-inoculated plants was similar to that of non-inoculated controls.

4.4.3 Quantitative detection of *Verticillium in-planta*

Genomic DNA of *V. dahliae* was detected in roots and stems of plants inoculated with MBVd31 and Vd1396-9 or co-inoculated with *V. tricorpus* isolates. In contrast, *V.*

tricorpus gDNA was detected only in roots of plants inoculated with MBVt22 and MBVt36 alone, and Vd1396-9+MBVt36 (Table 4.3). Samples collected from plants inoculated with *V. dahliae* isolate Vd1396-9 yield up to 18 times more gDNA than when plants were inoculated with the isolate MBVt31.

Table 4.2 Quantification of *V. dahliae* and *V. tricorpus* genomic DNA in potato root and stem.

Treatment	***V. dahliae*** (pg gDNA g^{-1})				***V. tricorpus*** (pg gDNA g^{-1})			
	Roots	**Stem**			**Roots**	**Stem**		
		Lower	Middle	Upper		Lower	Middle	Upper
NW-Control [a]	0±0 [b]	0±0	0±0	0±0	0±0	0±0	0±0	0±0
W-Control [a]	0±0	0±0	0±0	0±0	0±0	0±0	0±0	0±0
MBVt22	0±0	0±0	0±0	0±0	1±1	0±0	0±0	0±0
MBVt36	0±0	0±0	0±0	0±0	19±8	0±0	0±0	0±0
MBVd31	193±60	137±43	0±0	0±0	0±0	0±0	0±0	0±0
Vd1396	1287±387	2305±1282	690±191	361±86	0±0	0±0	0±0	0±0
MBVd31+MBVt22	142±34	180±135	1±1	0±0	0±0	0±0	0±0	0±0
Vd1396-9+MBVt22	1554±407	898±250	551±210	165±95	0±0	0±0	0±0	0±0
Vd1396-9+MBVt36	742±351	634±104	321±248	146±90	1±1	0±0	0±0	0±0

[a] Non-inoculated control plants: non wounded (NW) and wounded (W)
[b] Values are means ±1 standard error.

4.5 DISCUSSION

The absence of vascular discoloration and wilt symptoms in potato plants inoculated with *V. tricorpus* indicated that these isolates of *V. tricorpus* from Manitoba were nonpathogenic to potato cv. Russet Burbank under the assay conditions used. This finding agrees with previous studies documented by Davis et al. (2000), Robinson et al.

(2006) and Ebihara et al. (2003) who reported that potato plants inoculated with *V. tricorpus* showed little to no disease symptoms.

The presence of *V. tricorpus* in plants is not always associated with pathogenicity as suggested by Huisman (1988). He reported colonization of cotton and tomato roots by *V. tricorpus*, but not infection of the vascular tissue in cotton. In the current study, plants inoculated with *V. tricorpus* did not show vascular discoloration, despite presence of the fungus in the root, albeit, at very low levels.

Although *V. tricorpus* isolates did not cause external or internal symptoms of Verticillium wilt of potato in the current study, recovery of *V. tricorpus* gDNA from roots of the inoculated potato plants confirmed that *V. tricorpus* effectively colonized potato roots. This phenomenon has been associated with an endophytic life-history (Tyvaert et al. 2014). This is a common behavior for a diverse group of fungi that colonize roots without causing visible damage, while increasing abiotic and biotic stress tolerance (Rodriguez et al. 2009). Verticillium wilt suppression have been associated with the presence of fungal endophytes (Narisawa et al. 2002), by conferring resistance against root infection of pathogenic *Verticillium* species (Tyvaert et al. 2014).

Verticillium tricorpus isolates that behave as endophytes could potentially be used as biological control against *V. dahliae*. Indeed, pre-inoculation of cauliflower plants with *V. tricorpus* has controlled Verticillium wilt produced by *V. longisporum* (Tyvaert et al. 2014). In that study, root infection by *V. longisporum* was significantly reduced in

cauliflower roots colonized by *V. tricorpus*, suggesting that defense-related events taking place in roots could be involved in the cross-protection. Similar mechanisms have been observed in cv. Russet Burbank with the antagonistic bacteria *P. fluorescens* DF37 and *B. pumilus* M1 which induce accumulation of rutin, a secondary metabolite that reduce growth and sporulation of *V. dahliae* (El Hadrami et al. 2011).

The *V. dahliae* isolate MBVd31, collected from a potato commercial field in Manitoba (Chapter 3), was less pathogenic than the *V. dahliae* isolate Vd1396-9, which was shown to be highly pathogenic in an early study (Uppal et al. 2007). The symptoms produced by MBVd31 on potato cv. Russet Burbank are consistent with the typical symptoms of Verticillium wilt observed on commercial potato fields, such as uneven chlorosis of lower leaflets and noticeably longer stems, which eventually die and remain conspicuously erect in contrast to healthy plants (Rowe and Powelson 2002; Rowe et al. 1985). Interestingly, co-inoculation of MBVd31 (*V. dahliae*) and MBVt22 (*V. tricorpus*) reduced the internode length, suggesting some potential beneficial effect of *V. tricorpus* against Verticillium wilt symptoms. This observation may be an indication of the possibility of *V. tricorpus* isolates were beneficial against *V. dahliae*, as previously suggested by Davis et al. (2000) and Robinson et al. (2007). Nevertheless, the symptoms observed in the plants inoculated with MBVd31 compared to the isolate Vd1396-9, both *V. dahliae* isolates, suggest the isolate MBVd31 has a low pathogenicity on potato. This could also indicate that the potential suppressive effect of *V. tricorpus* may also depend on the pathogenicity of the *V. dahliae* isolate.

In summary, the findings reported here suggest that *V. tricorpus* in commercial fields in Manitoba is not pathogenic to potato. Although the *V. tricorpus* isolates were able to establish in the roots at very low levels, it did not colonize stem and leaf tissue, as *V. dahliae* did. Some reduction of Verticillium wilt symptoms in plants co-inoculated with MBVd31 and MBVt22 suggested a potential beneficial effect of *V. tricorpus* against isolates of *V. dahliae* that are not highly pathogenic. However, this observation requires further investigation. Moreover, it remains unclear what mechanisms could be involved. It may be possible that early inoculation with *V. tricorpus* could trigger plant defense mechanisms in potato, or at least give the *V. tricorpus* fungus time to colonize roots, and compete for space, which ultimately could limit potential infection sites for *V. dahliae*. Therefore, for this to occur, *V. tricorpus* colonization of root would need to occur before that of *V. dahliae*.

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CHAPTER 5

5. GENERAL DISCUSSION

Verticillium wilt is a major disease of potato (Johnson and Dung 2010). In Manitoba, Verticillium wilt is a serious threat to the potato industry, as 80 % of the potato productive area is planted with the cultivar cv. Russet Burbank, which is a processing potato cultivar moderately susceptible to the causal pathogen *V. dahliae* (Jansky and Rouse 2000). Among potato growers and industry, it is believed that Verticillium wilt is one of the main reasons for lower yields than in other potato growing regions (Dr. M. Tenuta, personal communication). Potato growers are looking for ways to reduce Verticillium wilt pressure and to increase potato yield. The hypotheses evaluated in a series of experiments were: 1) single applications of composted cattle manure or composted separated pig slurry solids, or the soil fumigant metam sodium can effectively suppress Verticillium wilt and increase marketable yield in Manitoba potato fields; 2) propagule density of *V. dahliae* should be a good predictor of Verticillium wilt severity; however, the traditional wet plating does not quantify propagule density of *V. dahliae* in soil accurately, due to the occurrence of other microsclerotia-forming *Verticillium* species, such as *V. tricorpus,* which may not be pathogenic to potato.

The results of this thesis provide new information that will contribute to the management of Verticillium wilt and to improve potato yield in Manitoba, with either soil fumigants or organic amendments. Additionally, the information presented from this thesis will contribute to better understanding of the relationship between Verticillium wilt severity and the propagule density of *V. dahliae*, and how other microsclerotia-forming *Verticillium* species, such as *V. tricorpus*, can affect such relationships.

5.1 COMPOSTED MANURES, POTENTIAL CONTROL OF VERTICILLIUM WILT

In our field studies, the overall effect of the single applications of three different rates of composted cattle manure or composted separated pig slurry solids on frequency of *Verticillium* and Verticillium wilt severity varied with different composts, application rates and potato cultivars. This is expected, as the disease suppressive effect of compost under field conditions is very variable, depending on factors such as target pathogen, feedstock-origin, application-rate, production batches and the differences in climate and soil type where the compost is applied (Bonanomi et al. 2007; Litterick et al. 2004; Noble and Coventry 2005; St. Martin 2015; Yadessa et al. 2010). Perhaps, another cause for such variability is that the effect of organic amendments on disease also depend on the time elapsed since incorporation (Papavizas and Lumsden 1980). For example, in very intensive worked and cultivated fields, the suppressive effects of compost have been observed one year after application (St. Martin 2015), which could be the case for the potato fields used in our studies. Developing disease suppression by compost takes time,

as it only occurs after the soil conditions and composition of the microbial communities have changed (Escuadra and Yoshimiki 2008; Yadav et al. 2015).

In our current studies, applications of composted cattle manure at 20 Mg ha^{-1} and composted separated pig slurry at 40 Mg ha^{-1}, reduced Verticillium wilt severity (AUWPC) in cv. Russet Burbank. Surprisingly, increasing the rate of application to 80 Mg ha^{-1} did not improve the suppressive effect of the composts. Although the disease suppressive effect of compost generally increased with rate of application (Noble and Coventry 2005), increasing the rate of application can also result in neutral effect or enhance disease severity (St. Martin 2015). This also suggests that growers interested in using compost, will not need to use such high rate that it could pose problems such transportation cost, groundwater and surface water pollution or the transference of heavy metals to the soil (St. Martin 2015; Stone et al. 2004). Nevertheless, it remains unclear what application rate of the composted manure is needed to reduce Verticillium wilt or improve potato yield consistently.

Our studies also provide evidence that the composted separated pig slurry solids and composted cattle manure have potential for reducing Verticillium wilt in potato. However, it is also necessary to study the interaction rate and timing of application that could make the suppressive effect consistent. Multi-year applications could bring more predictable improvements in Verticillium wilt suppression and potato yield. However, multi-year application studies are needed to test this hypothesis. Multi-year application of compost studies report more predictable improvements in yield, quality, and disease

suppression (Litterick et al. 2004; Stone et al. 2004), and yield stability compared with conventional systems supplemented only with synthetic fertilizer (Mallory and Porter 2007). These studies should also elucidate if there is a residual, cumulative or delayed suppressive effect with consecutive applications of compost, and which application rates maximize that effect.

The development of Verticillium wilt symptoms in cv. Russet Burbank was related to availability of phosphorus and nitrogen in soil late in season in 2012 and 2013. Similar response was observed with application of liquid dairy manure or synthetic fertilizer in other studies (Curless et al. 2012). This could suggest that potato plants may benefit from the available phosphorus and nitrogen during maturity when photosynthesis decreases, tuber growth slows and vines die. For instance, nitrogen availability at maturity can delay vine senescence (Lambert et al. 2005), which could benefit potato yield, as well. Compost can delay the natural decline of photosynthesis of leaves, associated with the increase of leaf nitrogen (Gent et al. 1999). However, it is still unknown if compost can prevent the photosynthetic decline due to Verticillium wilt.

While our studies point to the importance of nitrogen and phosphorus on the suppression of Verticillium wilt, the suppressive mechanism is not clear. Nutrients present in compost can either have a direct effect on the plant or support the activity of antagonistic microorganisms involved in the biological control of pathogens (Hoitink and Fahy 1986). Some of the suggested mechanisms involved in disease suppression by compost are competition for nutrients by beneficial microorganisms, activation of disease-resistance

genes in plants by microorganisms, and improvement of plant nutrition and vigour, leading to enhanced disease resistance (Hoitink and Boehm 1999; Noble and Coventry 2005). These mechanisms do not involve reduction of pathogen inoculum necessarily (Noble and Coventry 2005). In fact, our previous study reported that incorporation of composted cattle manure reduced Verticillium wilt incidence and maintained the same propagule density for the duration of the study (2 years) (Molina et al. 2014). Likewise, compost application could stimulate the activity of saprophytic fungi such *V. tricorpus*, which could reduce colonization of the pathogen or possibly, activate defense-related events, as reported in cauliflower infected with *V. longisporum* (Tyvaert et al. 2014). Therefore, it is hypothesized that improvement of nutrient availability and changes in the microbial activities are possibly involved, but further examination of the mechanisms associated whit this suppression is needed.

5.2 EFFICACY OF RELATIVELY LOW APPLICATION RATE OF METAM SODIUM AGAINST VERTICILLIUM WILT

Soil fumigation with metam sodium is a common practice for the management of Verticillium wilt (Pasche et al. 2014; Rowe and Powelson 2002; Tsror et al. 2005). Although Manitoba growers are interested in using metam sodium, hoping to increase yield and fulfill production demands, they are also looking to consider environmental and economic factors before applying the soil fumigant. Soil fumigants are generally non-specific and deadly to a large proportion of soilborne pathogens, as well as to beneficial microorganisms (Kinkel 1993). Therefore, the results presented here will contribute to

reduce the risk of volatile emission and non-target exposure of this toxic biocide, and ultimately to improve sustainability of potato production.

In our studies, the application of metam sodium at rates 0.8x and 1.2x of the recommended rate (467 L ha^{-1}), at a single depth of 20 cm and at soil temperature between 4 to 7 °C, reduced *Verticillium* propagule density, frequency of *Verticillium* and severity of Verticillium wilt in cv. Russet Burbank. Surprisingly, higher rates generated no additive benefits for disease suppression or potato yield. The application of metam sodium at a low rate (374 L ha^{-1}), effectively reduced *Verticillium* propagule density up to 40%, and increased potato yield in cv. Russet Burbank up to 24%. Our results agree with those obtained with a similar low application rate of metam sodium (373 L ha^{-1}), that reduced Verticillium wilt and increased tuber yield and quality (Pasche et al. 2014). Our findings offer information to help potato growers to make an economic and environmentally sound decision if using metam sodium for the control of Verticillium wilt, as growers usually apply 500 L ha^{-1}, which is within the product label-rates from 410 to 670 L ha^{-1}.

Although soil fumigation with metam sodium (374 L ha^{-1}) reduced propagule density at the beginning of the potato season and Verticillium wilt severity, and increased potato yield, propagule density increased at the end of the season. Soil fumigation can help to reduce population of soilborne pathogens, but it cannot eradicate them completely (Thornton et al. 1993). Pathogen levels in fumigated soils are likely to increase again to damaging levels, possibly after one season of susceptible crop (Kinkel 1993). Therefore,

it is highly recommended for potato growers willing to use metam sodium, to do so in conjunction with appropriate soil sampling and accurate quantification of *V. dahliae* levels, to determine if yield could be at risk. Further studies studying the residual effect of metam sodium application in commercial fields on *Verticillium* propagule density, potato yield and even symbiotic relationships of microorganisms with other crops, such as mycorrhiza and nitrogen fixation, will improve our understanding of the impact of soil fumigation.

5.3 OVERSTIMATION OF *V. DAHLIAE* PROPAGULE DENSITY WITH THE TRADITIONAL WET PLATING

In this study, the relationship between *Verticillium* propagule density and the severity of Verticillium wilt was often absent or even proportionally indirect, when using the wet planting method (Figure 3.6B). In fact, the inverse relationship between *Verticillium* propagule and frequency of *Verticillium* (Figure 2.4B) was unexpected because the density of *Verticillium* propagules levels at the end of the season was approximately 10 times greater than the initial density before potato planting, according to the wet plating quantification method. *Verticillium* propagule density data suggested that the traditional wet plating method does not estimate *V. dahliae* accurately, as fields with low propagule density were more severely affected by Verticillium wilt symptoms than fields with high propagule density (Chapter 2). The results presented in Chapter 2 suggested that propagules from other *Verticillium* species contributed to overestimation of *V. dahliae* levels.

This is particular important for potato growers, as the traditional plating method is commonly used to measure *V. dahliae* propagule density. In this study, the wet plating method did not quantify propagule density of *V. dahliae* accurately. Control measures against *V. dahliae*, such as soil fumigation with metam sodium or cultural practices (organic amendments), have to be planned and implemented before the crop is planted. So, the ability to identify and evaluate the disease risk based on propagule density is extremely important and necessary. Otherwise, potato growers could incur unnecessary higher productions costs, while attempting to control overestimated levels of *V. dahliae* inoculum. Therefore, the wet plating method needs to be replaced with a more reliable and accurate method that allows proper identification and quantification of *V. dahliae* in Manitoba potato soils.

5.4 MOLECULAR METHOD FOR THE QUANTIFICATION OF *V. DAHLIAE* AND OTHER *VERTICILLIUM* SPECIES

Detection and quantification of soilborne pathogens using molecular methods, such as real-time PCR have overcome the drawbacks of the traditional plating methods used for the diagnosis of several potato pathogens, due to their high level of sensitivity and specificity (Atallah et al. 2007; Bilodeau et al. 2012; Boine et al. 2014; Cullen et al. 2001, 2002). In this study, an accurate, rapid, and reliable real-time PCR tool based on published primers was optimized and evaluated for detection of *V. dahliae* in potato fields from Manitoba. The selected real-time PCR-based assay exhibited greater accuracy and sensitivity and was much faster than the traditional plating method. This real-time PCR assay detected and quantified *V. dahliae* inoculum in artificially and naturally

infested soils and potato plants (Chapters 3 and 4). Therefore, this molecular tool offers many applications, from quantification of *V. dahliae* inoculum in soil to diagnosis of Verticillium wilt in potato plant.

The applicability of these results for the potato industry is very important. Potato growers can now have an accurate tool to quantify the actual density of *V. dahliae* propagule inoculum and determine the risk of damage caused by Verticillium wilt. These results also complement our findings about the best application rate for metam sodium, which prove to be effective at 374 L ha^{-1} (Chapter 2). These results will help growers to make a more economically and environmentally sound decision about whether or not to use metam sodium.

The relationship between the amount of *V. dahliae* inoculum and Verticillium wilt severity, was detected when *V. dahliae* inoculum was quantified with the real-time PCR assay (Chapter 3). In contrast, we observed that the total *Verticillium* propagule density (cfu g^{-1}) measured with the traditional plating method did not realate with the extent of the Verticillium wilt symptoms observed in cv. Russet Burbank-2013 (Chapter 2 and 3). These results support the value of the real-time PCR assay quantifying *V. dahliae* inoculum density and suggest that the real-time PCR assay for the quantification of *V. dahliae* inoculum in soil can serve as a predictive tool for Verticillium wilt in potato fields.

In addition to the *V. dahliae* real-time PCR assay, real-time PCR assays were selected for the detection and quantification of *V. tricorpus* and *V. longisporum* in soil from Manitoba. Real-time PCR assays were also specific and sensitive for the detection of the pathogens in soil and plant (Chapter 3). Overall, these assays proved to be suitable tools to detect as little as 0.2 and 0.1 pg of gDNA of *V. dahliae* and *V. tricorpus*, respectively (Chapter 2). These results agree with the minimum detection levels reported for similar real-time PCR assays for *V. dahliae* in potato (Pasche et al. 2013) and in other susceptible hosts such as olives (Gramaje et al. 2013). These real-time PCR-based assays could potentially be used in diagnostics for the presence of *V. dahliae*, *V. tricorpus* and *V. longisporum* in soils as well as in potato plants and their respective host plants, which could be valuable in maintaining sanitation during the establishment of new plantings, by preventing the movement and use of infected seed.

5.5 *MAJOR VERTICILLIUM* SPECIES PRODUCING MICROSCLEROTIA IN MANITOBA

The real-time PCR assay contributed to the identification of three different *Verticillium* species producing microsclerotia in soil from seven different rural municipalities, which represent the major potato production regions in Manitoba (Chapter 2 and 3). Morphological and molecular characterization confirmed the identity of the species *V. dahliae*, *V. tricorpus* and *V. klebahnii*. *Verticillium dahliae* was cosmopolitan at all sampled fields, while *V. tricorpus* was the second most commonly found microsclerotia-forming *Verticillium* species in Manitoba. The microsclerotia colonies of the two species were difficult to distinguish, when soil was plated on semi-selective media (Chapter 3).

The fact that several *Verticillium* species can produce microsclerotia colonies on plated soil shows that the total propagule density quantified using the traditional plating method does not correspond to *V. dahliae* propagules exclusively, as previously suspected during the evaluation of compost and metam sodium. The lack of relationship between propagule density and the development of Verticillium wilt symptoms, or the negative relationship between the propagule density (cfu g^{-1}) quantified with the traditional plating method and the development of Verticillium wilt symptoms, was probably as a result of the presence of *V. tricorpus* (Chapter 3). This suggested that the current wet plating method is not sufficiently specific to differentiate propagule density of *V. dahliae* from other *Verticillium* species producing microsclerotia, and currently present in Manitoba soils, such as *V. klebahnii*, *V. tricorpus* (Chapter 2), and *V. longisporum*, which was recently found in Manitoba fields (Desjardins et al. 2015).

5.6 PRESENCE OF NONPATHOGENIC *V. TRICORPUS* IN POTATO SOILS IN MANITOBA

In our studies, real-time PCR assay confirmed that *V. tricorpus* is present in commercial potato fields in Manitoba. Further pathogenicity test on potato, using two random *V. tricorpus* isolates showed that *V. tricorpus* isolates do not produce Verticillium wilt symptoms or vascular discoloration in inoculated potato plants. The results suggested that *V. tricorpus* isolates collected in Manitoba are weak or nonpathogenic to potato.

Co-inoculation of *V. tricorpus* and *V. dahliae* isolates reduced the development of Verticillium wilt symptoms produced by a less aggressive isolate (Chapter 4). This suggests some potential beneficial effect of *V. tricorpus* against Verticillium wilt development. However, the role of *V. tricorpus* in commercial potato soils needs to be further elucidated. The real-time PCR assay used in this study may help to evaluate the possible association of this species with disease-suppressive soils and/or the potential of isolates of this species to serve as biological control against *V. dahliae* and *V. longisporum*, as suggested in previous studies (Davis et al. 2000; Robinson et al. 2007; Tyvaert et al. 2014).

5.7 RECOMMENDATIONS

Quantification of *V. dahliae* propagule density in soil using the real-time PCR method should be considered as a potential replacement of the traditional plating method in Manitoba. Adopting the method we used, potato growers could have more accurate information that could help them to better predict Verticillium wilt intensity and to incorporate disease management practices for Verticillium wilt such soil fumigation. Adequate disease management requires identification of the causal organism and accurate quantification of propagule density, and therefore a reliable and dependable detection method must be used.

The application of metam sodium at 374 L ha^{-1} reduced *Verticillium* inoculum and increased potato yield in cv. Russet Burbank. However, it is unknown if growers will need to re-apply the soil fumigant and at what rate, to accomplish similar effect in future

years. Additionally, further studies using high throughput sequencing-base metagenomics will improve our understanding of the impact of metam sodium application on soil microbial communities, including beneficial populations.

Single applications of composted cattle manure or composted separated pig slurry solids for the control of Verticillium wilt did not have a consistent suppressive effect on Verticillium wilt. Although it is believed that multi-year applications of compost may be a better fit for compost to develop consistent disease suppression in soil, studies need to be conducted to validate this hypothesis. Alternatively, compost can be co-inoculated with biological control such as *P. fluorescens* DF37, which have proven to be effective against *V. dahliae* in Manitoba (Uppal et al. 2008).

Manitoba growers are looking for more sustainable potato production systems. Therefore, management strategies that are conducive to the development of disease-suppressive soil need to be evaluated in multi-year experiments. Studies with successive applications of organic amendments such as composted cattle manure and composted separated pig slurry solids should allow more consistent and predictable improvements in yield, quality and disease suppression. Such studies will help to understand the mechanisms associated with disease suppression in soils treated with composted animal manures.

Phosphorus nutrition appears to play a role in regulating tuber set. High levels of available phosphorus in soil increased the overweight marketable yield, but at the

expense of the bonus and regular marketable categories, which showed an inverse relationship with available P. This may be worth studying further, as reduction in row spacing could help to gain more tuber yield within the regular and bonus marketable weight categories. Additionally, it will be interesting to explore if conditions conducive to tuber bulking occur in plots amended with compost and/or commercial fertilizer.

Further studies involving the study of *V. dahliae* in Manitoba need to consider the presence of *V. tricorpus* and *V. klebahnii* in the soil. Although the *V. tricorpus* isolates seem to be nonpathogenic to potato, it is still unknown what role *V. tricorpus* or V. klebahnii could be playing in relation with *V. dahliae*.

Verticillium tricorpus isolates found in Manitoba are not pathogenic to potato. However, they were found colonizing the roots of potato plants and reducing Verticillium wilt symptoms development when co-inoculated with *V. dahliae*. Further studies involving early inoculation with *V. tricorpus* may help to uncover what mechanism may be involved in the suppression of Verticillium wilt.

The real-time PCR-based assays used in this study should find wide application in studies such as competitiveness or fungal survival ability of *Verticillium* species, effect of crop rotation on the population dynamic of *V. dahliae* and *V. tricorpus*, fungal and pathogen survival ability in soils. Future research, using real-time PCR assays should aim to understand how the populations of these *Verticillium* species have shifted and the

factors driven such changes, and ultimately, the effect on wilt and early dying, and yield of potato.

Additionally, *Verticillium tricorpus* exhibits a greater genetic diversity than *V. dahliae* (Korolev and Katan 1999; Pegg and Brady 2002). It may be possible that the *V. tricorpus* isolates at a given site (whether from plant or soil), belong to more than one vegetative compatibility group. It may be possible that vegetative compatibility can be used to determine whether pathogenic, nonpathogenic and antagonistic isolates of *V. tricorpus* belong to dissimilar sub-population within the specie, but additional research is necessary to test this hypothesis.

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APPENDICES

Appendix I. Nutrients management plan during the study for each trial.

Year	Trial	Fertilization [a]		
2012		Fall 2011[b]	Spring 2012 [a]	Fertigation
	cv. Russet Burbank-2012			
	Site A	336 kg ha^{-1} K_2O as 0-0-60;	145 kg N ha^{-1} as 44-0-0 (Broadcast). 64 kg N ha^{-1} as 46-0-0; 33 kg N and 111 kg P_2O_5 ha^{-1} as APP; 15 kg N and 20 kg S ha^{-1} as ATS; 0.4 kg N ha^{-1} as 7-0-0; 0.6 kg B ha^{-1} as Boron-10% (Banding).	6.4 kg N ha^{-1} as 28-0-0
	Site B	None	21 kg N ha^{-1} as 46-0-0; 25 kg N ha^{-1} as 44-0-0; 228 kg K_2O ha^{-1} as 0-0-60; 6 kg N and 5.4 kg S ha^{-1} as 20.5-0-0-24 (Broadcast)	50 kg N ha^{-1} as 28-0-0
	Site C	56 kg ha^{-1} N as 46-0-0; 5 kg N and 24 kg ha^{-1} P_2O_5 as 11-52-0; 31.5 kg N and 37 kg S ha^{-1} as 20.5-0-0-24; 264 kg K_2O ha^{-1} as 0-0-60.	18 kg N ha^{-1} and 58 kg P_2O_5 ha^{-1} as APP; 12.5 kg N ha^{-1} and 17 kg S ha^{-1} as ATS (Banding).	67 kg N ha^{-1} as 28-0-0
	cv. Norland			
	Site F	224 kg ha^{-1} K_2O as 0-0-60	21.5 kg N and 24.5 kg S ha^{-1} as 21-0-0-24; 95 kg N ha^{-1} as 46-0-0 (Broadcast). 9 kg N and 30 kg ha^{-1} P_2O_5 as APP; 1.5 kg ha^{-1} Zn as Liquid Zn (Banding).	N/A
	Site G	220 kg ha^{-1} K_2O as 0-0-60;	23 kg N and 26 kg S ha^{-1} as 21-0-0-24; 98 kg ha^{-1} N as 46-0-0; (Broadcast). 9.3 kg ha^{-1} N and 31.5 kg ha^{-1} P_2O_5 as APP;	N/A
	Site H	97 kg ha^{-1} N as 46-0-0; 14 kg N ha^{-1} and 67 kg ha^{-1} P_2O_5 as 11-52-0; 246 kg ha^{-1} K_2O as 0-0-60; 2.2 kg ha^{-1} Zn as F420G; 1 kg ha^{-1} Cu as F220G.	18 kg N ha^{-1} and 58 kg P_2O_5 ha^{-1} as APP; 12.5 kg N ha^{-1} and 17 kg S ha^{-1} as ATS (Banding).	N/A
2013		Fall 2012 [a]	Spring 2013 [a]	Fertigation
	cv. Russet Burbank-2013			
	Site D	206 kg ha^{-1} N as 46-0-0.	98 kg N ha^{-1} as 44-0-0; 27 kg N and 93 P_2O_5 kg ha^{-1} as 10-34-0; 17 kg N and 23 kg S ha^{-1} as ATS; 0.6 kg B ha^{-1} as Boron-10%; 1 kg N and 1.2 kg Zn ha^{-1} as Zinc20 16-0-0 (Banding)	12 kg N ha^{-1} as 28-0-0
	Site E	None	41.5 kg N ha^{-1} as 46-0-0; kg ha^{-1}; 33 kg N and 38 kg S ha^{-1} as 21-0-0-24; 270 kg ha^{-1} of K_2O as 0-0-60 (Broadcast). 26 kg ha^{-1} of N and 79 kg ha^{-1} P_2O_5 as APP (Banding).	24 kg N ha^{-1} as 28-0-0

[a] Commercial products: ESN: Environmentally Smart Nitrogen (44% nitrogen), Urea (46-0-0), 28-0-0: ammonium nitrate (UAN), 15-0-0-20: ammonium thiosulfate (ATS); 0-0-60: potassium chloride, 10-34-0: ammonium polyphosphate (APP), F420G: Nexus-F420G (granular 20% zinc oxy-sulfate), F220G: Nexus-F220G (20% copper, 6% zinc).

[b] Broadcast application in fall before the fields planted to potato, and broadcast before planting or Banding at planting.

Appendix II. Effect of composted cattle manure, composted separated pig slurry solids, and the soil fumigant, metam sodium on petiole nutrient analysis of trials planted to cv. Russet Burbank in 2012 and 2013.

Year	Trials	Treatments	NO_3-N	P	K	S	Ca	Mg	Na	Zn	Fe	Mn	Cu	B
			ppm			%					ppm			
2012	All	**Control**	11179±1464[a]	0.35±0.04 b	8.9±0.5	0.28±0.02	0.8±0.06	0.7±0.1	0.03±0	28±2	46±3	125±27	2.6±0.5	32±2
		VAP40	12059±969	0.34±0.03 b	8.6±0.5	0.26±0.02	0.8±0.07	0.7±0.1	0.03±0.01	26±2	42±3	129±33	2.5±0.2	31±1
		VAP60	13359±1450	0.33±0.03 b	9.3±0.7	0.26±0.02	0.8±0.08	0.7±0.1	0.03±0.01	24±2	47±4	129±26	2.2±0.2	31±1
		CCM20	13714±1243	0.34±0.03 b	9.1±0.5	0.25±0.01	0.8±0.06	0.7±0.1	0.03±0	27±1	42±3	118±25	2.6±0.3	31±1
		CCM40	12783±1534	0.35±0.03 b	8.7±0.5	0.26±0.01	0.8±0.05	0.7±0.1	0.03±0.01	27±2	45±3	97±18	2.7±0.3	30±1
		CCM80	12408±1256	0.35±0.02 b	9.1±0.4	0.25±0.01	0.8±0.05	0.6±0.1	0.03±0	27±2	44±4	98±21	2.7±0.2	31±1
		FERT	12589±1374	0.46±0.04 a	8.5±0.5	0.24±0.01	0.8±0.08	0.7±0.1	0.03±0	26±2	47±4	124±26	2.4±0.4	30±1
	Site A		9089±670 B	0.48±0.02 A	7.8±0.3 B	0.29±0.01 A	0.9±0.03 A	0.5±0.1 B	0.02±0	25±1 B	58±1 A	152±7 A	2.1±0.1 B	35±1 A
	Site B		16906±504 A	0.34±0.01 B	10.6±0.2 A	0.22±0 B	0.6±0.02 C	0.4±0.1 B	0.03±0	22±1 B	37±1 B	179±12 A	2.2±0.1 B	27±0 B
	Site C		10997±401 B	0.28±0.01 C	7.9±0.2 B	0.27±0.01 A	0.8±0.03 B	0.9±0.1 A	0.04±0	31±1 A	41±2 B	24±1 B	3.5±0.2 A	30±0 B
		Treatment [b]	ns	0.0001	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
		Site	0.004	0.001	0.002	0.001	0.001	0.001	ns	0.007	0.003	0.001	0.004	0.004
		Treatment x Site	ns	ns	ns	ns	ns	ns	ns	na	ns	ns	ns	ns
2013	All	**Control**	12828±1166	0.4±0.03 b	10.2±0.5	0.22±0.02	0.9±0.1	0.6±0.1	0.05±0.01	53±5 ab	58±9	111±14	2.3±0.3	27±2
		CSS20	13015±897	0.4±0.02 b	9.6±0.3	0.21±0.01	0.8±0	0.6±0.1	0.05±0.01	56±6 ab	51±6	102±11	2.4±0.3	26±1
		CSS40	13557±901	0.5±0.03 ab	10.2±0.2	0.21±0.01	0.8±0	0.6±0.1	0.06±0.01	55±5 ab	48±3	92±5	2.5±0.3	25±1
		CSS80	12944±1583	0.6±0.02 a	10.1±0.3	0.22±0.01	0.8±0	0.6±0.1	0.07±0	59±6 a	52±5	90±7	2.8±0.3	26±1
		FERT	13154±1436	0.5±0.04 ab	9.9±0.3	0.21±0	0.9±0	0.6±0.1	0.05±0.01	48±7 b	57±9	99±10	1.9±0.3	26±1
	Site D		14370±614	0.6±0.02 A	9.8±0.28	0.19±0.01	0.9±0.04	0.5±0.02 B	0.05±0	68±3 A	67±4 A	115±7 A	1.9±0.2 B	27±1
	Site E		12105±821	0.4±0.02 B	10.2±0.12	0.22±0	0.8±0.02	0.7±0.04 A	0.06±0	41±2 B	39±1 B	86±4 B	2.8±0.2 A	25±0
		Treatment [b]	ns	0.001	ns	ns	ns	ns	ns	0.05	ns	ns	0.07	ns
		Site	ns	0.006	ns	ns	ns	0.02	ns	0.002	0.001	0.005	0.008	ns
		Treatment x Site	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

[a] Values are means ±1 standard error. Within columns means followed by the same letter are not significant different (P >0.05) according to the Bonferroni's multiple comparison test.

[b] Treatment significant probability. ns, not significant (P >0.05). Trends are indicated where P values were between 0.05 and 0.10.

Appendix III. Water soluble nitrate-N (NO_3^--N), Olsen extractable-P measured before planting, mid-season and before harvest of potato cv. Russet Burbank established in 2012 and 2013.

Year	Trials	Treatments	Planting [a]			Mid-season			Harvest		
			NH_4-N	NO_3-N	P	NH_4-N	NO_3-N	P	NH_4-N	NO_3-N	P
			mg kg^{-1}								
2012	**All**	**Control**	2±0 [b]	11±3	36±6	14±4	33±3	55±6 b	4±1	5±1 ab	62±5 bc
		Vapam40	3±0	13±3	36±5	18±3	31±3	51±7 b	4±0	4±0 b	56±6 c
		Vapam60	3±0	12±3	36±5	16±3	32±4	57±6 b	4±0	5±1 ab	64±7 bc
		CCM20	2±0	12±3	37±5	12±2	32±1	62±7 b	4±0	6±1 a	67±6 bc
		CCM40	2±0	12±3	37±5	12±2	34±3	74±5 b	4±0	5±1 ab	75±8 bc
		CCM80	2±0	12±3	37±5	11±1	36±3	103±9 a	3±0	5±0 ab	87±8 ab
		FERT	2±0	12±3	37±5	17±4	34±4	114±9 a	4±0	6±0 a	107±11 a
	Site A		2±0	8±0 B	15±0 B	13±2 B	29±2	58±8 B	4±0	4±0	52±5 B
	Site B		2±0	3±0 C	50±2 A	22±2 A	31±1	83±4 A	3±0	6±0	84±3 A
	Site C		2±0	24±1 A	41±1 A	7±0 B	39±2	78±7 AB	4±0	5±0	83±7 A
		Treatment [e]	ns	ns	ns	ns	ns	0.001	ns	0.004	0.002
		Site	ns	0.001	0.002	0.004	ns	0.02	ns	ns	0.001
		Treatment x Site	ns	ns	ns	ns	ns	ns	ns	ns	ns
2013	**All**	**Control**	7±3	14±4	29±6	6±0	21±5	59±12 c	10±3	9±2	67±13 c
		CSS20	6±1	15±4	26±4	6±0	21±5	70±15 c	8±3	8±1	74±14 bc
		CSS40	6±2	16±5	31±5	6±0	23±6	83±12 bc	8±2	9±1	91±12 bc
		CSS80	4±1	11±3	34±7	9±2	21±5	138±22 a	10±3	8±2	131±20 a
		FERT	5±1	11±3	31±5	9±3	18±4	105±16 ab	9±2	7±1	95±16 b
	Site D		4±1	4±0 B	18±1 B	8±2	12±1 B	55±6 B	3±0 B	5±0 B	56±4 B
	Site E		6±1	22±2 A	41±3 A	6±0	29±3 A	125±10 A	15±1 A	10±1 A	125±9 A
		Treatment [c]	ns	ns	ns	ns	ns	0.001	ns	ns	0.001
		Site	ns	0.001	0.001	ns	0.002	0.001	0.001	0.01	0.001
		Treatment x Site	ns	ns	ns	ns	ns	ns	ns	ns	ns

[a] Samples were collected immediately before planting, at mid season and before harvest.

[b] Values are means ±1 standard error. Within columns means followed by the same letter are not significant different (P >0.05) according to the Bonferroni's multiple comparison test.

[c] Treatment significant probability. ns, not significant (P >0.05). Trends are indicated where P values were between 0.05 and 0.10.

Appendix IV. Morphological characteristics of reference *Verticillium* species.

Strain code	Source	Host	Morphological characteristics				Identity
			Conidia	Microsclerotia	Chlamydospore	Resting mycelium	
			Width – Length (µm)				
V_104b	Dr. Daayf, Canada	Potato	4.2 - 6.3	Absent	Absent	Absent	*V. albo-atrum*
107724	CCFC, Canada	Potato	3.1 - 6.3	Absent	Absent	Absent	*V. albo-atrum*
177114	CCFC, Canada	Potato	3 - 6.9	Absent	Absent	Absent	*V. albo-atrum*
177115	CCFC, Canada	Potato	2.7 - 6	Absent	Absent	Absent	*V. albo-atrum*
216604	CCFC, Canada	Potato	3.1 - 6.3	Absent	Absent	Present	*V. albo-atrum*
AT3	Dr. Dobinson, Canada	Potato	2.7 - 5.2	Absent	Absent	Absent	*V. albo-atrum*
Vd1396-9	Dr. Daayf, Canada	Potato	2.7 - 5.9	38.2 - 60	Absent	Absent	*V. dahliae*
241194	CCFC, Canada	Tomato	3 - 6.3	41.6 - 60	Absent	Present	*V. dahliae*
Dvd-E6	Dr. Dobinson, Canada	Eggplant	2.6 - 6.1	41.7 - 59.9	Absent	Absent	*V. dahliae*
Dvd-3	Dr. Dobinson, Canada	Potato	2.9 - 6.5	41.3 - 58.7	Absent	Absent	*V. dahliae*
Dvd-P2	Dr. Dobinson, Canada	Potato	2.4 - 6	45.1 - 58.1	Absent	Absent	*V. dahliae*
Dvd-S100	Dr. Dobinson, Canada	Soil	2.7 - 5.7	39.3 - 59.7	Absent	Absent	*V. dahliae*
226890	CCFC, Canada	Soil	2.1 - 4.4	19.3 - 27.7	5.6 - 6.5	Present	*V. nigrescens*
226891	CCFC, Canada	Soil	2.2 - 4	16.1 - 22.9	Absent	Absent	*V. nigrescens*
Dvt-3	Dr. Dobinson, Canada	Unknown	3.3 - 5.6	58.9 - 90.8	6.5 - 7.9	Present	*V. tricorpus*
VD624	Dr. Subbarao, California	Cauliflower	3.5 - 8.4	43 - 62.5	Absent	Absent	*V. longisporum*
VD348	Dr. Subbarao, California	Cauliflower	3.5 - 8.7	43.2 - 62.2	Absent	Absent	*V. longisporum*
243377D	AAFC, Canada	Canola	3.5 - 8.7	44.9 - 61.7	Absent	Present	*V. longisporum*
243377E	AAFC, Canada	Canola	3.4 - 8.6	44.3 - 61.6	Absent	Present	*V. longisporum*
243378D	AAFC, Canada	Canola	3.2 - 8.2	38.7 - 53.5	Absent	Absent	*V. longisporum*
243378E	AAFC, Canada	Canola	3.1 - 7.5	32.4 - 44.6	Absent	Absent	*V. longisporum*

Appendix V. Morphological characteristics of *Verticillium* isolates obtained from Manitoba potato fields.

Strain code	Host[a]	Manitoba Region	Morphological characteristics				Name according to morphology	Real-time PCR assays[b]		Sequence Identity
			Conidia	Microsclerotia	Chlamydospore	Resting mycelium		*V. dahliae*	*V. tricorpus*	
			Width – Length (µm)							
MBVd02	Plant	Dufferin	3.0 - 4.9	62.0 - 88.3	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd03	Plant	Thompson	3.0 - 5.3	44.5 - 79.8	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd06	Plant	Elthon	2.8 - 5.7	56.9 - 88.5	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd07	Plant	Elthon	2.5 - 5.1	51.7 - 83.3	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt10	Plant	Elthon	3.5 - 5.7	60.4 - 96.8	6.1 - 8.1	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd13	Plant	Portage la Prairie	2.8 - 5.5	55.6 - 92.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd14	Plant	Portage la Prairie	3.0 – 6.0	47.1 - 86.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt22	Plant	North Cypress	3.5 - 7.1	70.6 - 97.6	5.3 - 6.8	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd23	Plant	Stanley	2.5 – 5.0	51.1 - 86.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd25	Plant	Rhineland	3.1 - 6.2	48.6 - 83.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd28	Plant	North Cypress	1.9 - 3.9	47.4 - 83.5	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd29	Plant	North Folk	2.5 - 4.9	49.0 - 84.8	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt30	Plant	North Folk	3.5 - 5.3	65.2 - 97.9	7.7 – 8.0	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd31	Plant	North Folk	2.7 - 5.4	46.3 - 79.3	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt32	Plant	North Folk	3.1 - 5.7	66.3 - 109.4	7.5 - 8.6	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd49	Plant	North Cypress	2.6 - 5.2	54.9 - 88.3	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd50	Plant	North Cypress	2.7 - 5.4	54.2 - 86.7	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt57	Plant	North Cypress	3.5 - 5.6	67.8 - 102.9	6.6 – 8.0	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt58	Plant	Elthon	3.6 - 6.4	65.8 - 108.4	6.3 - 7.5	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd60	Plant	Portage la Prairie	2.5 - 5.6	43.5 - 79.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd61	Plant	Portage la Prairie	2.5 - 5.5	46.2 - 81.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd75	Plant	Elthon	2.7 - 5.9	51.7 - 87.1	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVd81	Plant	Elthon	3.2 - 5.3	54.8 - 87.7	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVd01	Soil	Dufferin	2.8 - 4.7	65.6 - 89.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBGn04	Soil	Thompson	3.1 - 6.3	-	5.6 - 7.8	Present	*V. tricorpus*	-	-	*G. nigrescens*
MBVd05	Soil	Elthon	3.4 - 6.6	59.2 - 90.8	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd08	Soil	Elthon	2.0 - 3.8	56.1 - 87.7	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd09	Soil	Elthon	2.5 - 4.9	54.5 - 86.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd11	Soil	North Folk	3.4 - 6.9	47.4 - 71.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*

MBVd12	Soil	Portage la Prairie	2.6 - 5.2	49.9 – 76.0	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd15	Soil	Stanley	3.0 - 6.1	48.7 - 83.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd16	Soil	North Cypress	3.1 - 6.3	44.3 - 85.7	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd17	Soil	North Cypress	2.6 - 5.2	44.4 - 88.3	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd18	Soil	Stanley	2.9 - 5.8	44.7 - 84.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd19	Soil	Rhineland	2.1 - 4.1	44.4 - 83.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd21	Soil	Stanley	2.3 - 4.6	44.0 - 76.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd24	Soil	Stanley	3.1 - 6.2	54.5 - 88.7	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd26	Soil	North Cypress	3.0 - 5.9	50.8 - 88.3	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd27	Soil	North Cypress	2.8 - 5.7	49.1 - 84.5	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd33	Soil	North Folk	2.7 - 5.5	51.3 - 85.6	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVk34	Soil	North Folk	2.8 - 5.6	61.5 - 99.8	6.6 - 9.5	Present	*V. tricorpus*	-	+	*V. klehbanii*
MBVd35	Soil	Stanley	2.3 - 4.6	47.2 - 79.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt36	Soil	North Folk	3.3 - 5.7	60.1 - 91.4	7.1 - 7.6	Present	*V. tricorpus*	+	+	*V. tricorpus*
MBVt37	Soil	North Folk	3.1 - 5.8	64.1 - 122.7	7.0 - 7.1	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt38	Soil	North Folk	3.5 - 5.5	65.1 - 116.6	7.6 - 8.4	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt39	Soil	North Folk	3.4 - 5.3	66.7 - 115	6.8 - 7.5	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt40	Soil	North Folk	3.1 - 5.4	64.1 - 97.2	7.9 - 8.5	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt41	Soil	North Folk	3.3 - 5.5	62.5 - 93.9	6.1 - 8.3	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt42	Soil	North Folk	3.1 - 5.8	64.0 – 92.0	7.2 - 8.3	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt43	Soil	North Folk	3.1 - 4.3	64.8 – 93.0	5.2 - 6.4	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt44	Soil	North Folk	3.3 - 6.7	67.1 - 87.3	5.9 - 8.8	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd45	Soil	North Cypress	2.9 - 5.7	57 - 85.2	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd46	Soil	North Cypress	2.5 - 5.1	54.4 - 84.6	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd47	Soil	North Cypress	2.9 - 5.6	52.6 - 85	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd48	Soil	North Cypress	2.0 - 4.2	55.7 - 88.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd51	Soil	North Cypress	2.6 - 5.2	53 - 83.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd53	Soil	North Cypress	2.5 – 5.0	52.9 - 85.5	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd54	Soil	North Cypress	2.9 - 5.7	52.6 - 87	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd55	Soil	North Cypress	2.7 - 5.4	50.1 - 84.8	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd56	Soil	North Cypress	2.5 – 5.0	48.7 - 82.1	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd59	Soil	Portage la Prairie	2.7 - 5.8	42.7 – 80.0	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd62	Soil	Rhineland	2.8 - 5.9	44.0 - 81.5	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd63	Soil	Rhineland	2.3 - 5.8	45.5 - 80.6	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*

MBVd64	Soil	North Folk	2.6 - 5.4	41.4 - 73.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd65	Soil	Stanley	2.9 - 5.9	54.7 - 82.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd67	Soil	North Cypress	2.5 - 5.2	46.4 - 81.9	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVt69	Soil	North Cypress	3.1 - 5.7	66.5 - 101.5	6.5 - 7.2	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt70	Soil	North Folk	2.9 - 5.2	60.5 - 95.0	6.5 - 7.7	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd71	Soil	North Cypress	3.0 - 5.1	48.6 - 85.5	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*
MBVd72	Soil	North Cypress	2.9 - 4.8	51.6 - 89.4	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVt73	Soil	North Cypress	3.0 - 5.2	58.6 - 90.8	6.4 - 7.6	Present	*V. tricorpus*	-	-	*V. tricorpus*
MBVd74	Soil	Stanley	2.7 - 5.8	50.1 - 86.6	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVd76	Soil	Stanley	2.7 - 5.8	55.1 - 94.6	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVd77	Soil	Stanley	2.3 - 5.2	50.5 – 81.0	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVd78	Soil	Stanley	2.5 - 5.5	45.7 - 81.6	Absent	Absent	*V. dahliae*	+	+	*V. dahliae*
MBVt79	Soil	North Folk	2.9 – 5.0	64.2 - 103	6.5 - 7.9	Present	*V. tricorpus*	-	-	*G. nigrecens*
MBGn80	Soil	North Folk	3.0 - 6.4	0 - 0	5.8 - 7.7	Present	*V. tricorpus*	-	-	*V. tricorpus*
MBVd82	Soil	North Cypress	3.9 - 7.2	62.5 - 101.2	5.9 - 8.1	Present	*V. dahliae*	+	+	*V. dahliae*
MBVt83	Soil	North Cypress	3.2 - 5.5	69.8 - 104.4	6.6 - 7.8	Present	*V. tricorpus*	-	-	*V. tricorpus*
MBVt84	Soil	North Cypress	2.8 - 4.7	62.2 - 95.9	6.7 - 7.8	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVt86	Soil	North Folk	3.3 - 6.1	67.7 - 104.5	6.8 - 7.8	Present	*V. tricorpus*	-	+	*V. tricorpus*
MBVd89	Soil	North Cypress	2.5 - 5.6	49.9 - 82.4	Absent	Absent	*V. dahliae*	+	-	*V. dahliae*

a Isolates collected from either plant or soil.

b (+) indicates isolate amplified with protocol, and (-) indicates not amplification